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Síntese de Peptídeos em fase sólida de “seqüências difíceis” e estudos estrutura-função de peptídeos bioativos.

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À Deus, que nos momentos mais difíceis, sempre me orientou e me deu forças para continuar.

À minha família, Lucimar, Sabrina e Dudu, que sempre me encheram de amor, compreensão e apoio.

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obrigado pela educação e apoio.

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amigo.

“Não bastam estrelas brilhando, se não
tivermos olhos para alcançá-las”

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ABREVIATÓES

AA - Aminoácido

AAA - Análise de aminoácidos

ACN - Acetonitrila

AN - Receptor eletrônico

BAR - Benzidrilamino-resina

Boc - terc-Butiloxicarbonil

BOP - hexafluorofosfato de benzotriazolil-N-oxi-tris(dimetilamino)fosfônio

Bzl - Benzil

DCC - Diclóhexilcarbodiimida

DCM - Diclorometano

DIC - Diisopropilcarbodiimida

DIEA - Diisopropiletilamina

DMF - Dimetilformamida

DMSO - Dimetilsulfóxido

DN - Doador eletrônico

EtOH - Etanol

Fmoc - 9-fluorenilmetiloxicarbonil

HAc - Ácido acético

HATU - 1,1,3,3-tetrametiluroniohexafluorofosfato de O-(7-azabenzotriazol-1-il)

HFIP - 1,1,1,3,3,3-Hexafluorisopropanol

HOAt - 1-hidróxi-7-azabenzotriazol

HOBt - 1-hidróxibenzotriazol

IV - Espectroscopia no infravermelho

MBAR - Metilbenzidrilamino-resina

MeOH - Metanol

PIP - piperidina

RMN - Ressonância magnética nuclear

RPE - Ressonância paramagnética eletrônica

SPFS - Síntese de peptídeos em fase sólida

TFA - Ácido trifluoracético

Toac - Ácido 4-amino-2,2,6,6-tetrametilpiperidina-N-óxido-4-carboxílico

1. Apresentação

A nossa vida científica pode ser dividida em duas etapas, uma ligada propriamente ao estudo da metodologia da “Síntese de Peptídeos em Fase Sólida - SPPS”, e outra aos estudos estrutura/atividade de peptídeos e proteínas. Desta forma, esta tese de livre docência está também separada nestas duas vertentes.

No estudo da SPPS avaliamos os fatores envolvidos nas etapas de acoplamento e de clivagem. A etapa de acoplamento, a mais problemática desta metodologia, permitiu a análise dos fatores que afetam a solvatação (inchamento) do grão de resina, bem como o uso de resinas com elevado grau de substituição, enquanto a etapa de clivagem foi direcionada ao uso de diferentes resinas e meios reacionais.

A segunda parte desta tese apresenta estudos envolvendo a dualidade estrutura/função de peptídeos. Entre os diversos estudos realizados, destaque foi dado a citolisina “esticolisina II”. Nesta parte o objetivo principal é a determinação do mecanismo de ação destas moléculas bem como a obtenção de substâncias mais ativas.

Finalizando, estamos incluindo nesta tese os trabalhos científicos publicados, relacionados ao texto aqui exposto.

2. Síntese de Peptídeos em Fase Sólida – SPPS.

As proteínas podem ser consideradas como as biomoléculas mais importantes dos processos celulares, participando de quase todas as etapas do ciclo celular. Entre as funções que as proteínas participam podemos citar: 1) o controle das etapas de replicação e expressão gênica; 2) a atuação como catalisadores; 3) a regulação do metabolismo energético através das enzimas reguladoras; 4) em animais, a identificação e destruição de moléculas invasoras, fazendo parte do sistema imune; e 5) estrutural: como o colágeno, fibras musculares; entre miríades de outras.

Moléculas com a mesma estrutura química das proteínas, mas de tamanho menor, são chamadas de peptídeos. O ponto de divisão entre estas duas classes de moléculas é dúbio, podendo um peptídeo possuir de 30 até 100 moléculas de aminoácidos antes de ser denominado como sendo uma proteína, no entanto, o valor médio de 50 é o mais comumente encontrado. Os peptídeos apresentam por si só atividade biológica, agindo como hormônios e sinalizadores moleculares, entre outros. Atualmente uma grande quantidade de peptídeos é encontrada como toxinas em animais e bactérias.

O fato de as proteínas e peptídeos possuírem estrutura química parecida – resíduos de aminoácidos ligados por ligação amida, diferindo apenas no número de aminoácidos que os compõem, torna a síntese química destas moléculas semelhante para ambas.

O primeiro pesquisador que estudou a síntese peptídica foi Emil Fisher, em 1901, que descreveu a obtenção do primeiro dipeptídeo, sendo creditado a ele a origem do termo peptídeo. Durante as décadas seguintes, a química de peptídeos teve grande avanço, sendo introduzidos grupos protetores e métodos de acoplamentos mais eficazes. Apesar destes avanços, e a obtenção de peptídeos como a oxitocina, vasopressina, entre outros, a síntese destas seqüências era um processo complexo e altamente laborioso, durando de semanas a meses. Visando obter peptídeos de maneira mais rápida e eficiente, e aproveitando a grande quantidade de informação obtida sobre a estrutura e composição dos polímeros no início da década de 50, Bruce Merrifield desenvolveu a síntese de peptídeo em fase sólida, publicando o primeiro artigo sobre esta técnica em 1963 (MERRIFIELD, 1963).

Esta técnica nos anos posteriores a sua introdução teve um grande avanço, com a introdução de novos protetores, agentes acoplantes, procedimentos de clivagem e, finalmente, automação, proporcionando um grande avanço nos estudos biológicos nos últimos trinta anos.

Esse método de síntese encontra-se publicado em diversas revisões (ALBERICIO, 2000; ATHERTON.E.; SHEPPARD, 1989; BARANY; MERRIFIELD, 1980; GREGG, 1997; STEWART; YOUNG, 1984) e baseia-se no crescimento, resíduo por resíduo, da cadeia peptídica presa covalentemente pelo seu aminoácido carboxi-terminal a sítios reativos existentes em um suporte sólido (resina). O método da fase sólida compõe-se basicamente de duas estratégias principais: uma emprega o grupamento ácido-lábil terc-butiloxicarbonila (Boc) como protetor do α -amino grupo e derivados benzílicos (Bzl) para a proteção da maioria das cadeias laterais de resíduos de aminoácidos tri-funcionais. Alternativamente, a segunda opção utiliza os grupamentos 9-fluorenilmetiloxicarbonil (Fmoc-base lábil) e terc-butílicos (tBu), respectivamente.

Tanto na estratégia Boc/Bzl quanto na Fmoc/tBu, o aminoácido carboxi-terminal é ligado covalentemente à resina através de uma ligação éster para obtenção de peptídeos com carboxilatos livres, ou de uma ligação amida para a obtenção de peptídeos com extremidade α -carboxamida após a clivagem final em fluoreto de hidrogênio anidro (HF) ou em ácido trifluoroacético (TFA), respectivamente.

O método da fase sólida apresenta, como principal vantagem, a obtenção de uma grande quantidade de seqüências peptídicas diferentes em um curto espaço de tempo. Isso ocorre principalmente devido a dois fatores:

1. Automação do processo de síntese, que permite, principalmente pela insolubilidade da resina em todos os solventes utilizados na química de peptídeos, que todos os ciclos sintéticos ocorram em um único frasco de reação contendo uma placa porosa filtrante. Essa placa irá reter a resina desde o início até o final da síntese da seqüência desejada, evitando-se, portanto, a troca do frasco de reação, simplificando bastante o processo e evitando perdas do produto.
2. Eliminação de todos os solventes, reagentes e subprodutos, das diversas etapas do ciclo sintético, por simples filtração, permitindo o uso de excesso de solventes e reagentes.

Apesar destas vantagens, muitas dificuldades ainda são encontradas na SPPS. Um dos problemas ainda encontrado é a síntese dos chamados “peptídeos difíceis” (Kent, 1988). Estes peptídeos possuem a característica de se agregarem entre si ou com outras cadeias peptídicas, dificultando a difusão de reagentes e solventes no interior do grão causando a ocorrência de seqüências com uma ou mais deleções (sem 1 ou mais

aminoácidos). Este efeito parece ser dependente de problemas conformacionais e/ou agregações inter- e intra-moleculares das cadeias peptídicas em crescimento ancoradas à estrutura polimérica. Estas agregações são seqüência-dependente, sendo que a extremidade N-terminal reativa do peptídeo pode ficar impedida momentaneamente, dificultando a reação de acoplamento do aminoácido seguinte (PENNINGTON; BYRNES, 1994). Acoplamentos com 96% de eficiência podem gerar na síntese de um peptídeo com 31 resíduos uma queda no rendimento de 79%, sendo que este valor diminui para 6% quando esta etapa ocorre com eficácia de 99,8%. Apesar de serem seqüência-dependente, tem sido observado que a maior parte dos acoplamentos incompletos ocorre entre os resíduos 12 a 20 da seqüência. Este fenômeno é atribuído a propensão da formação de β -estruturas entre as cadeias peptídicas no interior do grão de resina em peptídeos com este tamanho (BARON e LOZE, 1978; KENT, 1985; PILLAI e MUTTER, 1981). Desta forma, um fator que é fundamental não só para a etapa de acoplamento, mas para todas as etapas envolvidas na SPFS, é a solvatação do grão de resina (PUGH et al., 1992). A efetiva solvatação é essencial para a acessibilidade dos agentes acoplantes, aminoácidos e demais reagentes aos sítios ativos da resina. Na busca de solventes mais eficientes para a SPFS inúmeras tentativas foram apresentadas, entre estas podemos citar o uso de sais caotrópicos (HENDRIX et al., 1990; THALER et al., 1991) e de álcoois fluorados como o TFE (YAMASHIRO et al., 1976) e o HFIP (MILTON et al., 1990). Na prática a determinação das condições apropriadas de solvatação não é uma tarefa fácil, pois as propriedades físico-químicas das resinas sofrem alteração durante o crescimento da cadeia peptídica. O poliestireno contendo 1% de divinilbenzeno é uma das resinas mais utilizadas e possui característica apolar, solvatando bem em diclorometano. No entanto, com o aumento do teor de aminoácidos ligados a ela, esta pode sofrer modificações em seu inchamento passando a solvatar bem em solventes apolares (figura 1).

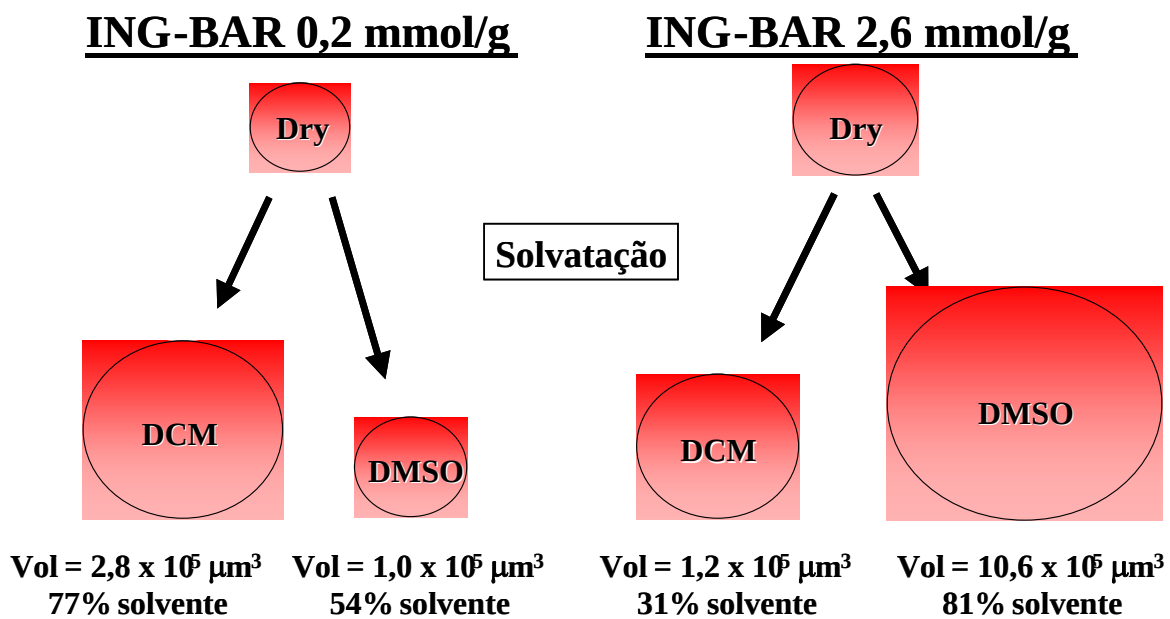


Figura 1: Inchamento da resina BAR, em DCM e DMSO, com baixo e alto teor do tripeptídeo ING.

Uma maneira de se evitar a agregação entre as cadeias polipeptídicas é o uso de resinas com baixo grau de substituição. Estas resinas, devido ao menor número de sítios por grão, aumentam a acessibilidade dos reagentes e solventes ao grupo amino que irá sofrer a reação, diminuindo as agregações intercadeias (PENNINGTON; BYRNES, 1994).

O aumento de temperatura também é utilizado como uma maneira de elevar a eficiência de acoplamento. A elevação da temperatura diminui as interações fracas (ligação de hidrogênio, iônicas, dipolares, etc) intercadeias e intracadeias, aumentando a acessibilidade aos sítios reativos da resina (RABINOVICH e RIVIER, 1994; VARANDA e MIRANDA, 1997). No entanto, o aumento da eficiência de acoplamento pode ser compensado pelo aparecimento de um maior número de subprodutos, tais como, racêmicos e aminoácidos amidas desidratados (asparagina e glutamina) (LLOYD et al., 1990; TAM;1985; TAM e LU, 1995)

Somada aos facilitadores acima, a melhoria dos métodos de acoplamento também tem sido estudada. Apesar de os primeiros agentes acoplantes desenvolvidos para a SPFS estarem em uso até o momento, como é o caso do DCC (diciclohexilcarbodiimida – Figura 2) na presença ou não do HOBt (hidroxibenzotriazol – figura 3) (KÖNIG e GEIGER, 1970), outros têm sido introduzidos neste tipo de síntese. Um exemplo é o DIC (diisoprilcarbodiimida) (ALBERT; HAMILTON, 1995) que substitui com vantagens o composto DCC, pois forma como subproduto a diisopropiluréia, mais solúvel que a diciclohexiluréia formada pelo DCC. Sais de urônio, como HBTU (hexafluorofosfato de 2-(1-H-benzotriazol-1-il)-1,1,3,3-tetrametilurônio) (DOURTOGLOU et al., 1978) e TBTU (Tetrafluoroborato 2-(1-H-benzotriazol-1-il)-1,1,3,3-tetrametilurônio) (KNORR et al., 1989) ou de fosfônio como o PyBOP (hexafluorofosfato de (benzotriazol-1-il-oxi)-tris-(pirrolino)fosfônio) (CASTRO et al., 1975) também têm sido utilizados na melhoria da SPPS (FIELDS et al., 1991). Um passo adiante no uso dos sais acima foi o desenvolvimento dos azabenzotriazóis por Carpino (ALBERICIO et al., 1997; CARPINO, 1993; CARPINO e EL-FAHAN, 1995; COIN et al., 2006), e do cloreto de HOBt (hidroxibenzotriazol) (DI FENZA e ROVERO, 2002; SABATINO et al., 2002) que mostraram ser mais eficientes que seus antecessores.

A figura 4 mostra a estrutura química de alguns destes ativadores utilizados na etapa de acoplamento na SPFS.

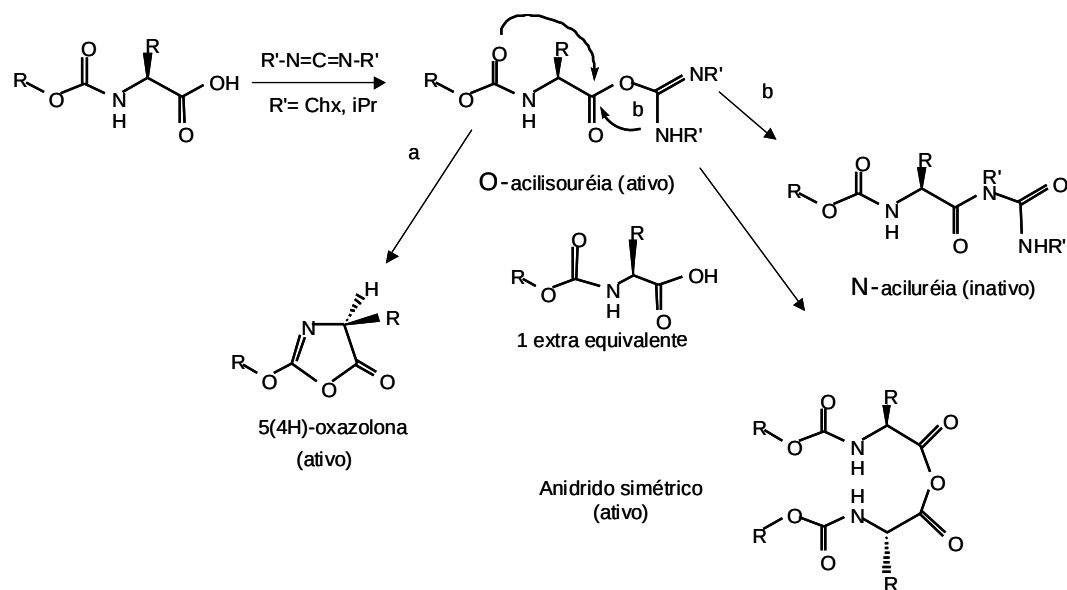


Figura 2: Mecanismo de formação da ligação peptídica através do uso de carbodiimidas.

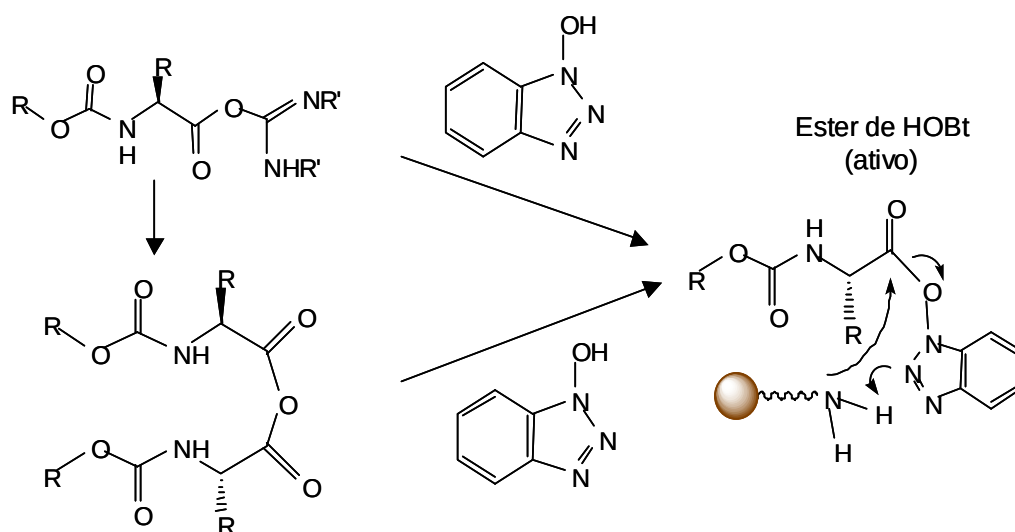


Figura 3: Mecanismo de formação da ligação peptídica através do uso de carbodiimidas na presença de HOBt.

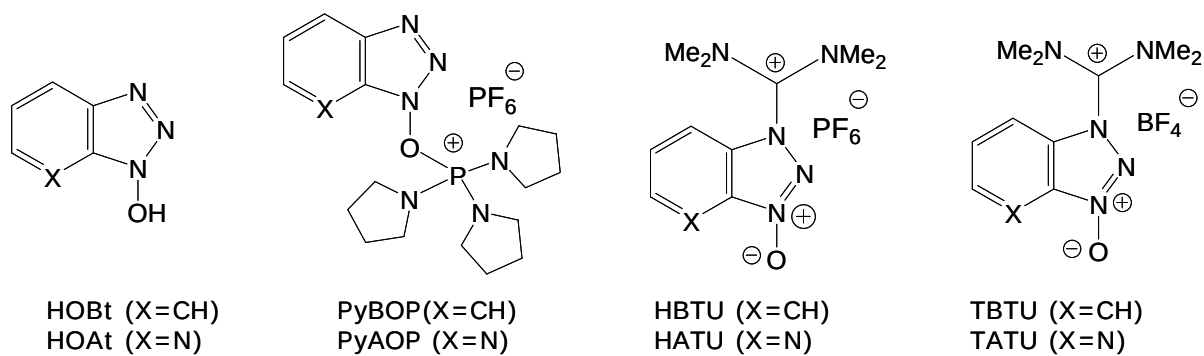


Figura 4: Estruturas químicas do HOBt e HOAt e de seus respectivos sais de fosfônio e urônio.

Entre os diversos métodos utilizados nos estudos acima e na determinação dos problemas encontrados durante a síntese, podemos citar a espectroscopia no infravermelho (IV) (HENKEL e BAYER, 1998; MILTON e MILTON, 1990; YAN e SUN, 1998), a ressonância paramagnética eletrônica (RPE) (VAINO et al., 2000), a RMN (FITCH et al., 1994; VALENTE et al., 2005; WARRASS et al., 2000), e o grau de solvatação destas peptidil-resinas, determinado por medidas microscópicas dos grãos de resina (medida indireta da agregação (CILLI et al., 1996)).

Um outro problema, ainda encontrado, é a obtenção de pequenas quantidades de peptídeos quando da utilização desse método, limitada pelo tamanho dos frascos de reação utilizados nos sintetizadores automáticos (principalmente os de síntese múltipla) e pelo baixo conteúdo de grupos funcionais das resinas utilizadas. O uso de resinas com grau de substituição cerca de 3 a 6 vezes maiores do que o normalmente encontrado (0,5 mmol/g) pode, no entanto, solucionar esse problema, além de proporcionar um gasto menor de solventes e tempo para a obtenção da mesma quantidade de peptídeo. Além disso, se analisarmos do ponto de vista da técnica de “peptide library” (ANDREWS et al., 1994; GALLOP et al., 1994; GORDON et al., 1996), um grão de resina com uma quantidade maior de peptídeos por grão pode ser de extrema importância tanto na interação da enzima-peptídeo quanto na determinação da sequência de aminoácidos.

Visando avaliar em pormenores a utilização de resinas com alto grau de substituição na síntese peptídica em fase sólida e os fatores que afetam as etapas de acoplamento e clivagem, resolvemos avaliar, durante boa parte de nossa vida profissional, as propriedades físico-químicas das peptidil-resinas, durante e após a síntese.

3. Atividades de Pesquisa posteriores à obtenção do Título de Doutor no estudo da SPFS.

Nesta parte da tese são colocadas as várias etapas da carreira científica desenvolvidas inicialmente no Departamento de Biofísica da UNIFESP e posteriormente no Departamento de Bioquímica e Tecnologia Química do Instituto de Química de Araraquara da UNESP. Estas atividades estão colocadas em ordem cronológica e de estudo, a partir de 1998. Desta forma, os artigos publicados dos projetos e colaborações realizadas estão colocados como anexos, juntamente com uma breve descrição.

1) Anexo 1 - Correlation between the mobility of spin labeled peptide chains and resin solvation: an approach to optimize the synthesis of aggregating sequences. *Journal of Organic Chemistry*, v. 64, p. 9118 - 9123, 1999.

Inicialmente continuamos a estudar a síntese de peptídeos em fase sólida através da RPE, em colaboração com o Prof. Dr. Clovis R. Nakaie, visando complementar os dados obtidos durante a tese de doutorado. Desta forma em 1999 publicamos um trabalho completo sobre a utilização da RPE no estudo da síntese de peptídeos em fase sólida. Neste trabalho consolidamos nosso grupo na utilização da RPE no estudo da SPFS e na utilização do TOAC na síntese peptídica como um todo. Os dados obtidos compararam a mobilidade da cadeia peptídica obtida por RPE com o inchamento obtido através de microscopia direta (medida direta do grão de resina) e com os valores de rendimento da etapa de acoplamento em diferentes tipos de solventes. Neste trabalho mostramos que a solvatação do grão de resina é importante para a eficiência da etapa de acoplamento e que esta pode ser acompanhada pela RPE através de parâmetros simples obtidos do espectro, tais como os valores de W_0 e W_{-1} . Estas variáveis representam as larguras dos picos de campo médio e alto dos espectros, sendo que o W_0 (Figura 5) mostrou melhor correlação com a eficiência de acoplamento. Um outro ponto positivo deste trabalho foi o de mostrar que utilizando solventes adequados, a utilização de resinas com alto teor de sítios por grão é possível de uso.

Este trabalho mostrou também a importância da viscosidade no rendimento da etapa de acoplamento, propriedade, até aquele momento, desprezada em estudos envolvendo a SPFS.

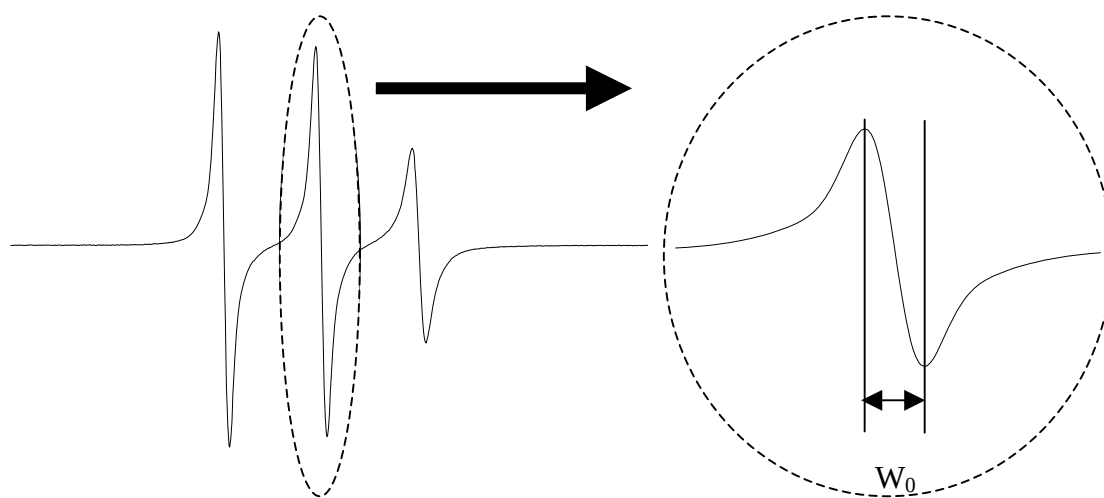


Figura 5: Espectro de RPE, mostrando a obtenção do parâmetro W_0 .

2) Anexo 2: Importance of the solvation degree of peptide-resin beads for amine groups determination by the picric acid method. J Braz Chem Soc. , v. 11, p. 474 - 478, 2000.

Durante o desenvolvimento do trabalho colocado no anexo 1 foi possível observar a ineficácia da metodologia do ácido pícrico (GISIN, 1972; HANCOCK et al., 1975) para a determinação do teor de acoplamento em diversas peptidil-resinas estudadas. Nesta metodologia, a resina, com amino grupos previamente desprotonados, é submetida ao tratamento com solução de ácido pícrico em DCM, ocorrendo a ligação do amino-grupo com este ácido na proporção 1:1. O excesso é eliminado por diversas lavagens com DCM para a remoção de vestígios de ácido pícrico livre, e a resina é tratada com TEA 10% (v/v) em DCM para retirar totalmente os íons picrato ligados aos amino grupos. A absorbância da solução obtida é então medida em 358 nm, e a concentração de amino-grupos livres é obtida. Desta forma, resolvemos avaliar quais as etapas deste método eram as responsáveis pela falha nos resultados obtidos. Os estudos realizados comprovaram que a baixa solvatação, principalmente na etapa de ligação entre o íon picrato e o amino-grupo da peptidil-resina, era a responsável pelo baixo valor obtido quando comparado com o teórico. A simples mudança do solvente DCM para um mais polar, como o DMF, resolveu este problema; no entanto, mudanças dos solventes nas etapas de lavagens também foram necessárias para o perfeito funcionamento deste método.

3) Anexo 3: Effect of temperature on peptide chain aggregation: an EPR study of model peptidyl-resins. Tetrahedron Letters, v. 42, p. 3243 - 3246, 2001.

Continuando os estudos envolvendo os fatores que afetam a etapa de acoplamento na SPFS, resolvemos estudar, através da RPE, o efeito da temperatura na mobilidade das cadeias peptídicas no interior do grão de resina. Neste trabalho mostramos que o aumento de temperatura diminui a quantidade de interações inter/intra-moleculares aumentando a mobilidade da cadeia como um todo. Nos espectros obtidos foi possível verificar a diminuição de uma população mais imóvel com o aumento da temperatura. Desta forma, mostramos que não é somente o aumento da energia cinética a responsável pela melhoria no acoplamento, mas também a desagregação dos peptídeos dentro do grão de resina.

4) Anexo 4: Solvation of Polymers as Model for Solvent Effect Investigation: Proposition of a Novel Polarity Scale. Tetrahedron., v. 58, p. 4383 - 4394, 2002.

Em 1996 publicamos o primeiro artigo relacionando o inchamento de resinas e peptidil-resinas com um parâmetro de polaridade (CILLI et al., 1996) obtido através da soma de dois outros parâmetros: o poder receptor (AN) e o poder doador eletrônico (DN) do solvente, introduzidos por Gutmann e colaboradores (GUTMANN, 1976; MAYER et al., 1975; PARKER et al., 1978). Estes parâmetros representam a eletrofilicidade (AN) e nucleofilicidade (DN) do solvente, e eram empregados até aquele momento separadamente, para explicar várias reações químicas ou interações soluto-solvente. Supondo que as interações entre os peptídeos são fortemente afetadas pelo teor de grupamentos N-H (eletrofílico) e C=O (nucleofílico) das ligações peptídicas, resolvemos na época testar a somatória dos termos AN e DN, na proporção 1:1, como um possível novo parâmetro empírico de polaridade de solventes. Além do enfoque de interesse mais amplo no campo da química em geral, procuramos também encontrar regras mais claras de solvatação de resinas e que, portanto, facilitariam a predição dos melhores solventes a se empregar para a síntese de cada classe de peptidil-resinas.

Este trabalho foi complementado com novos dados, totalizando um total de 11 modelos diferentes, concluindo que a soma dos termos (AN+DN) pode realmente ser considerado um novo parâmetro de polaridade. Destacamos que, nos estudos de solvatação com os modelos estudados, este novo parâmetro mostrou melhor correlação com o inchamento que outros parâmetros largamente utilizados para este fim, como o parâmetro de solubilidade de Hildebrand's - " δ " (HILDEBRAND, 1949). Este parâmetro também permitiu o entendimento do motivo de algumas misturas de solventes não incharem a resina como o esperado, de acordo com os parâmetros de polaridade. Estes solventes, por possuírem valores de AN e DN altos, preferem interagir entre si, à resina. Como exemplo, podemos citar os solventes TFE e DMSO, com alto AN e DN, respectivamente, que na proporção 1:1 causam baixo inchamento na resina.

5) Anexo 5: Determination of site-site distance and site concentration within polymer beads: A combined swelling-electron paramagnetic study. *Journal of Organic Chemistry*, v. 70, p. 4561 - 4568, 2005.

Neste trabalho a microscopia direta foi utilizada na determinação de vários parâmetros relacionados ao grão de resina, sendo estes dados confirmados por RPE. A estratégia utilizada envolveu a medida dos diâmetros dos grãos de resina e peptidil-resinas na forma seca e solvatada, cálculos dos respectivos volumes, números de sítios ativos por grão de resina, distância entre os sítios e concentração destes grupos no interior do grão de resina. A confirmação da coerência dos cálculos foi realizada através da comparação destes valores com o início do aparecimento da interação “spin-spin” descrita na literatura (TRÄUBLE e SACKMANN, 1972) para compostos paramagnéticos através de RPE.

Com a determinação destes parâmetros, estas variáveis foram relacionadas ao teor de acoplamento obtido para o acoplamento de um aminoácido. A análise desta correlação mostrou que, quanto maior a concentração no interior do grão de resina e menor a distância entre os grupos reativos, menor o rendimento de acoplamento.

6) Anexo 6: Study of the effect of the peptide loading and solvent system in SPFS by HRMAS-NMR. *Journal of Peptide Science*, v. 11, p. 556 - 563, 2005.

Através da RPE, como descrito nos trabalhos anteriores, foi possível analisar os efeitos de diferentes meios e condições na mobilidade da extremidade N-terminal da peptidil-resina marcada com o ácido 2,2,6,6-tetrametilpiperidina-1-oxil-4-amino-4-carboxílico (Toac). Como é nesta região que acontece o acoplamento do aminoácido seguinte da sequência que está sendo sintetizada, estes estudos forneceram uma ferramenta importante para a avaliação da extremidade amino-terminal do peptídeo. No entanto, visando avaliar em maiores detalhes a agregação do peptídeo como um todo e não apenas na extremidade N-terminal, resolvemos partir para outras técnicas espectroscópicas de análise. A RMN foi a técnica escolhida. A análise conjunta dos dados obtidos por meio destas duas técnicas poderia permitir o entendimento de como os aminoácidos se agregam no interior do grão de resina, bem como para o desenvolvimento de ferramentas para a diminuição destas interações.

Desta forma utilizamos, novamente, além das resinas comerciais, resinas de partida que contenham elevados teores de sítios reativos. A explicação para o uso deste tipo de resina se baseia na maximização das interações intercadeias dentro do grão, facilitando a determinação das agregações existentes. De uma maneira geral, a restrição do movimento das cadeias peptídicas leva à redução e distorção da intensidade e conseqüente alargamento dos picos obtidos por RMN. O uso da RMN na SPFS trouxe resultados similares aos obtidos utilizando a técnica de RPE. As peptidil-resinas com baixo teor de sítios por grão solvaram melhor em solventes apolares, neste caso o CDCl_3 , e as resinas com alto grau de substituição solvaram melhor em solventes polares como o DMSO. Um resultado interessante foi o obtido para a BAR de 1.6 mmol/g, que mostrou alto grau de mobilidade em DMSO, inclusive superior ao da resina de 3,0 mmol/g, indicando um ponto onde o ganho de solvatação provocado pelo aumento de polaridade da resina chega a um máximo. Este dado talvez indique um grau de substituição limite na qual se deva trabalhar; acima deste valor os números de cadeias no grão estão em uma quantidade que a solvatação eficiente fica inviável.

O uso da técnica de T2 com tempo de relaxação se mostrou eficiente na visualização da população mais imóvel, indicando assim uma nova metodologia no estudo da agregação da SPFS.

As comparações dos dados obtidos por RMN e RPE mostraram correlação, comprovando que a adição da sonda paramagnética TOAC não afeta negativamente o estudo de agregação de peptidil-resinas.

7) Anexo 7: EPR investigation of the influence of side chain protecting groups on peptide-resin solvation of the Asx and Glx model containing peptides. Tetrahedron Letters, v. 48, p. 5521 - 5524, 2007.

Apesar do grande avanço da SPFS e da sedimentação dos dois principais protocolos utilizados nesta metodologia, uma questão ainda permanece: qual dos dois protocolos é o mais adequado? O Boc/Bzl ou o Fmoc/tBu? Desta forma, resolvemos contribuir com esta discussão avaliando a influência dos diferentes aminoácidos e protetores das cadeias laterais dos resíduos tri-funcionais, normalmente utilizados durante a SPFS, na agregação das cadeias peptídicas dentro do grão de resina. Para este intento empregamos a ressonância paramagnética eletrônica (RPE) utilizando como modelos as seqüências XAAAA e XING, onde o grupo X foi o aminoácido em análise na ausência e na presença dos protetores normalmente encontrados na SPFS para os resíduos tri-funcionais. Os resultados obtidos mostraram que a escolha do melhor protocolo para a SPFS pode ser mesmo seqüência dependente. No entanto, seqüências contendo os aminoácidos Asn e Gln protegidos com o grupo Xan (química Boc/Bzl) e na forma isento de protetor se mostraram mais vantajosas para a SPFS por induzir uma menor agregação intercadeias nas resinas de baixo GS. Por outro lado, nas resinas de alto GS, a forma sem protetor foi a mais interessante, ou por apresentar maior mobilidade intercadeias, ou por ter um custo mais reduzido. Para os aminoácidos Asp e Glu o protetor tBu foi o mais adequado por induzir menor agregação no interior do grão. Desta forma, para a síntese de peptídeos contendo estes aminoácidos, o ideal seria utilizar a química Fmoc/tBu com os aminoácidos amidas desprotegidos em sua cadeia lateral.

8) Anexos 8 e 9: 8) Comparative Investigation of the Cleavage Step in the Synthesis of Model Peptide Resins: Implications for N- α -9-fluorenylmethyloxycarbonyl-Solid Phase Peptide Synthesis. Chem. and Pharm. Bull., v. 55, p. 468 - 470, 2007 e 9) Evaluation of the TFMSA/TFA/Thioanisole Cleavage Procedure for Application in Solid-Phase Peptide Synthesis. Chem. Pharm. Bull., v. 49, p. 1089 - 1092, 2001.

Uma outra etapa da síntese estudada foi a da clivagem do peptídeo da resina nos dois protocolos empregados na SPFS. Nestes estudos mostramos que o tipo de resina, bem como os tempos de clivagem devem ser fixados dependendo da resina e do primeiro aminoácido ligado à resina. A escolha errada da resina pode acarretar uma perda razoável do rendimento final da síntese.

4. Atividades de Pesquisa posteriores à obtenção do Título de Doutor no estudo da relação estrutura/função de peptídeos.

Em um outro campo de pesquisa, mas ainda relacionado com a SPFS, participamos de vários estudos relacionando a estrutura com a função de peptídeos.

Atualmente, dezenas de peptídeos estão sendo utilizados como fármacos, outras dezenas mais estão em fase de registro e outras centenas em testes clínicos e pré-clínicos avançados (ALBERICIO, 2004). Os peptídeos sintéticos utilizados para fins terapêuticos movimentam cerca de 13 bilhões de dólares em um mercado que cresce 10% ao ano (VERLANDER, 2002).

Resumidamente, os objetivos destes trabalhos foram determinar os requisitos estruturais essenciais e relacioná-los com a elucidação de seus mecanismos de ação, gerando informações que sirvam de base para a concepção de novas drogas.

1) Anexos 10 a 15: 10) First synthesis of a fully active spin-labeled peptide hormone Febs Lett., v. 446, p. 45 - 48, 1999; 11) Comparative EPR and fluorescent conformational studies of fully active spin labeled melanotropic peptides. Febs Lett., v. 497, p. 103 - 107, 2001; 12) Synthesis and pharmacological properties of TOAC-labeled angiotensin and bradykinin analogs. Peptides, v. 23, p. 65 - 70, 2002; 13) Conformational Studies of Toac-Labeled Bradykinin analogues in model membranes. Lett. Pept. Sci., v. 9, p. 83 - 89, 2002; 14) Monitoring the chemical assembly of a transmembrane bradykinin receptor fragment: correlation between resin solvation, peptide chain mobility and rate of coupling. Europ. J. Org. Chem., v. 21, p. 3686 - 3694, 2002; 15) Conformational basis for the biological activity of toac-labeled angiotensin II and bradykinin. EPR, circular dichroism and fluorescence studies. Biopolymers, v. 74, p. 389 - 402, 2004.

Os primeiros trabalhos deste tópico foram relacionados à síntese de peptídeos contendo o marcador paramagnético TOAC. Nestes trabalhos os peptídeos foram marcados com este marcador paramagnético, visando observar a viabilidade de seu uso em estudos estrutura/função.

Estes estudos permitiram a obtenção de peptídeos ligados a este composto e mantendo em parte a sua atividade biológica, viabilizando a utilização da RPE em estudos estrutura/função. Nestes trabalhos a nossa contribuição foi relacionada à síntese destes compostos e estudos utilizando a RPE. Ressaltamos que, apesar de hoje a síntese destes peptídeos ser largamente descrita, na época, a metodologia teve que ser adequada a este tipo de composto.

2) Anexos 16 e 17: 16) Conformational flexibility of three cytoplasmic segments of the Angiotensin II AT(1a) receptor. *J. Pept. Sci.*, v. 8, p. 23 - 35, 2002; 17) Synthesis and immunological activity of a branched peptide carrying the T-cell epitope of gp43, the major exocellular antigen of *Paracoccidioides brasiliensis*. *Scandinavian Journal of Immunology*, v. 59, p. 58 - 65, 2004.

Visando ainda contribuir para o desenvolvimento científico no Brasil, realizamos colaborações no sentido de sintetizar, purificar e caracterizar peptídeos obtidos através da SPFS para estudos estrutura/função de moléculas peptídicas para outros grupos de pesquisa.

Ressaltamos, no entanto, que a colaboração não se resumiu a simples aplicação da SPFS, mas também passou pela discussão do design das moléculas e dos experimentos a serem realizados.

Na obtenção do peptídeo gp 43, além da síntese de um peptídeo na forma de árvore de lisina, estudos de solvatação também foram realizados. Ressaltamos que este peptídeo de 56 resíduos foi um dos maiores já sintetizados pelo nosso grupo de pesquisa.

3) Anexos 18 e 19: 18) Model peptides mimic the structure and function of the N-terminus of the pore-forming toxin Sticholysin II. Biopolymers, v. 84, p. 169 - 180, 2006; 19) Correlations Between Differences In Amino Terminal Sequences and Different Hemolytic Activity of Sticholysins. Toxicon, 2007 – in press.

Toxinas marinhas têm despertado a atenção de cientistas devido ao envolvimento em intoxicações humanas e impactos sócio-econômicos que tem ocorrido em algumas regiões do globo. A elucidação da estrutura química e de seu mecanismo de ação é muito importante, não somente para o entendimento da base molecular do mecanismo de ação, mas também no desenvolvimento de medidas preventivas, tais como, a detecção, determinação e métodos terapêuticos. A *Stichodactyla helianthus* é uma anêmona relativamente abundante nos mares de Cuba e possui entre seus mecanismos de defesa duas actinoporinas. Duas delas são as STI e a STII, que são polipeptídios básicos, e apresentam pesos moleculares na ordem de 20 KD (GOMEZ et al., 1986). Estas moléculas são polipeptídeos básicos de PI = 9,2 e 9,8, respectivamente, e apresentam pesos moleculares da ordem de 20 kDa (GOMEZ et al., 1986). Ambas apresentam alta atividade hemolítica tendo um mecanismo de lise que passa pela formação de um poro oligomérico toroidal com um raio funcional da ordem de 1 nm originado pela agregação provável de 3 ou 4 monômeros (figura 2); (LOS RIOS et al., 1999; TEJUCA et al., 2001; LOS RIOS et al., 1998). Elas são homólogas em 93% de sua sequência de aminoácidos, sendo observadas três substituições não-conservativas e nove substituições conservativas-semiconservativas. Isto indica que ambas são isoformas da mesma hemolisina (HUERTA et al., 2001; LANIO et al., 2001). No entendimento do mecanismo de ação destas citolisinas, a região com maior potencial de estudo é a amino-terminal. Experimentos de N-truncamento com outra hemolisina relacionada (63% de identidade), Equinatoxina II (Eq II), isolada da anêmona *Actinia equina*, sugerem que esta região fica inserida na membrana lipídica (ANDERLUH et al., 1997), hipótese corroborada pela determinação de sua estrutura tridimensional (MANCHENO et al., 2003). Somado a isto, o fato de ser a região mais variável de citolisinas de anêmonas torna esta região um segmento interessante para o estudo da estrutura/função destas citolisinas (ANDERLUH et al., 1997; ANDERLUH e MACEK, 2002). Ainda nesta extremidade estão situadas boa parte das diferenças entre a St II e a St I. A estas modificações é atribuída a maior atividade hemolítica da St II em relação a St I, medida tanto em termos de saída de potássio interno de eritrócitos quanto da cinética de hemólise (MARTINEZ et al., 2001). Esta diferente capacidade de formação de poros entre as

esticolisinas tem sido explicada pela diferença de cargas na região N-terminal, o que reduziria a velocidade de inserção da St I, diminuindo, assim, sua capacidade hemolítica. A comparação da sequência N-terminal (primeiros 30 resíduos) destas toxinas revela, após o alinhamento, quatro substituições, três delas não-conservativas: ácido glutâmico na posição 2 (St I) por alanina, ácido aspártico na posição 9 por alanina, glicina na posição 23 por ácido glutâmico e uma semi-conservativa: ácido glutâmico na posição 16 por glutamina. Além disso, a St I exibe um resíduo de serina extra localizado como posição N-terminal (HUERTA et al., 2001)

Os resultados obtidos até agora por nosso grupo (anexo 1) e colaboradores mostraram que um peptídeo contendo os 30 aminoácidos desta região apresenta atividade hemolítica, embora muito menor que a da proteína original. No entanto, o poro formado pelo peptídeo apresentou mesmo tamanho que a proteína original, mostrando mesma função. Os dados obtidos também mostraram que a presença dos 10 primeiros resíduos é importante para a sua função, sendo que a ausência deste segmento hidrofóbico diminui a atividade biológica da molécula.

Tendo em vista que as principais alterações que diferenciam as proteínas STI e STII estão na região amino-terminal (últimos 30 resíduos), o anexo 2 teve como um dos objetivos comparar os fragmentos amino-terminais destes polipeptídeos, bem como a importância da polaridade da extremidade amino-terminal.

Para o desenvolvimento deste trabalho foram sintetizados peptídeos carboxiamidas, e correspondente às regiões N-terminais da St I e da St II. O estudo destes peptídeos permitiu avaliar quais das modificações são mais importantes para explicar a diferença de atividade entre a St II e a St I, pois os fragmentos com 20 resíduos possibilitam avaliar as modificações somente dos aminoácidos 15 e 22 da St II e 16 e 23 da St I, enquanto os contendo 30 e 31 resíduos permitiram analisar as demais alterações desta região da proteína. O último peptídeo sintetizado permitiu avaliar a influência da serina na atividade hemolítica da St I. Os peptídeos com 20 resíduos da St I e St II mostraram a mesma atividade hemolítica. Este dado mostra que as alterações Glu por Gly e Gln por Glu não afetam a atividade hemolítica dos mesmos. Já os peptídeos com 30 e 31 resíduos da St II e St I, respectivamente, mostraram diferenças acentuadas em suas atividades hemolíticas, indicando que a diferença de atividade pode estar relacionada à região de 1 a 10 da sequência. O peptídeo da St II foi mais ativo que o da St I. O peptídeo St I 2-31 apresentou atividade hemolítica similar à sequência St I 1-31, indicando que a presença da serina na extremidade N-terminal não é isoladamente um dos fatores que afeta negativamente a formação do poro pelo peptídeo.

4) Anexo 20: Combinatorial synthesis and directed evolution applied to the production of α -helix forming antimicrobial peptides analogues. *Current Protein and Peptide Science*, v. 7, p. 473 - 478, 2006.

Atualmente o uso indiscriminado e não criterioso dos antibióticos contra diversos processos infecciosos tornou-se um grave problema na área da saúde. Este procedimento tem levado a um aumento progressivo no número de cepas com elevado poder patogênico e que adquirem resistência aos antibióticos tradicionais. Assim sendo, há necessidade urgente do desenvolvimento de novas drogas contra esses microorganismos (DONADIO et al., 2002b; DONADIO et al., 2002a). Uma fonte de pesquisa, purificação e caracterização de novas moléculas ativas é a fauna e flora brasileira, pois o Brasil apresenta uma das maiores diversidades do mundo. Neste contexto, um dos campos que mais crescem é a procura e aplicação de peptídeos bioativos, particularmente os antimicrobianos (PAMs), devido ao elevado potencial de suas aplicações terapêuticas (CUDIC e OTVOS, 2002).

As aplicações terapêuticas dos peptídeos bioativos (ANDREU et al., 1998) incluem o tratamento de infecções bacterianas, fúngicas, virais, incluindo o tratamento do câncer. Os peptídeos possuem a vantagem de apresentar baixa ou nenhuma atividade colateral, tornando-se, assim, excelentes candidatos a serem empregados como drogas alternativas aos atuais antibióticos (HANCOCK e CHAPPLE, 1999; HANCOCK e DIAMOND, 2000). Estudos recentes mostraram que a ação dos PAMs não está limitada apenas à destruição de microorganismos invasores. Desta forma, esses peptídeos desempenham uma diversidade de funções no mecanismo de defesa do hospedeiro, aumentando assim a eficácia do sistema imunológico quer seja em vertebrados, invertebrados ou plantas. Dentre as principais ações dos PAMs num processo infeccioso, destacam-se: I) destruição inicial das bactérias invasoras e geração de estímulos antiinflamatórios; II) indução da liberação de histaminas pelos mastócitos, iniciando o processo de vasodilatação; III) neutralização das endotoxinas e inibição de proteases que agem no local da infecção, etc (FÁZIO, 2005; HANCOCK e DIAMOND, 2000).

Neste sentido, existe um grande interesse no entendimento do mecanismo de ação desses peptídeos, uma vez que a elucidação dos mesmos permitiria o desenvolvimento de novos agentes farmacológicos e o aumento de suas eficiências biológicas. Para o entendimento do modo de ação destes peptídeos, modificações estruturais em sua

seqüência primária e estudos conformacionais variando-se o pH, força iônica, além da interação com membranas naturais e artificiais são alguns dos estudos realizados.

Apesar da grande diversidade da Biota brasileira e da importância dos PAMs, poucos são os grupos no Brasil que trabalham nesta área (LEITE et al., 2005; MENDES et al., 2004; MENDES et al., 2005; SFORCA et al., 2005). Desta maneira é importante o estudo dos peptídeos da BIOTA brasileira.

Visando a um melhor entendimento do modo de ação destas substâncias e à busca de moléculas mais ativas que os peptídeos nativos de origem nacional, começamos uma nova linha de pesquisa.

Esta linha de pesquisa é realizada em colaboração com a Profa. Dra. Mariana S. Castro, da UnB. Esta colaboração em seu estágio inicial já rendeu uma publicação que é um pequeno “review” sobre a síntese combinatória e sua aplicação no desenvolvimento de peptídeos antimicrobianos com estrutura em α -hélice.

5. Considerações finais

As atividades relatadas neste texto são indicativos seguros da qualidade dos trabalhos que estamos realizando desde a inserção na carreira científica, mostrando a nossa capacidade de investir na proposição de novos trabalhos e em novas metodologias quando necessárias. Ressaltamos que estas etapas sempre foram acompanhadas por estudantes que, por seus desempenhos e esforços, já mostram frutos em seus desenvolvimentos profissionais e acadêmicos. Enfim, acreditamos que nossos esforços e dedicação poderão ajudar o Brasil a se tornar um centro de excelência em Bioquímica e Biotecnologia.

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Anexo 1

Correlation between the Mobility of Spin-Labeled Peptide Chains and Resin Solvation: An Approach To Optimize the Synthesis of Aggregating Sequences¹

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Resin solvation properties affect the efficiency of the coupling reactions in solid-phase peptide synthesis. Here we report a novel approach to evaluate resin solvation properties, making use of spin label electron paramagnetic resonance (EPR) spectroscopy. The aggregating VVLGAAIV and ING sequences were assembled in benzhydrylamine-resin with different amino group contents (up to 2.6 mmol/g) to examine the extent of chain association within the beads. These model peptidyl-resins were first labeled at their N-terminus with the amino acid spin label 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid (Toac). Their solvation properties in different solvents were estimated, either by bead swelling measurement or by assessing the dynamics of their polymeric matrixes through the analysis of Toac EPR spectra, and were correlated with the yield of the acylation reaction. In most cases the coupling rate was found to depend on bead swelling. Comparatively, the EPR approach was more effective. Line shape analysis allowed the detection of more than one peptide chain population, which influenced the reaction. The results demonstrated the unique potential of EPR spectroscopy not only for improving the yield of peptide synthesis, even in challenging conditions, but also for other relevant polymer-supported methodologies in chemistry and biology.

Introduction

Almost four decades after its introduction, Merrifield's Nobel prize awarded solid-phase peptide synthesis (SPPS) technique² still presents some drawbacks. Among these, one persistent challenge concerns the synthesis of strongly aggregating sequences assembled in highly substituted resins. Despite the economical advantages of this protocol, it enhances chain association inside the resin matrix, substantially impairing coupling reactions during chain

growth, as expected for any solid-supported chemical process. Among several factors, the success of the SPPS is markedly dependent on the degree of solvation of the peptide chain throughout the polymeric matrix.

With the aim of searching for correlations between peptidyl-resin solvation and physicochemical properties of the solvating system, an initial attempt, based on resin swelling measurements in a volumetric flask,³ led to the proposition of a contour solvation plot relating the resin degree of solvation and the two components of Hildebrand's solubility parameters (δ and δ_h).⁴ Alternatively, other investigators used the strategy of microscopic measurement of bead size to estimate resin solvation properties.^{5,6} With the same objective, we evaluated⁷ the swelling of model peptidyl-resins, varying the polarity as well as the amount of the resin-bound sequence in ca. 30 solvent systems encompassing the entire polarity scale. Using this approach, it was possible to verify that each type of peptidyl-resin displayed a specific solvation profile, facilitating the choice of solvent for optimal synthesis conditions. Moreover, since this solvation study was in fact an investigation of solute–solvent interactions, where each peptidyl-resin was a special model of a heterogeneous and complex type of solute, we were able to propose⁷ a novel solvent polarity parameter based on

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(1) Abbreviations for amino acids and the nomenclature of peptide structure follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* 1971, 247, 997). Other abbreviations are as follows: BHAR = benzhydrylamine-resin; Boc = *tert*-butoxycarbonyl; Bzl = benzyl; 2-BrZ = 2-bromobenzyloxycarbonyl; DCM = dichloromethane; DIEA = diisopropylethylamine; DMF = *N,N*-dimethylformamide; DMSO = dimethyl sulfoxide; EPR = electron paramagnetic resonance; EtOH = ethanol; FT-IR = Fourier transform-infrared; HFIP = hexafluoro-2-propanol; HOBt = 1-hydroxybenzotriazole; Fmoc = 9-fluorenylmethyloxycarbonyl, HPLC = high-performance liquid chromatography; MeOH = methanol; NMP = *N*-methylpiperidinone; NMR = nuclear magnetic resonance; PSA = preformed symmetrical anhydride; SPPS = solid-phase peptide synthesis; TBTU = 2-(1-*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEA = triethylamine; TFA = trifluoroacetic acid; TFE = trifluoroethanol; Toac = 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid.

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electron acceptor (AN) and electron donor (DN) properties of the solvent.⁸

To further improve the knowledge of the molecular events taking place as a function of solvation inside the resin beads, spectroscopic techniques can provide more direct information regarding the conformational features of peptide chains inside the polymer matrix. For this purpose a great variety of spectroscopic techniques, such as CD,⁹ FTIR,¹⁰ and NMR,¹¹ have been employed in the peptide synthesis field. In this context, electron paramagnetic resonance (EPR) spectroscopy¹² is clearly the method of choice to obtain relevant information concerning the microenvironment and the dynamics of solvated peptide chains attached to the polymer. To our knowledge, the first attempt to monitor peptide solvation during peptide synthesis was made recently in our laboratory.¹³ In this study, the paramagnetic amino acid derivative 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid (Toac),¹⁴ introduced into the peptide synthesis field either as the *tert*-butyloxycarbonyl (Boc)^{15,16} or as the 9-fluorenylmethyloxycarbonyl (Fmoc)¹⁷ N^α -protected derivative, was used to label model peptidyl-resins. When compared to more flexible paramagnetic compounds,^{18,19} this spin label offers the advantage of binding more rigidly to the system under study as a consequence of its C^α -tetrasubstituted cyclic structure containing both the ligand site and the paramagnetic center. This stereochemistry renders this probe more sensitive to conformational properties of the structure to which it is attached. Owing to these features, its use has been largely expanded, encompassing investigations of peptide conformation in singly and doubly labeled sequences,²⁰ of membrane protein fragments,²¹ and of biologically active peptides.^{22,23}

In the present work we describe, for the first time in the SPPS field, an evaluation of the relationship between EPR spectral parameters and the rate of coupling reactions of model peptidyl-resins labeled with the Toac spin probe. Following preliminary work,¹³ peptides with strong self-association tendency were deliberately chosen since the use of this type of sequence may facilitate the detection in the EPR spectra of relevant conformational features spread throughout the resin matrix. In addition to varying the sequence, the degree of peptide loading was also altered to obtain variable degrees of chain association. The benzhydrylamine-resin (BHAR) introduced for the synthesis of α -carboxamide peptides²⁴ was selected as the solid support for peptide growth, and differently substituted resin batches were synthesized under strictly controlled conditions.²⁵ The use of these BHAR batches allowed the synthesis of peptidyl-resin with peptide content varying from 6% to almost 70% (weight/weight).

Results and Discussion

The well-known aggregating sequences VVLGAAIV and ING, corresponding to the 291–298 segment of the murine H-2K protein²⁶ and to the 72–74 segment of the acyl carrier protein,²⁷ respectively, were synthesized bound to increasingly substituted BHAR (from 0.2 to 2.6 mmol/g) using the Boc/Bzl chemistry. The calculated peptide contents (by amino acid analysis) of the VVLGAAIV–BHAR were 14% and 68% for BHAR batches of 0.2 and 2.6 mmol/g substitution degrees, respectively. For ING–BHAR the peptide contents were 6%, 16%, and 47% when 0.2, 0.6, and 2.6 mmol/g substituted BHAR was used, respectively. All peptidyl-resins were labeled with Fmoc-Toac at their N-terminal portion as previously described.¹³ To avoid spin–spin exchange interactions, which broadens the EPR line shapes,¹² the labeling was kept as low as possible.¹³ Moreover, the low labeling protocol allows the physicochemical and steric perturbations to be kept due to the introduction of the spin probe at a minimum, decreasing their influence on the solvation characteristics of the peptidyl-resin under investigation. Before swelling studies were performed or EPR spectra were obtained, a small portion of each peptide sequence was cleaved off the resin with anhydrous HF^{2a,b} to check if the material was homogeneous. The purity of all cleaved crude peptides, estimated by analytical HPLC, was ca. 90%. Results from amino acid analyses and mass spectra were also consistent with the expected peptide sequences.

Figure 1 displays the EPR spectra of VVLGAAIV–BHAR (14% peptide content) swollen in various solvent systems. As can be seen, chain mobility decreased in the order 10% HFIP/DCM < NMP < 50% TFE/DCM < DCM < DMF < “magic mixture”²⁸ < DMSO, as indicated by the progressive line broadening of the EPR spectra. In the

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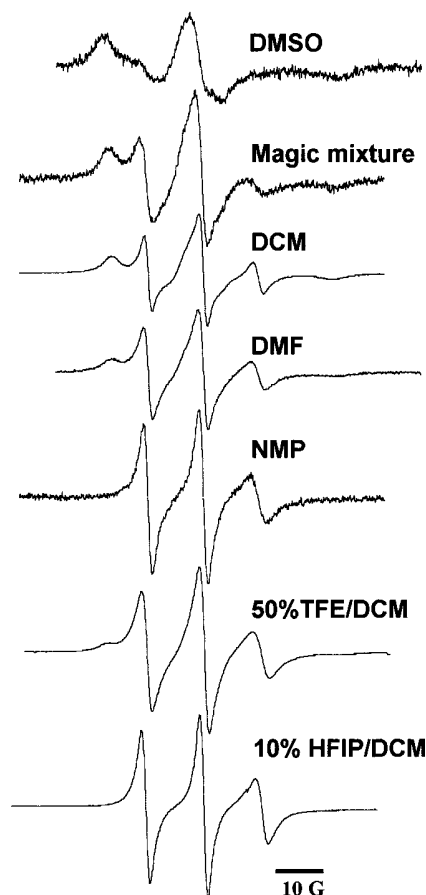


Figure 1. EPR spectra of VVLGAAIV-BHAR (14% peptide content) swollen in different solvents.

poorest solvated conditions (DMSO and the magic mixture), a very broad (powder) spectrum, typical of spin probe immobilization on the EPR time scale, is observed. The spectra of resin solvated in the magic mixture, DMF, DCM, and 50% TFE/DCM display two components, one with broad lines and the other with narrow lines, corresponding to strongly and weakly immobilized spin label populations, respectively. As the solvation increases, both the separation between the outer extreme for the broad line spectra ($A_{\max}^{12}$, Figure 1) and the line width of the narrow line spectra (as monitored by the low- and high-field peaks in Figure 1) decrease, indicating an increasing degree of motion of both populations. In addition, the broad line component progressively disappears, being essentially undetectable in NMP and in 10% HFIP/DCM. The separations between the outer extremes in the spectra of the strongly immobilized population in Figure 1 are (DMSO) 64.0 G, ("magic mixture") 62.1 G, (DMF) 61.9 G, and (DCM) 60.6 G. The center field peaks in the spectra displaying two components contain the contribution of both populations, strongly and weakly immobilized. Nevertheless, it can be seen that, as the proportion of the weakly immobilized population increases, as well as the overall mobility, the line width of the center peak (W_0) decreases. The values of W_0 are given to the right of the spectra. The spectra in Figure 1 show that, although proposed²⁸ as an efficient chain-dissociation solvent system for so-called "difficult sequences", the magic mixture composed of DCM/DMF/NMP (1:1:1), 1% Triton, and 4 N ethylene carbonate was

Table 1. Correlation among Swelling Degree, Peak Line Width of the Central Field Resonance (W_0), and the Coupling Rate^a on VVLGAAIV-BHAR (14% and 68% Peptide Content)

solvent	solvent volume within a bead ^b (%)	W_0 (G)	coupling yield ^c (%)
VVLGAAIV-BHAR (14% Peptide Content)			
DCM	67	2.6	51
DMF	63	3.0	45
DMSO	49	8.9	05
VVLGAAIV-BHAR (68% Peptide Content)			
DCM	39	8.8	06
DMF	34	7.6	02
DMSO	60	6.6	25
10% HFIP/DCM	72	4.1	27

^a Coupling of Boc-Val. ^b [(Swollen volume - dry volume)/swollen volume] $\times$ 100. ^c Coupling yield after 15 min with the PSA method. Concentration of acylating components: 2.5×10^{-3} M (1.5 molar excess over the amine component for a 68% peptide content), 1.0×10^{-3} M (equimolar proportion to the amine component for a 14% peptide content).

not capable of improving the solvation of the hydrophobic sequence used in this study. The results confirm the aggregating tendency of this sequence,²⁶ a higher chain mobility only being attained when the resin is swollen in NMP and in well-known β -sheet structure-disrupting solvent systems, such as mixtures of polyfluorinated alcohols (HFIP and TFE) and DCM.

Table 1 shows the correlation among the rate of the coupling reaction and the peptide-loaded resin degree of swelling and chain mobility estimated by the line width of the EPR central field peak in DCM, DMF, and DMSO. The yield of acylation using Boc-Val was measured because valine corresponds to the subsequent residue of the murine H-2K protein fragment under study.²⁶ It is noteworthy that the higher the swelling of resin beads (percentage of bead volume occupied by the solvent),⁷ the narrower the EPR central field peak line width (W_0) and the faster the coupling reaction (DCM > DMF > DMSO). These data demonstrate that the efficiency of the solid-supported coupling reaction strongly depends on chain mobility, which is, in turn, affected by the swelling degree of the peptidyl-resin.

Table 1 also presents the swelling - W_0 - coupling rate values measured for highly-peptide-loaded VVLGAAIV-BHAR (68% peptide content). The EPR spectra of this peptidyl-resin are given in ref 13. Chain mobility decreased in the order 10% HFIP < DMSO < NMP $\approx$ DMF $\approx$ DCM. When compared to the lowly-peptide-loaded resin, there is a clear inversion of solvation, strong chain immobilization being observed for the highly-peptide-loaded resin in DCM, whereas an increased rate of tumbling is seen in DMSO. These relevant spectral changes can be interpreted in terms of the dominant influence of the apolar polystyrene-1% divinylbenzene BHAR matrix in the lowly-peptide-loaded case, whereas the polar characteristics of the peptide, in particular, the peptide bond, tend to invert this trend as the amount of resin-bound peptide chains increases. The data in Table 1 for the heavily-VVLGAAIV-loaded resin confirm the correlation between the efficiency of the solid-supported coupling reaction and the rate of chain motion as determined by the degree of resin solvation. Much faster coupling was measured in DMSO and 10% HFIP/DCM than in the traditional solvent DCM or DMF. Table 1 shows that the rates of coupling were rather similar in

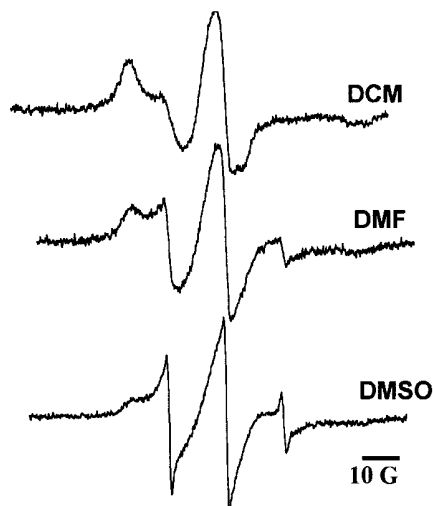


Figure 2. EPR spectra of AAIV-BHAR (53% peptide content) swollen in DCM, DMF, and DMSO.

Table 2. Correlation among Swelling Degree, Peak to Peak Line Width of the Central Field Resonance (W_0), and the Coupling Rate^a on AAIV-BHAR (53% Peptide Content)

solvent	solvent volume within a bead ^b (%)	W_0 (G)	coupling yield ^c (%)
DCM	38	4.6	11
DMF	48	3.2	27
DMSO	72	1.6	49

^a Coupling of Boc-Gly. ^b [(Swollen volume - dry volume)/swollen volume] $\times$ 100. ^c Coupling yield after 15 min with the PSA method. Acylating components in 2.5×10^{-3} M concentration and with 1.5 molar excess over the amine component of the peptidyl-resin.

DMSO and in 10% HFIP/DCM, although the chain mobility was clearly higher in the latter solvent. This result is likely due to partial consumption of the activator induced by the polyfluorinated alcohol during the acylating reaction.

The C-terminal tetrapeptide fragment of the VVL-GAAIV sequence was also assembled in highly substituted BHAR and studied with regard to swelling degree, EPR spectra, and kinetics of coupling. The purpose of this investigation was to verify whether the assembly of a highly aggregating sequence coupled with the use of a highly substituted resin (53% peptide content) can facilitate chain association, usually reported to occur more frequently after coupling the fifth amino acid of the peptide sequence.²⁹ The EPR spectra of the AAIV-resin (Figure 2) indicated significant chain aggregation as evinced by the strongly immobilized spectrum in DCM. Much before coupling the fifth residue, the strong electron donor DMSO promoted higher solvation of the shorter sequence. The results in Table 2 confirm once more the direct relationship between coupling rate and chain solvation/mobility, previously demonstrated for the lowly- and highly-loaded VVLGAAIV-resins.

EPR spectra of the ING-BHAR with peptide contents of 6%, 16%, and 47% swollen in different solvents are shown in Figures 3–5, respectively. Spectral alterations

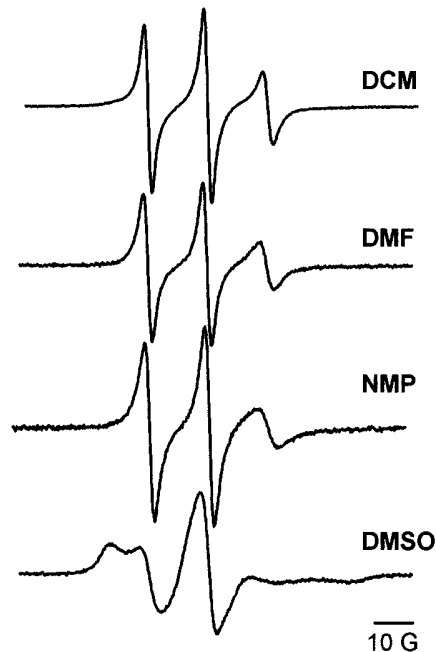


Figure 3. EPR spectra of ING-BHAR (6% peptide content) in DCM, DMF, NMP, and DMSO.

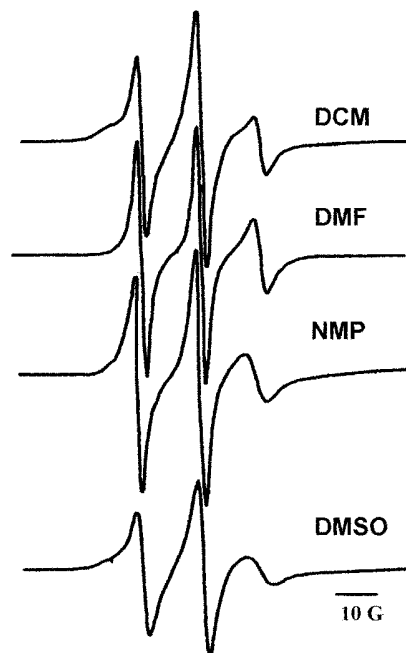


Figure 4. EPR spectra of ING-BHAR (16% peptide content) in DCM, DMF, NMP, and DMSO.

as a function of the amount of resin-bound peptide chains are observed when the polarity of the solvents is taken into account. The proposed (AN + DN) polarity scale⁷ yields values of 21.4, 40.6, 42.6, and 49.1 for DCM, DMF, NMP, and DMSO, respectively. In accordance with this scale, the lowest-peptide-loaded (6%) ING-BHAR exhibited improved solvation and, therefore, chain mobility in DCM due to the dominant influence of the apolar polystyrene structure. In contrast, a powder spectrum was observed when the strong electron donor DMSO was used (Figure 3). This solvation tendency was completely inverted in the case of the highest-peptide-loaded ING-

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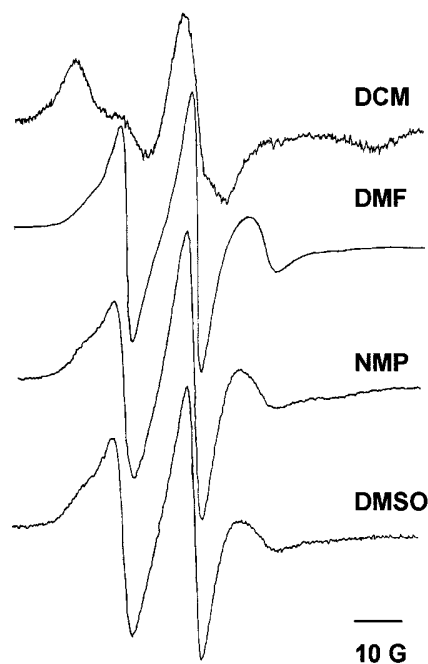


Figure 5. EPR spectra of ING-BHAR (47% peptide content) in DCM, DMF, NMP, and DMSO.

Table 3. Correlation among Swelling Degree, Solvent Viscosity, Peak Line Width of the Central Field Resonance (W_0), and the Coupling Rate Reaction^a on ING-BHAR (6%, 16%, and 47% Peptide Contents)

solvent	solvent volume within a bead ^b (%)	W_0 (G)	viscosity (25 °C) (cP)	yield of coupling ^c (%)
ING-BHAR (6% Peptide Content)				
DCM	77	1.9	0.400	86
DMF	70	2.3	0.796	68
NMP	73	3.0	1.666	45
DMSO	54	4.1	2.000	20
ING-BHAR (16% Peptide Content)				
DCM	76	2.1	0.400	84
DMF	78	2.0	0.796	83
NMP	77	2.6	1.666	74
DMSO	78	2.9	2.000	46
ING-BHAR (47% Peptide Content)				
DCM	31	^d	0.400	01
DMF	81	3.3	0.796	38
NMP	83	3.8	1.666	28
DMSO	81	4.0	2.000	24

^a Coupling of Boc-2BrZ-Tyr. ^b [(Swollen volume - dry volume)/swollen volume] $\times$ 100. ^c Coupling yield after 15 min with the PSA method. Concentration of acylating components: 2.5×10^{-3} M (1.5 molar excess over the amine component for 16% and 47% peptide contents), 1.0×10^{-3} M (equimolar proportion to the amine component for a 6% peptide content). ^d Powder spectra.

resin (Figure 5). Significant chain immobilization was detected for this peptidyl-resin in DCM, whereas the polar DMSO induced the best peptide chain solvation. The 16% peptide content ING-resin presented EPR spectra in the four solvents consistent with its intermediate loading (Figure 4).

Differently from the data for the VVLGAAIV and AAIIV resin-bound sequences, those for the ING-resin (Table 3) reveal the influence of the solvent viscosity upon chain motion (as estimated by W_0 values) and, as a consequence, upon the observed coupling rate. This influence was noticed mainly for ING-BHARs of 16% and 47% peptide content, where equivalent swelling degrees in

some solvents did not correlate with the values of W_0 and coupling rates. These findings indicate that, although some solvents may swell the resin to similar extent, the coupling is slower in the more viscous ones, emphasizing the importance of diffusional effects in polymer-supported reactions.³⁰

It is also worth noting that in some circumstances it was possible to detect the influence of additional factors on the efficiency of the coupling reaction. For instance, the similar coupling rate observed in DCM and DMF for the 16% peptide content ING-BHAR (Table 3) cannot be explained solely by swelling and viscosity effects, as the resin swells equally in both solvents, and the former is less viscous than the latter. The slower coupling in DCM is probably due to the well-known inadequacy of this apolar solvent to dissociate strong peptide chain interactions³¹ resulting from a large amount of interchain hydrogen bondings. This hypothesis is strengthened by a second spectral component due to a more immobilized chain population in the spectra in DCM (Figure 4).

In conclusion, the present work demonstrates the possibility of correlating EPR spectral parameters with factors that govern polymer-supported reactions during peptide synthesis. The acylation rate depends on peptide chain mobility, which is, in turn, influenced by a variety of factors such as the resin swelling degree, solvent viscosity, and solvent ability to disrupt aggregated peptide chains. The data showed that, under most of the conditions studied, aggregating sequences gave rise to composite EPR spectra. Under low loading conditions and in solvents (DMF, Figure 3) that promote disaggregation, only a single-component spectrum was observed. Moreover, we have previously shown³² that Toac-labeled peptide-free BHAR also gives rise to one component EPR spectra, irrespective of the solvent system used.

The present data reveal the unique sensitivity of spin label EPR spectra to monitor polymer-supported processes and point to the great potential of this approach in the expanding area of the use of polymeric materials for a variety of chemical and biological purposes. Finally, in terms of peptide synthesis methodology, the results evince that EPR spectroscopy can be a valuable tool in the selection of the best solvation conditions to overcome extreme difficulties such as the synthesis of strongly aggregating sequences in highly-peptide-loaded solid supports.

Experimental Section

Materials. *N*^α-*tert*-Butyloxycarbonyl (Boc)-Asn, -Ile, -Gly, -Val, -Leu, and -Ala were purchased from Bachem, Torrance, CA. Benzhydrylamine-resins (BHARs) were synthesized as previously reported²⁵ to obtain highly substituted resin batches. Solvents and reagents were purchased from Aldrich or Sigma Chemical Co. DMF was distilled (over P₂O₅ and ninhydrin under reduced pressure) before use. All solvents used for swelling studies were HPLC grade, and all chemicals met ACS standards.

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Peptide Synthesis. The peptides were synthesized manually accordingly to the standard Merrifield Boc/Bzl strategy.^{2a-c} Briefly, the α -amino group deprotection and neutralization steps were performed in 30% TFA/DCM (30 min) and in 10% DIEA/DCM (10 min). The synthesis scale was 0.4 mmol, and all Boc-amino acids were coupled with TBTU³³ in the presence of HOBt and DIEA (at 3-, 3-, and 4-fold excess over the amino component in the resin, respectively), using DMF or 20% DMSO/NMP for ING or VVLGAAIV syntheses. After 2 h, the qualitative ninhydrin test was performed to estimate the completeness of the coupling reaction; the recoupling procedure was done when the ninhydrin test was positive. Cleavage reactions were carried out with the low-high HF procedure.³⁴ The resin was rinsed with ethyl acetate and the peptide extracted in 10% acetic acid aqueous solution and lyophilized. In addition to the expected theoretical yield, the purity of the crude peptides was determined by high-performance liquid chromatography (HPLC). The HPLC conditions were 0.1 M NaH_2PO_4 (pH 7.0, solvent A) and acetonitrile/ H_2O (9:1, v/v, solvent B), linear gradient from 5% to 50% of B in 45 min, flow rate of 1.5 mL/min, and UV detection at 220 nm.

Measurement of Bead Swelling. Before use in peptide synthesis and microscopic measurement of bead size, all the amino-protonated BHAR batches (Cl^- form) were sized by being suspended in DCM and EtOH and being sifted in porous metal sieves to lower the standard deviations of resin diameter to about 4%. Swelling studies of these narrowly sized bead populations were performed and published elsewhere,⁵ with minor modification in some calculated swelling parameters.⁷ Briefly, 150–200 dry and swollen beads of each resin, allowed to solvate overnight, were spread over a microscope slide and measured directly at low magnification. Since the sizes in a sample of beads are not normally but log-normally distributed,

the central value and the distribution of the particle diameters were estimated by the more accurate geometric mean values and geometric standard deviations. All resins were measured with the amino groups in the unprotonated form obtained by 3×5 min TEA/DCM/DMF (1:4.5:4.5, v/v/v) washings followed by 5×2 min DCM/DMF (1:1, v/v) and 5×2 min DCM washings. Resins were dried under vacuum using an Abderralden-type apparatus and refluxed in MeOH.

EPR Studies. EPR measurements were carried out at 9.5 GHz in a Bruker ER 200 SRC spectrometer at room temperature (22 ± 2 °C) using flat quartz cells. Labeled peptidyl-resins were preswollen overnight in the solvent under study before the spectra were run. The magnetic field was modulated with amplitudes less than one-fifth of the line widths, and the microwave power was 5 mW to avoid saturation effects.

Determination of Coupling Yields. A thermostatic reaction vessel (at 25 °C) containing 50–100 μmol of the amino component of the peptidyl-BHAR was submitted to the coupling reaction with the proper Boc-amino acid derivative using the preformed symmetrical anhydride method (PSA),^{2a-c} generated in DCM at 0 °C (60 min). After this time, the reaction was stopped by filtration to remove the dicyclohexylurea precipitate, and the DCM filtrate was evaporated. The symmetrical anhydride generated was dissolved in the solvent under investigation and added to a reaction vessel containing peptidyl-resin preswollen in the same solvent. The coupling yield was monitored by the picric acid method;³⁵ each experiment was performed in duplicate.

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Anexo 2

Article

Importance of the Solvation Degree of Peptide-Resin Beads for Amine Groups Determination by the Picric Acid Method

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O clássico e importante método do ácido pícrico, usado nas áreas de bioquímica e química de polímeros para a quantificação de grupos aminos, foi escolhido neste trabalho como modelo para investigar a importância do grau de solvatação de grãos poliméricos durante o protocolo analítico. Verificou-se que este método, proposto há cerca de três décadas atrás, falha na quantificação de grupos amino de peptidil-resinas contendo sequência agregante e polar. Isto ocorre devido à solvatação insuficiente dos grãos quando apenas o CH_2Cl_2 é utilizado na etapa de ligação do ânion picrato e na subsequente etapa de lavagem. Demonstrou-se que a utilização nestas etapas, de soluções de $\text{CH}_2\text{Cl}_2/\text{DMF}$ (dimetilformamida) e de $\text{CH}_2\text{Cl}_2/\text{EtOH}$ permite uma determinação correta dos grupos amínicos de peptidil-resinas. Além da importância em si para o método da síntese de peptídeos em fase sólida, estes resultados representam também a primeira comprovação experimental da correlação quantitativa existente entre o grau de solvatação e a eficiência de um método analítico efetuado em grãos de resinas.

The classic and important picric acid method used in polymers biochemical and chemical fields of polymers for amine group quantification was chosen in this work as a model for evaluating the influence of the resin bead solvation during an analytical procedure. It was observed that this method, proposed almost three decades ago, failed to quantify amine groups of peptidyl-resin containing aggregating and polar sequence. This was due to inefficient solvation of resin beads when only CH_2Cl_2 was used for picrate anion binding and subsequent washing steps. It was demonstrated that the use of $\text{CH}_2\text{Cl}_2/\text{DMF}$ (dimethylformamide) and $\text{CH}_2\text{Cl}_2/\text{EtOH}$ solutions during these steps allows correct determination of peptidyl-resin amine groups. Besides the importance for the solid phase peptide synthesis methodology itself, these findings also represent the first quantitative demonstration of the relationship between solvation degree and the efficiency of a polymer-supported analytical method.

Keywords: picric acid method, peptide synthesis, polymer, polarity, resin

Introduction

It is well recognized that the success of any polymer-supported process depends upon the solvation degree of its polymeric matrix. This is particularly true for solid phase peptide synthesis^{1,2} where the better the solvation of resin beads, the higher the yield of the synthesis^{3,4}. In an attempt to further investigate this issue, we have proposed rules which might govern solvation of peptidyl-polymers varying the nature and the amount of peptide chains⁵. Based upon the correlation between solvation data of different peptide-resins (taken as solute models) in 28 solvent systems, which encompass entirely the polarity scale, it was also possible to propose, in this report, the (AN+DN) summation term as a novel polarity parameter. The AN and DN values⁶ represent the electron acceptor and donor properties of the solvent,

respectively and this alternative polarity term will be further used in the present report.

Spectroscopic methods have also been used for solvation studies including Fourier-transform infra-red⁷, nuclear magnetic⁸ and electron spin^{9,10} resonance methods for assessment of resin-bound peptide chains motions, using in the latter, a labeling strategy with a special paramagnetic amino acid-type probe¹¹. This effort in improving the polymer solvation knowledge has also recently led us to demonstrate that benzhydrylamine-resin (BHAR)¹², a phenylmethylamine group-containing copolymer of styrene-1%-divinylbenzene, so far used as the solid support for peptide synthesis, can be alternatively employed as a novel anion exchange resin for liquid chromatography^{13,14}.

Hence, aiming to better evaluate the importance of polymer solvation characteristics, the present work describes, for the first time, the quantitative influence of the bead solvation upon the effectiveness of a resin-supported

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analytical method. Because it is considered simple, non-destructive and very accurate, the picric acid method^{15,16} was chosen for this investigation. Besides its application for quantification of resin-bound amine groups in the polymer field, this analytical procedure, similarly to the ninhydrin procedure¹⁷, is also very useful for monitoring the critical amino acid coupling or peptide α -amine group deprotection reactions during the stepwise solid phase peptide synthesis cycles^{1,2}. The picric acid method is based on the 1:1 picrate-ammonium salt formation in the resin, after treatment with the picric acid. After exhaustive washing, the resin-bound picrate anion is usually eluted with 10% triethylamine (TEA)/CH₂Cl₂ and this picrate salt is spectrophotometrically measured at 358 nm using a molar absorptivity of 14,500 dm³ mol⁻¹ cm⁻¹ after dilution of the eluent with EtOH (more than 90%, v/v)¹⁵. As only the apolar CH₂Cl₂ is used in most steps of the original picric acid method, we envisioned that a peptidyl-resin which does not swell appropriately in CH₂Cl₂ might be taken as model for demonstrating the exact contribution of the bead solvation degree on the efficacy of solid-supported analytical method.

Experimental

Materials

All amino acids, except Gly, are of the L-configuration. N- α -tert-butyloxycarbonyl (Boc)-Asn, and -Ile were purchased from Bachem, Torrance, CA. The highly substituted 2.6 mmol g⁻¹ BHAR batch was synthesized according to previous report¹⁸. Solvents and reagents were purchased from Aldrich or Sigma Co. Triethylamine (TEA) and diisopropylethylamine were distilled over ninhydrin and dimethylformamide (DMF), over P₂O₅ and ninhydrin, under reduced pressure. All solvents used for swelling studies were HPLC grade.

Peptide synthesis

The peptides were synthesized manually accordingly to the standard Merrifield Boc/Bz strategy¹. Briefly, the α -amino group deprotection and neutralization steps were performed in trifluoroacetic acid (TFA), 30 % (v/v) in CH₂Cl₂ and TEA, 10 % (v/v) in CH₂Cl₂, respectively. The scale of synthesis was 0.2 mmol and all Boc-amino acids were coupled in DMF with benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) in the presence of N-hydroxybenzotriazole (HOBt) and diisopropylethylamine (with a 4- and 5- fold excess over the amino component in the resin, respectively). Boc-Asn was coupled in CH₂Cl₂/DMF (1:1) using diisopropylcarbodiimide and HOBt as acylating reagents (3-fold

excess). After double coupling with a two-hour reaction time each, the qualitative ninhydrin test¹⁹ was carried out to estimate the completeness of the reaction.

Swelling measurement of beads

Before use in peptidyl-resin synthesis and microscopic measurement of bead sizes, the amino protonated BHAR batches (Cl⁻ form) were exhaustively sized by the suspension in CH₂Cl₂, EtOH and sifted in pore metal sieves to lower the standard deviations of resin diameters to about 4 %¹⁸. From 150 to 200 dry and swollen beads of each resin, allowed to equilibrate overnight, were spread over a microscope slide and measured directly at low magnification. Since the sizes in a sample of beads are not normally but log-normally distributed, the central value and the distribution of the particle diameters were estimated by the more accurate geometric mean values and geometric standard deviations.²⁰ Resins were dried *in vacuo* using an Abderhalden-type apparatus with MeOH refluxing.

Results and Discussion

The ING [(72-74)-acyl carrier protein] fragment, known to be aggregating²¹ and also very polar according to a previous hydrophobic scale²² was selected as the model of peptidyl-resin which does not present good solvation in CH₂Cl₂ if synthesized in highly substituted resins (2.6 mmol g⁻¹ BHAR). Earlier results^{5,10} with this peptidyl-resin have shown that when its amine group extremity is in unprotonated form (after alkaline treatment), only 31% of its total swollen bead volume is occupied by CH₂Cl₂ against 83% measured in the polar aprotic DMF.

Table 1 summarizes the results of four experimental variations applied for the picric acid method protocol, each one carried out with the same amount of the ING-BHAR containing a total of 0.363 mmol of amine groups, previously determined with amino acid analysis. Protocol 1 shows the result obtained with the original picrate method where CH₂Cl₂ was used as the solvent for all steps of the procedure. The final amount of measured amine groups (0.108 mmol) was significantly lower than expected, thus suggesting that the failure in this standard protocol might be due to an incomplete solvation of the peptidyl-resin in some steps of the method.

To help interpreting this result, the Table 2 displays for comparison, the complete microscopic swelling data of ING-BHAR containing its amine groups in unprotonated and protonated (picrate salt) forms. The polarity values of CH₂Cl₂, its 1:1 mixture with DMF and EtOH, estimated by the (AN+DN) scale⁵ are also included in this table. The calculated percentage of total bead volume occupied by

CH_2Cl_2 decreased appreciably from 31% to only 6% when its amine groups are transformed from unprotonated to protonated form, thus indicating that the lower amount of amine group measured with protocol 1 may be at least in part, due to the strong shrinking of peptidyl-resin beads during the picrate binding step carried out in CH_2Cl_2 .

Noticeably, the result obtained with protocol 2 in Table 1 seems to confirm this hypothesis as the simple CH_2Cl_2 substitution with 1:1 CH_2Cl_2 /DMF increased the amount of measured picrate groups removed from the resin. The resin swelling in this more polar mixed solvent, characterized by (AN+DN) value of 32 against 21.4 for CH_2Cl_2 , increased from 6% to 73%, as shown in Table 2. However, despite of the improvement in this second protocol, the amount of measured ammonium groups (0.392 mmol) was now slightly higher than the correct value (0.363 mmol). One explanation for this result might be due to the low solvation of the picrate-resin component but now at the washing step made solely in CH_2Cl_2 , just after binding of the chromophore. This unfavorable swelling condition may induce incomplete release of excess unbounded picrate anion dispersed throughout the peptide-resin matrix, thus increasing slightly the measured ammonium group content of the experiment in the protocol 2.

In the light of this hypothesis, replacement of CH_2Cl_2 for 1:1 CH_2Cl_2 /EtOH during the washing step, following the picrate binding on the resin was tested, protocol 3. The enhanced solvation of the picrate-resin in this mixed solvent (42% against 6% in CH_2Cl_2 , Table 2), due to its higher polarity [(AN+DN) value of 45.3, Table 2], seemed to ensure the removal of the excess picric acid from the resin, allowing the correct ammonium group quantification with this protocol (0.363 mmol g^{-1}). The final solvation test (protocol 4) was carried out in order to check the need of CH_2Cl_2 replacement for CH_2Cl_2 /DMF mixture during the amine group TEA-neutralization process in the first step of the picric acid method. The reason was the lower swelling of this model peptidyl-resin in TEA solution in CH_2Cl_2 rather than in DMF (52% against 80% of bead swelling) measured previously⁵. However, no difference in the amine group quantification between these last two protocols (3 and 4) was observed, thus stressing that in this case, the small difference in swelling mentioned between these two solvent systems during the TEA treatment was not sufficient to affect the final result.

The validity of the use of the (AN+DN) term as a novel solvent polarity scale has been previously demonstrated by investigating the interaction (solvation) of different peptidyl-

Table 1. Comparative picric acid monitoring of ING-BHAR (2.6 mmol g^{-1}) with different solvating protocols.

Step	Protocol			
	1	2	3	4
Deprotonation	2 x 5 min TEA/ CH_2Cl_2	2 x 5 min TEA/ CH_2Cl_2	2 x 5 min TEA/ CH_2Cl_2	2 x 5 min TEA/DMF
Washing	6 x 2 min CH_2Cl_2	6 x 2 min CH_2Cl_2	6 x 2 min CH_2Cl_2	6 x 2 min CH_2Cl_2
Picrate binding	3 x 5 min 0.08 mol L^{-1} picric acid in CH_2Cl_2	3 x 5 min 0.08 mol L^{-1} picric acid in CH_2Cl_2 /DMF	3 x 5 min 0.08 mol L^{-1} picric acid in CH_2Cl_2 /DMF	3 x 5 min 0.08 mol L^{-1} picric acid in CH_2Cl_2 /DMF
Washing	10 x 2 min CH_2Cl_2	10 x 2 min CH_2Cl_2	10 x 2 min CH_2Cl_2 /EtOH	10 x 2 min CH_2Cl_2 /EtOH
Elution of the bound picrate	2 x 10 min TEA/ CH_2Cl_2	2 x 10 min TEA/ CH_2Cl_2	2 x 10 min TEA/ CH_2Cl_2	2 x 10 min TEA/ CH_2Cl_2
Washing	2 x 2 min CH_2Cl_2	2 x 2 min CH_2Cl_2	2 x 2 min CH_2Cl_2	2 x 2 min CH_2Cl_2
Amine group (mmol)	0.108	0.392	0.363	0.362

Table 2. Swelling degree of ING-BHAR (2.6 mmol g^{-1}) on unprotonated and protonated (picrate salt) forms.

Solvent	CH_2Cl_2			CH_2Cl_2 /DMF		CH_2Cl_2 /EtOH	
Polarity (AN+DN)	21.4			32.0		45.3	
Amine group	Diam. dry bead (μm)	Diam. Swollen bead (μm)	Vol. Solvent within bead (%) ^a	Diam. swollen bead (μm)	Vol. solvent within bead (%) ^a	Diam. swollen bead (μm)	Vol. solvent within bead (%) ^a
NH_2	79	89	31	131	78	100	52
NH_3^+ Picrate ⁻	88	90	06	135	73	94	42

^a [(swollen volume - dry volume)/swollen volume] x 100.

resins, taken deliberately as models of complex solutes, with several solvent systems.⁵ Due to its amphoteric character given by the summation of Lewis acid and Lewis base in a single term, this polarity parameter showed better correlation with the solvation degree of all peptidyl-resin assayed than other solvent properties known so far as the dielectric constant, the Dimroth-Reichardt $E_T(30)$ ²³ and AN and DN, isolated. Although not shown in that work, several other relevant solvent empirical properties such as Hildebrand's δ solubility parameter²⁴, Kamlet-Taft's π^* ²⁵ parameter and the Swain's²⁶ acidity (α) and basicity (β) scales that are equivalent to the AN and DN numbers, respectively, were also considered but none of them presented improved correlation with solvation data of peptidyl-resins, if compared with the proposed (AN+DN) number. We are currently examining other types of solvent-solute interactions using this amphoteric solvent parameter and the present work represents an example of its application as a polarity parameter for helping evaluate failures on the picric acid procedure.

To our knowledge, the present study represents the first quantitative solvation approach applied to a resin-supported analytical technique. The results confirmed the critical role of the solvation property of the polymeric matrix in an important procedure used in biochemistry and polymer chemistry areas. We demonstrated that, due to the poor solvation effect associated with conformational restraints of the resin-bound peptide chains, the original picric acid method, introduced almost three decades ago, is not capable of measuring correctly the amine group content of some peptide-loaded resins. It is demonstrated in the present work that this limitation is due basically to strong shrinking of beads in apolar condition which is often observed^{3,5} with resins containing strong aggregating and/or polar sequences. Moreover, in contrast to the belief that the main problem in this method might be related to insufficient removal of excess picric acid used¹⁶, the present report emphasizes instead that efficient resin solvation at the picrate-binding step is essential for the accuracy of this analytical method, considered superior to the ninhydrin procedure.²⁷

In summary, the present findings exposed relevant shortcomings in this well-known resin-supported analytical method. Care should be therefore geared towards any procedures or reactions carried out throughout resin matrix including for instance, not only the synthesis of other macromolecules such as oligonucleotides²⁸, but those involved in combinatorial chemistry²⁹ and solid-phase organic synthesis³⁰ strategies, intensively applied as examples of modern polymer application in chemical and biological fields.

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Anexo 3



Effect of temperature on peptide chain aggregation: an EPR study of model peptidyl-resins

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Abstract—The effect of temperature on the dynamics of peptide chains inside resin beads was monitored by electron paramagnetic resonance (EPR) spectroscopy. A two-component spectra was obtained for low and highly peptide-loaded model peptidyl-resins labeled with the paramagnetic amino acid 2,2,6,6-tetramethyl-piperidine-*N*-oxyl-4-amino-4-carboxylic acid (TOAC), indicating the presence of strongly and weakly immobilized populations. Increasing levels of chain disaggregation were observed with increasing temperature, leading in some cases to a complete disappearance of the more immobilized population. The present findings demonstrate that EPR spectral parameters are highly sensitive to the solvation properties of labeled sites inside the resin matrix and can be of great value for the understanding of polymer-supported processes or reactions. © 2001 Elsevier Science Ltd. All rights reserved.

Merrifield's solid-phase peptide synthesis method¹ has been intensively investigated as a convenient model to improve the knowledge of the physicochemical basis of processes occurring throughout a polymeric matrix. In this context, emphasis has been recently given to resin-supported combinatorial chemistry for the development of new drugs.^{2,3} Despite all these continuous efforts to optimize the peptide synthesis protocol, serious short-

comings have intriguingly persisted. They are mainly related to incomplete coupling during peptide growth caused by chain aggregation inside the bead.⁴ To overcome this problem alternative solid supports have been continuously proposed for peptide synthesis.^{5,6} In addition, attempts to establish the rules which govern resin solvation have been the object of several publications, as this property has been found to depend upon the

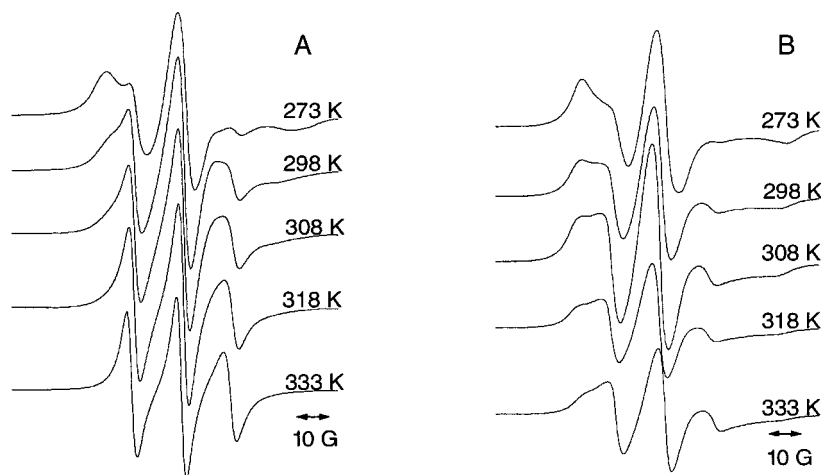


Figure 1. Effect of temperature upon the EPR spectra of TOAC-labeled low (A) and highly loaded (B) VQAAIDYING-BHAR in DMF.

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resin, peptide sequence and loading, as well as the solvent system.^{7,8} We and other groups have applied EPR spectroscopy to investigate the solvation properties of peptidyl-resins.^{9–11} The goal of the present report is to extend this strategy to examine the effect of temperature on the dynamics of model peptidyl-resin beads and its possible implications in the peptide synthesis methodology.

The VQAAIDYING (65–74) fragment of the acyl carrier protein¹² and the (CRF^{34–41}) corticotrophin releasing factor antagonist [NRKL(Nleu)EII]¹³ were synthesized through the conventional Boc/Bzl-solid-phase method¹⁴ using benzhydryl- and methylbenzhydrylamine-resins (BHAR and MBHAR, respectively), in low and highly substituted conditions (up to 3.0 mmol/g). The latter conditions were designed to promote chain association inside the resin beads. For EPR studies, similarly to the labeling strategy used for free peptides, the peptidyl-resins were labeled with the *N*-protected^{15,16} paramagnetic amino acid 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid (TOAC). In order to avoid spin–spin exchange interactions, which may broaden the EPR lines and to minimize possible physicochemical and steric perturbations, the extent of labeling was kept as low as possible. It

was also assumed that the TOAC-labeled peptide chains are dispersed homogeneously throughout the resin matrix and behave similarly to the unlabeled chains in all solvent systems tested. Samples were placed in flat quartz cells and EPR measurements were carried out at 9.5 GHz in a Bruker ER 200 spectrometer using a variable-temperature accessory. The temperature was monitored making use of a thermocouple. Labeled peptidyl-resin beads were pre-swollen in the solvent under study for 24 h. The samples were equilibrated at the desired temperature for approximately 5 min before running the spectra. The magnetic field was modulated with amplitudes less than one-fifth of the linewidths, and the microwave power was 5 mW to avoid saturation effects.

Fig. 1 displays the EPR spectra of low and highly peptide-loaded (30 and 83% peptide content, respectively) VQAAIDYING-BHAR swollen in dimethylformamide (DMF) in the 273–333 K temperature range. In most spectra two components are present: a broad one and a narrow one, due to strongly and weakly immobilized populations, respectively. As expected, the motion increased with increasing temperature, as indicated by the narrowing of the spectral lines correspond-

Table 1. Effect of temperature on the EPR spectra of low and highly loaded TOAC-labeled VQAAIDYING-BHAR swollen in DMF

Temperature (K)	BHAR (degree of substitution)					
	0.3 mmol/g			3.0 mmol/g		
	W_0 (G)	h_{-1}/h_0	$A_{\max}$ (G) ^a	W_0 (G)	h_{-1}/h_0	$A_{\max}$ (G) ^a
273	5.50	0.008	57.0	6.98	0.026	63.4
298	4.17	0.184	Nd	5.50	0.063	63.3
308	3.57	0.258	Nd	5.10	0.092	61.0
318	3.27	0.341	Nd	4.76	0.123	60.0
333	3.00	0.484	Nd	4.20	0.165	Nd

^a Nd, not determined.

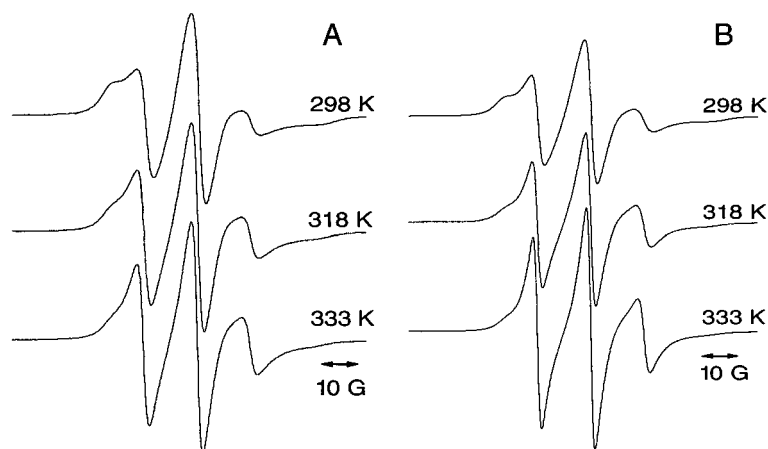


Figure 2. Effect of temperature on the EPR spectra of TOAC-labeled highly loaded NRKL(Nleu)EII-MBHAR in DMF (A) and in DMSO (B).

Table 2. Effect of temperature on the EPR spectra of highly loaded TOAC-labeled NRKL(Nleu)EII-MBHAR swollen in DMF and DMSO.

Temperature (K)	Solvent					
	DMF			DMSO		
	W_0 (G)	h_{-1}/h_0	$A_{\max}$ (G) ^a	W_0 (G)	h_{-1}/h_0	$A_{\max}$ (G) ^a
298	4.21	0.13	58.0	3.28	0.16	Nd
318	3.72	0.20	Nd	2.91	0.24	Nd
333	3.42	0.25	Nd	2.49	0.30	Nd

^a Nd, not determined.

ing to both populations. A clear difference in the solvation properties of the two resins is observed with increasing temperature. It can be noted that the heavily peptide-loaded resin presents a larger contribution of the more immobilized population (Fig. 1B), very likely as a consequence of a higher degree of chain association. In contrast, a progressive disappearance of the more immobilized component was observed with the low peptide-loaded resin (Fig. 1A). This component became essentially undetectable at 333 K. Table 1 presents the values of some EPR spectral parameters whose values can be correlated with the dynamics of labeled sites in the resin. We measured the central field peak linewidth (W_0), that contains the contribution of both populations, the ratio of heights of the high and mid-field lines (h_{-1}/h_0), where h_{-1} corresponds essentially to the weakly immobilized component and the separation between the outer extrema ($A_{\max}$), that corresponds essentially to the more immobilized component. The lower the values of W_0 and $A_{\max}$ and the higher the h_{-1}/h_0 ratio, the faster the motion of the labeled sites. Accordingly, a significant decrease in W_0 and $A_{\max}$ and an increase in h_{-1}/h_0 are observed as the temperature increases from 273 to 333 K. Table 1 also corroborates the differences between the low and highly peptide-loaded resins, emphasizing the challenge involved in synthesizing peptides in heavily chain loaded conditions.

Fig. 2 shows the EPR spectra of highly loaded NRKL(Nleu)EII-MBHAR (78% peptide content) swollen in DMF and in dimethylsulfoxide (DMSO) at different temperatures. A small, yet significant increase in chain mobility occurs in the latter solvent, as indicated by the greater contribution of the more mobile component in Fig. 2B and by EPR parameters shown in Table 2.

These results indicate that the latter solvent is more appropriate for the synthesis of this sequence, in heavily loaded conditions. Furthermore, the larger W_0 values at higher loading in the spectra of VQAAIDYING (Table 1) when compared to NRKL(Nleu)EII (Table 1), regardless of temperature, suggest that the former sequence is characterized by a more pronounced tendency to self-associate.

Taken together, the present findings point to the usefulness of increasing the temperature to improve the peptide synthesis yield, as already discussed.¹⁷ However,

care should be taken, since this effect seems to depend on several parameters, including the sequence to be assembled.^{18,19} Nevertheless, the evaluation of the effect of temperature on the dynamics of peptidyl-resins demonstrates that EPR can be used as a very sensitive tool to discriminate the structural characteristics of each specific sequence even inside very complex and heterogeneous media. Moreover, due to the spin probe location in the peptide sequence, the present approach is unique for the detection of different interchain association levels at the N-terminal region, where the extent of steric hindrance is critical for the success of the synthesis.

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Anexo 4

Solvation of polymers as model for solvent effect investigation: proposition of a novel polarity scale[☆]

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Abstract—A precise understanding of the polymer solvation effect has been considered crucial to many modern methods, but its dependence on the polarity of the medium is still not entirely established. To more thoroughly address this issue, the swelling degrees of polymers with a great variety of structures, taken as solute-models, were measured and correlated with the polarity of ca. 30 solvent systems. Relevant for any resin-supported methods, a characteristic solvation behavior of each class of polymeric material was detected. Moreover by interpreting the relationship between the large set of solute–solvent interaction data and the most solvent properties known so far, the sum of solvent electron acceptor (AN) and donor (DN) numbers, at a 1:1 proportion was suggested as an alternative and more accurate empirical solvent polarity scale. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

Although initial reports dealing with the influence of solvent in a determined interaction with solute molecules have been documented since the XIX century,¹ the acceptance of a single solvent polarity scale as the most appropriate for interpreting any solvent effect has not been achieved yet. Although the exact definition of solvent polarity is still elusive, it seems reasonable to consider that this property is related to the overall solvation capability of solvent, encompassing all possible nonspecific and specific intermolecular interactions with solute ions or molecules.² For decades, a great amount of experiments has allowed the proposition of some empirical polarity scales, most of them derived from experiments where single solute-

models are used to probe (spectrophotometrically, thermodynamically, kinetically, etc) the interaction with solvents of different polarities. A collection of excellent reviews is available regarding the solvent effect and polarity issues.^{2,3}

Differing conceptually from all those previous experiments, we have initiated⁴ an alternative solvent effect investigation, studying not only a single solute-molecule but instead, a set of cross-linked polymers with a great variety of characteristics. In this approach the solvation properties of these polymers, estimated by the swelling measurements of beads in solvents with different polarities, are correlated with various existing solvent polarity scales. Experimentally, this relationship is examined in a contour solvation curve where swelling degree versus solvent polarity values are plotted. The most accurate polarity parameter will be that one which reveals the best fit (less dispersion) in this curve regardless of the type of resin.

The swelling degrees of some model resins attaching peptide sequences in ca. 30 solvent systems which encompass broadly the polarity scale have been previously determined⁴ through microscopic measurement of dry and swollen bead sizes. As the solvation of peptide-resin might be influenced by the electrophilic and nucleophilic moieties of a peptide bond, namely N–H and C=O groups, respectively, we also attempted in that study, the correlation of peptide-resin solvation with the simple sum of both Gutmann's acidic, electrophilic (AN) and basic, nucleophilic (DN) numbers⁵ of each solvent. The AN number represents the dimensionless number expressing the acceptor property of a given solvent and is based on the solvent-dependent ³¹P NMR chemical shifts of

[☆] See ref. 44.

Keywords: polymer; resin; polarity; peptide; solvent.

Abbreviations: BHAR, benzhydrylamine-resin; Boc, *tert*-butoxycarbonyl; Bu, *tert*-butyl; Bzl, benzyl; CD, circular dichroism; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, *N,N'*-dimethylformamide; DMSO, dimethylsulfoxide; EPR, electron paramagnetic resonance; EtOH, ethanol; HOBt, 1-hydroxybenzotriazole; Fmoc, 9-fluorenylmethyloxycarbonyl; HPLC, high-performance liquid chromatography; IR, infrared; MeOH, methanol; NMP, *N*-methylpiperidinone; NMR, nuclear magnetic resonance; PEG, poly(ethylene glycol); PS–DVB, copolymer of styrene and divinylbenzene; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEA, triethylamine; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TOAC, 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid.

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Table 1. Solvent parameters^{2,5,10,23}

Entry	Solvent	(AN+DN)	ε	$E_T(30)$ (kcal/mol) ^w	δ (cal/mL) ^{1/2}	π^*	α	β	$(\alpha+\beta)$	$(\pi^*+\alpha+\beta)$
1	Toluene	3.4	2.4	33.0	8.90	0.54	0.00	0.11	0.11	0.65
2	DCM	21.4	8.9	40.7	9.70	0.82	0.13	0.10	0.23	1.05
3	Chloroform	27.1	4.7	39.1	9.30	0.58	0.20	0.10	0.30	0.88
4	NMP	40.6	33.0	42.2	11.30	0.92	0.00	0.77	0.77	1.69
5	DMF	42.6	36.7	43.8	12.10	0.88	0.00	0.69	0.69	1.57
6	DMSO	49.1	46.7	45.1	12.00	1.00	0.00	0.76	0.76	1.76
7	TFE	53.5	26.7	54.1	11.90	0.73	1.51	0.00	1.51	2.24
8	EtOH	69.1	24.3	51.9	12.70	0.54	0.86	0.75	1.61	2.15
9	MeOH	71.3	32.6	55.4	14.50	0.60	0.98	0.66	1.64	2.24
10	Formamide	63.8	109.5	55.8	19.20	0.97	0.71	0.48	1.19	2.16
11	50% TFE/Toluene	28.5	14.6	43.6	10.40	0.63	0.78	0.06	0.84	1.47
12	20% TFE/DCM	27.5	12.5	43.4	10.10	0.80	0.41	0.08	0.49	1.29
13	50% TFE/DCM	37.5	17.8	47.4	10.80	0.78	0.82	0.05	0.87	1.65
14	80% TFE/DCM	47.4	23.1	51.4	11.50	0.75	1.23	0.02	1.25	2.00
15	20% DMSO/NMP	42.3	35.7	42.8	11.40	0.94	0.00	0.77	0.77	1.71
16	50% DMSO/THF	38.6	27.1	41.3	10.60	0.79	0.00	0.66	0.66	1.45
17	65% NMP/THF	36.1	24.1	40.5	10.50	0.80	0.00	0.69	0.69	1.49
18	50% DCM/DMF	32.0	22.8	42.3	10.90	0.85	0.07	0.40	0.47	1.32
19	50% DCM/DMSO	35.3	27.8	42.9	10.90	0.91	0.07	0.43	0.50	1.41
20	50% MeOH/DMSO	60.2	39.7	50.3	13.30	0.80	0.49	0.71	1.20	2.00
21	50% TFE/DMF	48.1	31.7	49.0	12.00	0.80	0.76	0.35	1.11	1.91
22	50% TFE/DMSO	51.3	36.7	49.6	12.00	0.87	0.76	0.38	1.14	2.01
23	10% TEA ^a /DCM	25.1	8.3	39.8	9.20	0.75	0.12	0.16	0.28	1.03
24	10% TEA ^a /DMF	44.5	33.3	42.6	11.20	0.80	0.00	0.69	0.69	1.49
25	10% TEA ^a /DMSO	50.4	42.3	43.8	11.10	0.91	0.00	0.76	0.76	1.67
26	20% PIP ^a /DCM	25.1	8.3	39.7	10.00	0.72	0.10	0.29	0.39	1.11
27	20% PIP ^a /DMF	42.1	30.5	42.1	11.90	0.76	0.00	0.76	0.76	1.52
28	20% PIP ^a /DMSO	47.2	38.5	43.2	11.80	0.88	0.00	0.82	0.82	1.70

^a See Table 2 for values of TEA and PIP solvent parameters.

triethylphosphine oxide. Conversely, DN represents the solvent electron donor character and is correlated with the molar enthalpy value for the reaction of the donor solvent with SbCl₅ as a reference solute acceptor.

The initial results with the tested amphoteric (AN+DN) solvent term were promising, as it showed better correlation with the swelling degree of peptide-resins than the dielectric constant (ε) or even the Dimroth–Reichardt's $E_T(30)$ polarity parameter⁶ which is one of the most widely accepted in chemistry. This latter solvent scale is based on the measured transition energy (kcal/mol) for the longest wavelength of the absorption band of the model solute pyridinium *N*-phenoxide betaine dye.

As a function of these preliminarily findings, the main focus of the present study is on the development of a more complete investigation regarding the suitability of the (AN+DN) parameter as an alternative and more accurate polarity scale. Thus, a great amount of polarity parameters and also of model polymeric materials was carefully selected to further develop the present correlation approach. In regard to the examined solvent properties, besides the ε and $E_T(30)$ scales, Hildebrand's solubility parameter δ ,⁷ which has been to date the parameter of choice to correlate with the polymer solvation,⁸ was also investigated. It represents the measure of the (cohesive) energy required to separate solvent molecules from one another as a consequence of the need for accommodating solute molecules.

In addition to these examples of 'one-parameter' scales, (ε , $E_T(30)$ and δ), there are still others which suggest the

combination of two or more solvent properties simultaneously to better unravel solvent effect in a generic sense. The introduced (AN+DN) term is therefore representative of the so-called 'two-parameter' theory.⁹ In analogy, we also included in the present report the classical Kamlet–Taft's α , β and π^* parameters.¹⁰ The first two terms are related to the electrophilic (hydrogen donor) and nucleophilic (hydrogen acceptor) properties of the solvent and are obtained from studies of a set of solute–solvent interactions.^{2,10} Their values correspond to the average energy of the longest wavelength absorption peaks of the solute in the solvent molecules and transformed into a dimensionless scale which ranges from 0 to 1. Otherwise, the π^* parameter was proposed by the same authors suggesting that it may reflect the polarity/polarizability properties of the solvent.

Thus, paralleling the (AN+DN) scale to be evaluated, the sum of the α and β properties was also tested including or not the π^* parameter. Therefore the $(\alpha+\beta)$ and the $(\pi^*+\alpha+\beta)$ additive terms constitute together with ε , $E_T(30)$ and δ terms, the set of solvent properties to be compared with the (AN+DN) scale in their correlation with the swelling characteristics of polymers. The latter $(\pi^*+\alpha+\beta)$ summation term is representative of the 'multi-parameter' theory¹¹ and has often been applied to interpret many types of solute–solvent interactions.^{2,3,10} For the sake of simplicity the coefficients for each components of the $(\alpha+\beta)$ or of the $(\pi^*+\alpha+\beta)$ expressions will be considered as being 1 in this study. Otherwise, following a previous report,⁴ the (AN+DN) parameter will be tested comparatively in 1:1; 2:1 and 1:2 proportions between its two components.

Table 2. Solvent parameters^{2,5,10,23}

Solvent	AN	DN	ϵ	$E_T(30)$ (kcal/mol)	δ (cal/mL) ^{1/2}	π^*	α	β	$\alpha + \beta$	$\pi^* + \alpha + \beta$
<i>n</i> -Hexane	0	0	1.9	31.0	7.30	−0.04	0	0	0	−0.04
<i>n</i> -Heptane	0	0	—	31.1	7.40	−0.08	0	0	0	−0.08
Toluene	3.3	0.1	2.4	33.0	8.90	0.54	0	0.11	0.11	0.65
Benzene	8.2	0.1	2.3	34.3	9.20	0.59	0	0.10	0.10	0.69
Carbon tetrachloride	8.6	0	2.2	32.4	8.60	0.28	0	0.10	0.10	0.38
1,1-Dichloroethane	16.2	0	10.0	39.4	—	0.48	0.1	0.10	0.20	0.68
1,2-Dichloroethane	16.7	0	10.1	41.3	9.90	0.81	0	0.10	0.10	0.91
Nitrobenzene	14.8	4.4	34.8	41.2	10.00	1.01	0	0.30	0.30	1.31
Dichloroethylenecarbonate	16.7	3.2	10.1	41.9	—	—	—	—	—	—
DCM	20.4	1.0	8.9	40.7	9.70	0.82	0.13	0.10	0.23	1.05
Diethyl ether	3.9	19.2	4.2	34.5	7.40	0.27	0	0.47	0.47	0.74
Nitromethane	20.5	2.7	36.7	46.3	12.70	0.85	0.22	0.06	0.28	1.13
1,4-Dioxane	10.3	14.3	2.2	36.0	10.00	0.55	0	0.37	0.37	0.92
Ethyl acetate	9.3	17.1	6.0	38.1	9.00	0.55	0	0.45	0.45	1.00
Methyl acetate	10.7	16.3	6.7	38.9	—	0.60	0	0.42	0.42	1.02
Chloroform	23.1	4.0	4.7	39.1	9.30	0.58	0.20	0.10	0.30	0.88
Benzonitrile	15.5	11.9	25.2	41.5	8.40	0.90	0	0.37	0.37	1.27
Tetrahydrofuran (THF)	8.0	20.0	7.5	37.4	9.10	0.58	0	0.55	0.55	1.13
Acetone	12.5	17.0	20.7	42.2	9.60	0.71	0.08	0.43	0.51	1.22
Dimethoxyethane	10.2	20.0	7.2	38.2	8.30	0.53	0	0.41	0.41	0.94
Acetonitrile	18.9	14.1	36.0	45.6	11.90	0.75	0.19	0.40	0.59	1.34
Propylene CO ₃	18.3	15.1	65	46.6	—	0.83	0	0.40	0.40	1.23
Tributylphosphate	9.9	23.7	7.9	39.6	—	0.65	0	0.80	0.80	1.45
Sulfolane	19.2	14.8	43.3	44.0	—	0.98	0	0.39	0.39	1.37
Tetramethylene sulfone	19.2	14.8	—	44.0	—	—	—	—	—	—
4-Butyrolactone	17.3	18.0	12.6	44.3	—	0.87	0	0.49	0.49	1.36
Tetramethylurea	9.2	29.6	23.4	41.0	—	0.83	0	0.80	0.80	1.63
Trimethylphosphate	16.3	23.0	20.6	43.6	—	0.72	0	0.77	0.77	1.49
Piperidine (PIP)	0	40.0	5.8	35.5	11.10	0.30	0	1.04	1.04	1.34
NMP	13.3	27.3	33.0	42.2	11.30	0.92	0	0.77	0.77	1.69
Dimethylacetamide	13.6	27.8	37.8	42.9	10.80	0.88	0	0.76	0.76	1.74
DMF	16.0	26.6	36.7	43.8	12.10	0.88	0	0.69	0.69	1.57
Diethylacetamide	13.6	32.2	—	41.4	—	0.84	0	0.78	0.78	1.72
Pyridine	14.2	33.1	12.3	40.5	10.70	0.87	0	0.64	0.64	1.51
DMSO	19.3	29.8	46.7	45.1	12.00	1.00	0	0.76	0.76	1.76
Hexamethylphosphoramide	10.6	38.8	29.6	40.9	10.50	0.87	0	1.05	1.05	1.92
TFE	53.5	0.0	26.7	54.1	11.90	0.73	1.51	0	1.51	2.24
2-Phenylethanol	33.8	23.0	—	49.5	—	0.88	0.64	0.61	1.25	2.13
<i>N</i> -Methylformamide	32.1	27.0	182.4	54.1	16.10	0.90	0.62	0.80	1.42	2.32
Diethylamine	9.4	50.0	3.6	35.4	8.00	0.24	0.03	0.70	0.73	0.97
Benzyl alcohol	36.8	23.0	—	50.4	12.1	0.98	0.60	0.52	1.12	2.10
Ethylamine	4.8	55.5	6.2	—	—	—	—	—	—	—
Triethylamine (TEA)	1.4	61.0	2.4	33.3	7.40	0.14	0	0.71	0.71	0.85
Formamide	39.8	24.0	109.5	55.8	19.20	0.97	0.71	0.48	1.19	2.16
<i>t</i> -Butanol	27.1	38.0	12.5	43.7	10.50	0.41	0.42	0.93	1.35	1.76
1-Butanol	36.8	29.0	17.5	49.7	11.60	0.47	0.84	0.84	1.68	2.15
EtOH	37.1	32.0	24.3	51.9	12.70	0.54	0.86	0.75	1.61	2.15
2-Propanol	33.5	36.0	18.3	48.4	11.40	0.48	0.76	0.84	1.60	2.08
MeOH	41.3	30.0	32.6	55.4	14.50	0.60	0.98	0.66	1.64	2.24
Water	54.8	18.0	78.4	62.8	23.40	1.09	1.17	0.47	1.64	2.73
Acetic acid	52.9	20.0	6.2	51.7	10.10	0.64	1.12	0.45	1.57	2.21
Diaminoethane	20.9	55.0	—	42.0	—	0.47	0.13	1.43	1.56	2.03
Ethylenediamine	20.9	55.0	12.9	—	12.30	—	—	—	—	—
Formic acid	83.6	19.0	57.9	57.7	12.1	0.65	1.23	0.38	1.61	2.26
Trifluoroacetic acid (TFA)	105	0	8.2	—	10.60	0.50	—	—	—	—
Trifluoromethane sulfonic acid	129.1	0	—	—	—	—	—	—	—	—

As complement to this designed effort in searching for a novel and more suitable solvent polarity scale, the present investigation also further aimed at better understanding the factors that may govern the complex polymer solvation phenomenon. The large amount of swelling data to be here obtained would certainly be of value for the improvement, not only of the solid phase peptide synthesis¹² itself, but also for other polymer-supported methodologies such as the widely applied solid phase organic synthesis procedure¹³ often in association with the unique combinatorial chemistry experimental strategy.¹⁴

Needless to say, the fundamental aspect of all these experimental approaches lies in the fact that they all depend markedly on the efficiency of the solvation property of polymeric materials chosen as the solid support for their applications. In this context, it seems imperative that the selection of the model resins for the present study must be made judiciously. Thus, the set of cross-linked resins to be investigated in this report comprises a large amount of polymeric materials with different characteristics including their overall polarity, ionic form and the amount and type of backbone-attached ligands.

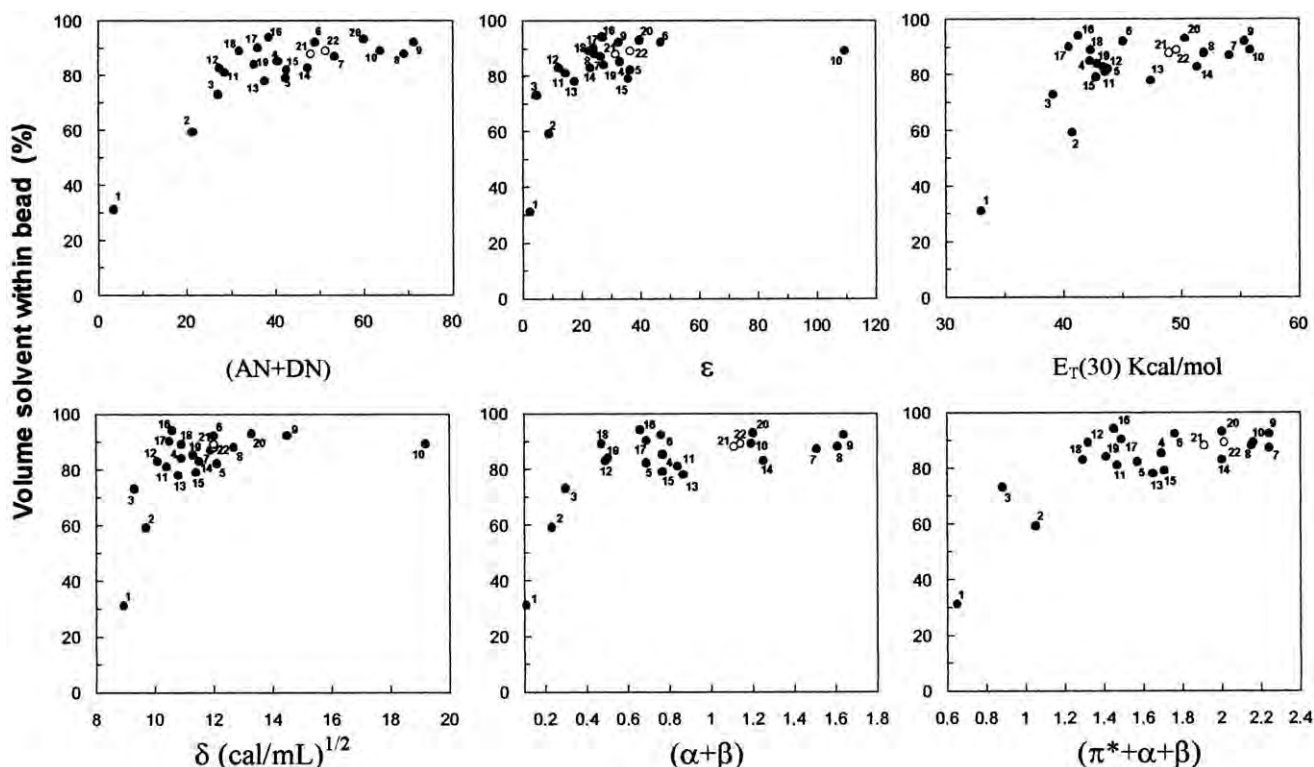


Figure 1. Swelling of resin (1), BHAR-NH₃⁺, 2.4 mmol/g as a function of solvent (AN+DN), ϵ , $E_T(30)$, δ , $(\alpha+\beta)$ and $(\pi^* + \alpha + \beta)$ values.

2. Results

The polymers selected for the present study are: resin (1), a very highly positively charged benzhydrylamine-resin (BHAR-NH₃⁺, 2.4 mmol ammonium group/g), a phenyl-methylamine group-containing copolymer of styrene and 1% divinylbenzene (PS-DVB), synthesized according to an earlier report;¹⁵ resin (2): the hydrophobic PS-DVB copolymer alone; resins (3) [Bu(DADP)₄-BHAR] and (4) [(NANP)₄-BHAR], the 1.4 mmol/g BHAR resin attaching either the (DADP)₄ sequence protected at Asp-side chains with the *tert*-butyl (Bu) groups or the unprotected and more polar (NANP)₄ segment, respectively, and both with a very high (about 70%, g/g) peptide-content. Resin (5) is an aminoalkyl-cross-linked polymer¹⁶ composed predominantly of polydimethylacrylamide matrix (SPAR-50, 0.6 mmol/g, from Advanced ChemTech Inc.) and that significantly differs from the others by containing an entirely hydrophilic network. Resin (6) is also a representative of a polystyrene-based copolymer but containing 2% of a polar cross-linking function (tetraethyleneglycol diacrylate) replacing the traditional divinylbenzene group. This resin, namely PS-TTEGDA, was synthesized according to a previous report¹⁷ and contains 0.7 mmol/g of amine functions in deprotonated form. Lastly, resin (7) is a 1% chloromethylated-PS-DVB but grafting poly-(ethylene-glycol) (PEG) group¹⁸ which increases the hydrophilicity of the polymer backbone. The PEG group may be derived to introduce amine groups in the polymer structure and the commercial presently studied resin contains 0.3 mmol/g amine groups [(TentaGel-SNH₂) or (TG)-resin], from Advanced ChemTech Inc.

The list of 28 single and mixed solvents that were used in the present study, most of them applied in different steps of the solid phase peptide synthesis,¹² together with the corresponding values of the six solvent polarity parameters are shown in Table 1. In addition the more complete Table 2 lists the values of solvent properties for 57 single solvents. The supplementary information section contains the set of measured swelling degrees of the seven resins determined in a microscope. The percentage of swollen bead volume occupied by the solvent ranged from a minimum of 7% (resin 4, toluene) to a maximum of 94% (resin 1, DMSO/THF). Similarly to that applied for the study of other empirical solvent properties^{8,19} and following a previous study,⁴ the equation below was used for the determination of values of solvent parameter for mixed solvents. In this equation, x_1 and x_2 are the solvent parameters for the two components of the mixture, and ϕ_1 and ϕ_2 are the corresponding volume fractions.

$$X_{1+2} = \phi_1 x_1 + \phi_2 x_2 \quad (1)$$

Figs. 1–3 show, as representative examples, the solvation profiles for resins 1–3 [(BHAR-NH₃⁺, PS-DVB and Bu(DADP)₄-BHAR, respectively], when their swelling data are correlated with (AN+DN), ϵ , $E_T(30)$, δ , $(\alpha+\beta)$ and $(\alpha+\beta+\pi^*)$ values. Irrespective of the resins examined the best correlation occurs with the solvent (AN+DN) term (Figs. 1(A), 2(A) and 3(A), respectively) than with other parameters. The also amphoteric $(\alpha+\beta)$ scale seems to follow, with a slightly poorer correlation, the good relationship observed when the (AN+DN) number is used. This

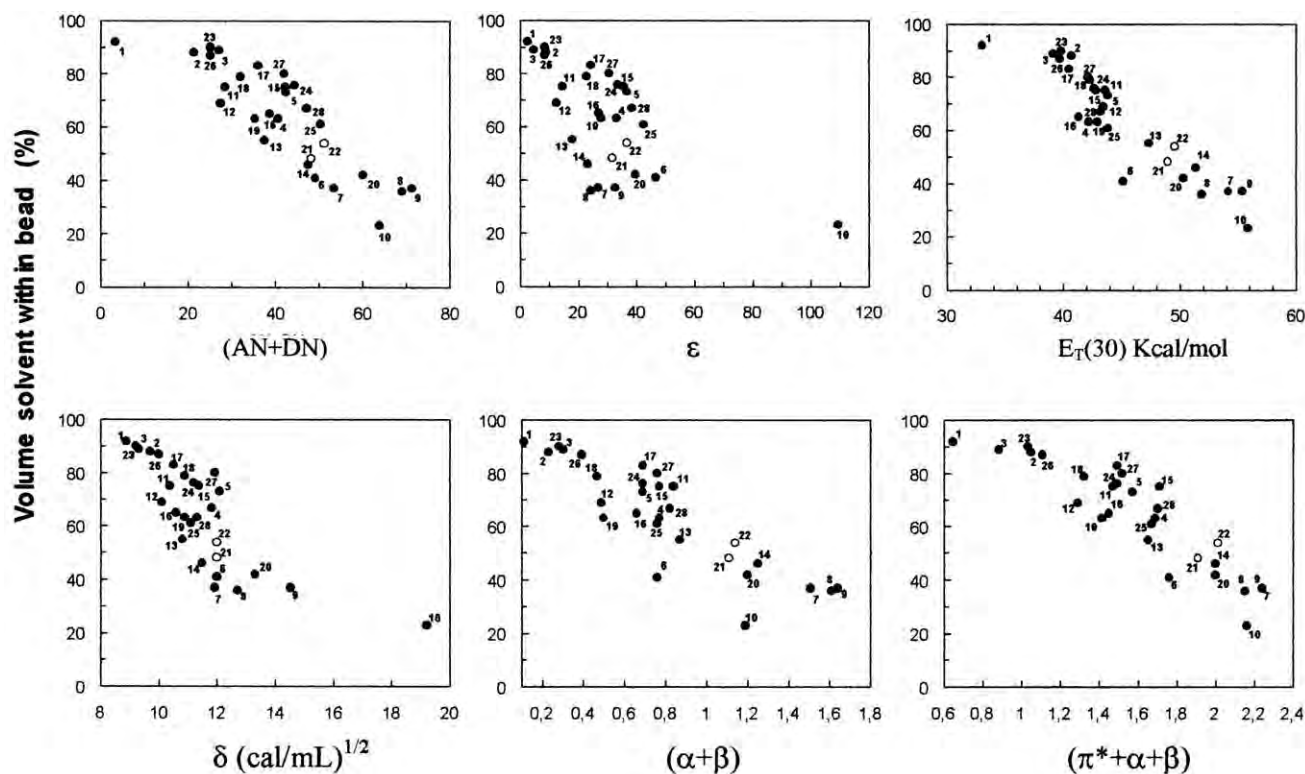


Figure 2. Swelling of resin (2), PS-DVB as a function of solvent (AN+DN), ϵ , $E_T(30)$, δ , $(\alpha+\beta)$ and $(\pi^*+\alpha+\beta)$ values.

trend is also observed in resins 4–7 and the corresponding four contoured solvation curves are collectively shown in Fig. 4. The complementary figures which reveal less accurate relationships between swelling data and other polarity

parameters for resins 4–7 are included in the supplementary information section.

The proportion between AN and DN numbers was also

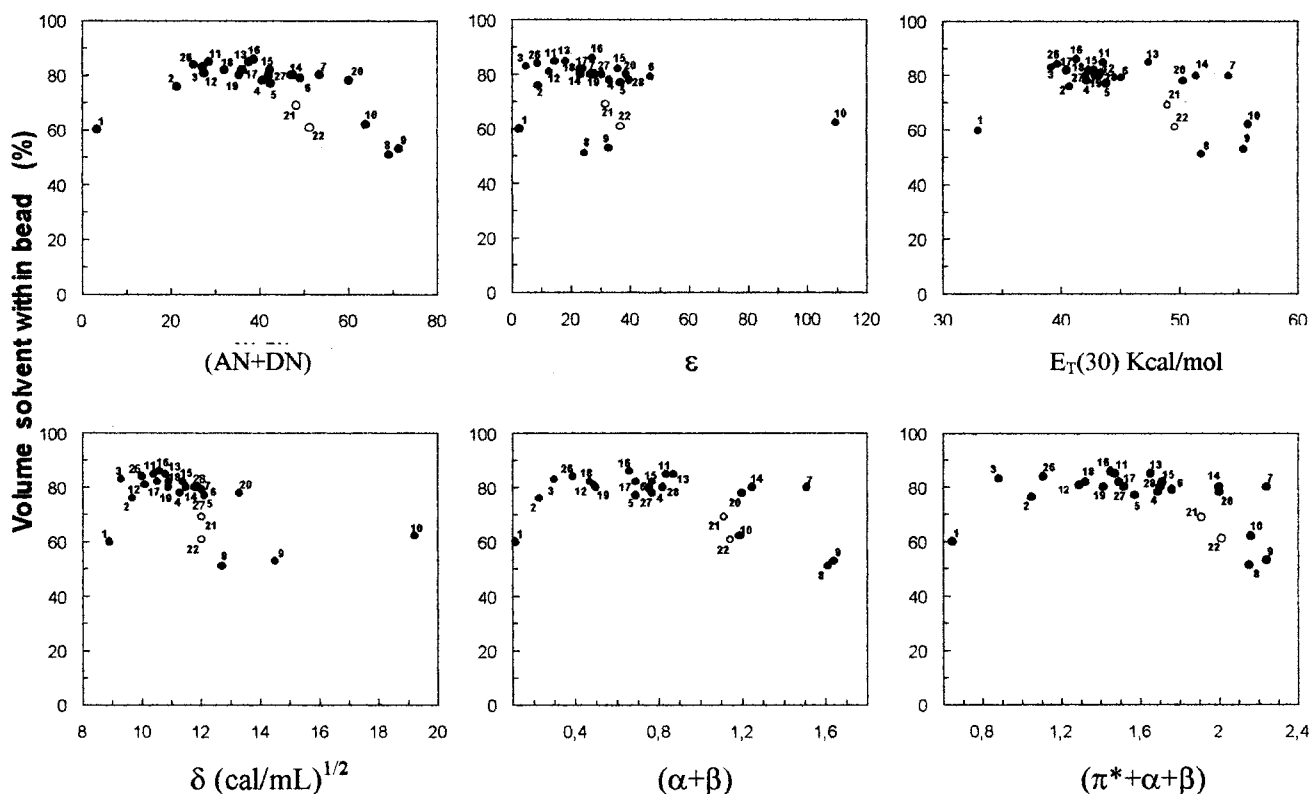


Figure 3. Swelling of resin (3), Bu(DADP)₄-BHAR, 1.4 mmol/g as a function of solvent (AN+DN), ϵ , $E_T(30)$, δ , $(\alpha+\beta)$ and $(\pi^*+\alpha+\beta)$ values.

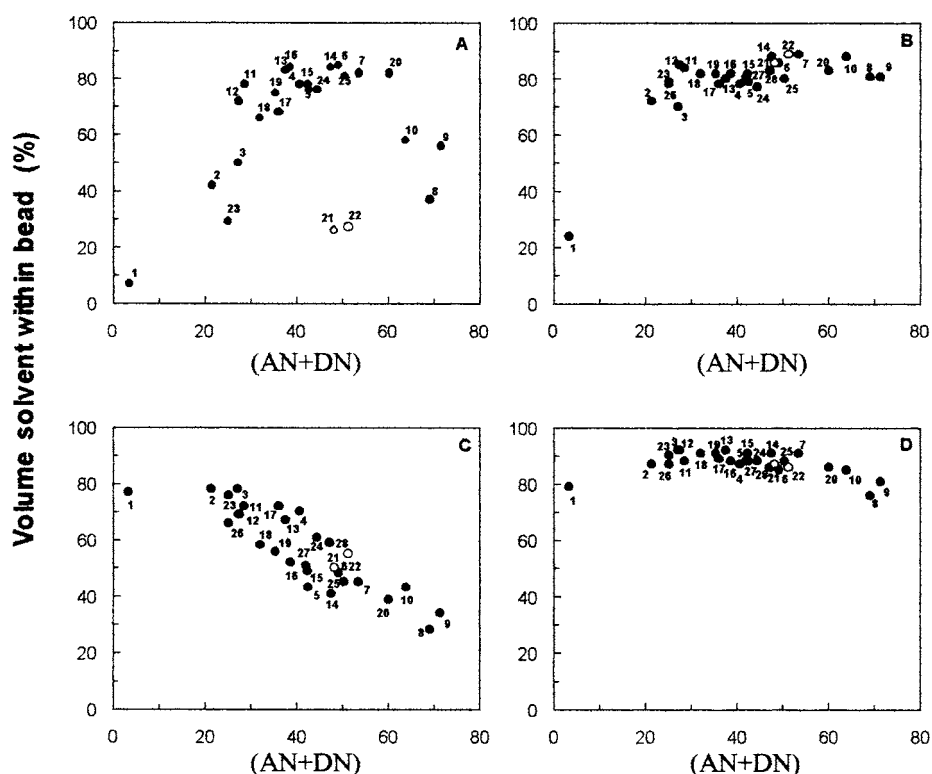


Figure 4. Swelling of resins (4), (NANP)₄-BHAR, 1.4 mmol/g (A), (5), SPAR-50, 0.6 mmol/g (B), (6), PS-TTEGDA, 0.7 mmol/g (C) and (7), TG, 0.3 mmol/g (D) as a function of solvent (AN+DN) values.

varied (2:1 and 1:2) but as previously observed with some peptide-resins,⁴ the correlations of all resins (1–7) with swelling data in these two proportions were weaker than that observed when in the practical 1:1 proportion (comparative figures in the supplementary information section). Finally, by analyzing the set of swelling versus polarity parameter values, the mixed solvents 21 and 22 (TFE/DMF and TFE/DMSO), represented by open circles, deviate from the average solvation curve only with (NANP)₄-BHAR (resin 4, Fig. 4(A)) and to a much lesser extent with Bu(DADP)₄-BHAR (resin 3, Fig. 3). Lower swelling degrees than those predicted by their polarity values are observed. This effect which occurs only with this type of mixed solvents was already described⁴ and therefore will be further discussed in terms of their heterogeneous composition.

As a second but also relevant goal of the present investigation, a clear difference in the solvation of each resins is observed depending on their structural characteristics (Figs. 1–4). The following maximum solvation regions in terms of the (AN+DN) values are detected for each resin: lower than 20 for the apolar PS-DVB and PS-TTEGDA (resins 2 and 6, respectively); above 40–50 for the polar BHAR-NH₃⁺ and SPAR-50 (resins 1 and 5, respectively). An intermediary position is occupied by Bu(DADP)₄-BHAR (resin 3) and (NANP)₄-BHAR (resin 4), which display enhanced solvation in solvent systems characterized by (AN+DN) polarity values centered near 40 (Fig. 3) and 50 (Fig. 4(A)), respectively. Interestingly, a different swelling behavior (Fig. 4(D)) was observed with the TG-resin (resin 7). This PEG-grafted polymer may be included in the intermediary position in terms of polarity

as its maximum solvation region occurs near 40. However differing from all others, this resin does not display a significant decrease in swelling regardless of the polarity value of the solvent system. The measured minimum swelling values are as high as 80% and thus, it is the only polymer which presents an excellent solvation irrespective of the polarity of the medium.

3. Discussion

3.1. The applied resin solvation versus solvent polarity approach

The differentiated approach herein applied for simultaneously examining resin solvation process and validity of different polarity resins was first based on the well known statement that polymers display maximum swelling in solvents with polarity similar to their polymeric backbone. This finding was for instance, verified when the Hildebrand' δ parameter was applied for correlating swelling of different types of polymeric materials.⁸ It means that the exact profile of the contoured solvation curve showing the relationship between swelling and polarity values will always depend on the physico-chemical characteristic of each polymer. As observed in all the figures of the present work and that will be further discussed in more details, those more hydrophobic or hydrophilic resins will have their maximum solvation regions in these figures shifted to the left or right sides of the polarity scale corresponding to the more apolar or polar regions, respectively. Alternatively a maximum solvation region located in the middle of this scale may be also observed when in the

Table 3. The (AN+DN) solvent scale

Solvent	(AN+DN)	Solvent	(AN+DN)
<i>n</i> -Hexane	0	<i>N</i> -Methyl-pyrrolidinone (NMP)	40.6
<i>n</i> -Heptane	0	Dimethylacetamide	41.4
Toluene	3.4	<i>N,N</i> -Dimethylformamide (DMF)	42.6
Benzene	8.3	Diethylacetamide	45.8
Carbon tetrachloride	8.6	Pyridine	47.3
1,1-Dichloroethane	16.2	Dimethylsulfoxide (DMSO)	49.1
1,2-Dichloroethane	16.7	Hexamethylphosphoramide	49.4
Nitrobenzene	19.2	Hexamethylphosphoramide	49.4
Dichloroethylenecarbonate	19.9	1,1,1-Trifluoroethanol (TFE)	53.5
Dichloromethane (DCM)	21.4	2-Phenylethanol	56.8
Diethyl ether	23.1	<i>N</i> -Methylformamide	59.1
Nitromethane	23.2	Diethylamine	59.4
Dioxane	24.6	Benzyl alcohol	59.8
Ethyl acetate	26.4	Ethylamine	60.3
Methyl acetate	27.0	Triethylamine (TEA)	62.4
Chloroform	27.1	Formamide	63.8
Benzonitrile	27.4	<i>t</i> -Butanol	65.1
Tetrahydrofuran (THF)	28.0	1-Butanol	65.8
Acetone	29.5	Ethanol (EtOH)	69.1
Dimethoxyethane	30.2	2-Propanol	69.5
Acetonitrile	33.0	Methanol (MeOH)	71.3
Propylene CO ₃	33.4	Water	72.8
Tributylphosphate	33.6	Acetic acid	72.9
Sulfolane	34.0	Diaminoethane	75.9
Tetramethylene sulfone	34.0	Ethylenediamine	75.9
4-Butyrolactone	35.3	Formic acid	102.6
Tetramethylurea	38.8	Trifluoroacetic acid (TFA)	105.0
Trimethylphosphate	39.3	Trifluoromethane sulfonic acid	129.1
Piperidine (PIP)	40.0		

case of resins containing intermediary polarity characteristic. Accordingly, Figs. 1–4 of the present report (and all those of supplementary information section) show examples of each of this type of solvation behavior.

This heterogeneity in terms of figures when swelling and polarity values are both correlated, regardless of the resin or of the solvent parameter selected, strongly evinces that the determination of a single linear or non-linear regression equation, with its corresponding correlation coefficient applicable for this complex relationship is not yet achieved and awaiting for a more complete investigation. One possibility to further clarify this complex problem may involve the simultaneous use of a variety of solvent parameters in a single equation (multi-parameter theory¹¹). To date, only a report²⁰ has suggested a linear relationship between the swelling degree of a polyurethaneimide-type resin with the $E_T(30)$ solvent parameter.²⁰ However the restriction of this approach only to few types of solvents that did not encompass entirely the polarity scale as done in the present work, hampered the visualization of a possible maximum solvation region for this type of polymer.

Taken together and despite this impossibility in determining any type of correlation coefficient for the swelling versus polarity values, the main assumption stated in this work is that, by examining visually the degree of dispersion of the large amount of data of the solvation curves, one can judiciously select the solvent property that seems to be the most appropriate for scaling polarity. Certainly this parameter will be that one which has displayed lesser dispersion (best fit) of data thoroughly all these correlation curves as a consequence of its higher sensitivity and accuracy towards resin–solvent interaction effect. To better validate this

effort, the set of resins investigated in this report comprised deliberately ionic and neutral polymers, those containing entirely hydrophilic or hydrophobic backbones, and also those with a mixed character given by the attachment of chemical groups or peptide chains, containing different polarities and amounts spread in their matrices. In our view this was basically the simple but valid criteria herein applied for interpreting the polymer solvation phenomenon and the potential of the alternative (AN+DN) polarity scale, comparatively to those already existing in the literature.

3.2. The (AN+DN) solvent term

In an excellent review²¹ regarding physicochemical properties of polymers for solid phase organic synthesis, the authors have suggested a more complete evaluation of the preliminarily proposed (AN+DN) solvent term mainly in comparison with the traditional Hildebrand's δ parameter. In our view, the large amount of presently accumulated data correlating the swelling behavior of a total of eleven types of polymers (four from a previous study⁴),[‡] most with known solvent properties, confirmed that the (AN+DN) term might be considered a novel empirical polarity scale. Of great relevance is the fact that it was unequivocally proven that this scale is more adequate to probe solvation of any type of polymers in comparison with the δ parameter. The better accuracy of the (AN+DN) term, when

[‡] As the correlation between swelling of BHAR–NH₂ (1.4 mmol/g), Bz(DADP)₄–BHAR (70% peptide-content), ING–BHAR (6 and 47%) resins with (AN+DN), in 2:1, 1:1, 2:1 proportions, and with ϵ and $E_T(30)$ terms were already described,⁴ the lacking correlation data of these resins with additional δ , ($\alpha+\beta$) and ($\pi^*+\alpha+\beta$) solvent parameters are now included in supplementary information section of this work.

Table 4. Binary correlations of solvent parameters

X/Y	(AN+DN)
ε	(AN+DN)=36.59+0.23 ε , $r=0.2994/n=49$
$E_T(30)$	(AN+DN)=−58.09+2.29 $E_T(30)$, $r=0.7374/n=52$
δ	(AN+DN)=−2.29+4.19 δ , $r=0.4961/n=41$
π^*	(AN+DN)=32.14+15.25 π^* , $r=0.1709/n=51$
$(\alpha+\beta)$	(AN+DN)=10.38+39.92 $(\alpha+\beta)$, $r=0.9251/n=50$
$(\pi^*+\alpha+\beta)$	(AN+DN)=−2.99+30.76 $(\pi^*+\alpha+\beta)$ $r=0.8607/n=50$

r =correlation coefficient of linear regression; n =number of solvents.

its components are, in 1:1 and not in other proportions, was also demonstrated regardless of the type of the polymeric material (corresponding figures in the supplementary information section). Table 3 shows the complete (AN+DN) polarity scale containing values of 57 solvents ranging from a minimum of 0 (hexane) to a maximum of near 130 (trifluoromethanesulfonic acid).

As complement, the linear correlations calculated between the (AN+DN) number and other solvent properties examined in this study were calculated and are shown in Table 4. The best correlation of the (AN+DN) scale occurs exactly with those representative of the two-parameter⁹ $(\alpha+\beta)$ or multi-parameter^{2,11} $(\pi^*+\alpha+\beta)$ solvent effect concepts. In these two cases the calculated correlation coefficients (r) with the (AN+DN) parameter are 0.92 and 0.86, respectively. In agreement with their comparative poorer fits observed in all the swelling versus polarity term plots, the two representatives of the one-parameter solvent effect theory [$E_T(30)$ and δ] display only fair relationships with the (AN+DN) values ($r=0.73$ and 0.49 , respectively). In addition, much more weak correlations are calculated using the remaining single parameters ε and π^* ($r=0.30$ and 0.18 , respectively).

Taken together one may state that those polarity scales which consider the solute–solvent interaction in a generic sense as an acid–base process, seem to be the most adequate. Accordingly, after the (AN+DN) scale, improved correlations are observed with the $(\alpha+\beta)$ and the polyfunctional $(\pi^*+\alpha+\beta)$ terms. One possibility to explain the slightly lesser adequacy of both these additive terms may be due to the fact that Kamlet–Taft's parameters are all an average of data from a great amount of solvent effect studies using several types of solute probes. This characteristic can be considered advantageous in some circumstances but may also be regarded as one of their weaknesses.²

In respect to other solvent parameters which show a more reduced correlation with the resin solvation data, one can speculate that it is due to the fact that none of them contain the important amphoteric character existing in the (AN+DN) number. A second possibility may lie in the inadequacy of the solute–probe molecule used for their determination as compared with those used for AN and DN scale properties. This might be the case of the Dimroth–Reichardt's $E_T(30)$ parameter. Of note is also the fair suitability of the Hildebrand's solubility parameter δ . These results might be partially credited to its less rigorous determination method, although some successful examples of good correlation between δ values of the polymer with those of the solvent have been reported.^{8,22}

In this case, as each resin has a characteristic polarity, its maximum solvation will occur with solvents with similar δ values.

The very weak correlation of the ε property with the swelling property of resins is indicated by the very low ($r=0.30$) correlation coefficient with the (AN+DN) term. This inadequacy is expected as this macroscopic solvent parameter only reflects the electrostatic interaction between solute and solvent molecules but not how effective is the alignment between both dipoles. Moreover, no other types of interaction are included in this solvent parameter thus precluding its acceptance as a suitable polarity scale.²³

To explain the better accuracy of the (AN+DN) term to access the solvation property of polymers one can hypothesize that besides its amphoteric character, an additional factor may be involved in the improved suitability of the selected probe molecules for scaling its two components. As stressed, the AN number reflects the electrophilic property of solvents and the triethylphosphine oxide is the probe chosen for its determination. This compound presents the same special and important requirements which can be important for its use as an appropriate probe:⁵ (i) the nucleus of the solute molecule is not close to the actual site of interaction (basic oxygen atom); (ii) the model-solute is a very strong base. This characteristic assures a high sensitivity of the phosphorus resonance to solvent change; (iii) the solute–solvent interaction always occurs at a well-defined site, namely, at the oxygen atom; the remaining coordination sites of the phosphorus atom are blocked off by inert alkyl groups.

In the other hand, the measurement of the nucleophilicity of solvents represented by the DN number depends on the SbCl_5 compound as probe model molecule. This solute also seems to be very appropriate as it fulfills the following requirements: (i) irrespective of the donor molecule, the adducts are formed in a 1:1 molar ratio; (ii) SbCl_5 is a very strong acceptor and this allows a reasonable degree of adduct formation even with very weak donors; (iii) the Sb–Cl bonds are difficult to be heterolyzed and ionization equilibrium can be neglected even in the case of interaction with strong donor molecules.

The notion of solvent polarity is often used to choose a solvent or to explain solvent effects. Because solute–solvent interactions depend on the structure of both components, the proposition of a universal solvent polarity scale seems to be very difficult and maybe unattainable. Thus in using the (AN+DN) scale one should always be aware of this inherent weakness. But despite this expected limitation in the search for this perfect polarity scale, the results herein described, based on the study of a set of different polymers combined with the selection of appropriate solvent systems, validate the (AN+DN) term as a novel, dimensionless and accurate polarity term. Obviously how widely applicable and adequate is this scale when other solute–solvent interactions are to be considered, is still an open issue and awaiting further research. In other areas, excepting chemistry (mainly in biology), the traditional but in most cases, inadequate mention of the dielectric constant as the universal polarity scale has been a very common practice for

decades. Thus one may hopefully expect that a more practical and acceptable polarity scale might appear for progressively correcting this type of conceptual misunderstanding.

3.3. Solvation property of polymers

To better validate the findings of the present polymer solvation study, some important experimental requirements were previously defined: (i) in addition to the large amount of solvent systems chosen for encompassing as broadly as possible the polarity scale, the set of examined polymers was characterized by their heterogeneity in terms of the type of the solute-models; (ii) before swelling measurements, the home-made resins were sifted in pore metal sieves to reduce the standard deviations of resin diameters as previously published;⁴ (iii) the microscopic measurement method^{15,24} of resin beads was applied due to its high accuracy and sensitivity; (iv) in the specific case of peptide-resins, the amount, polarity and aggregation tendency of the peptide sequence were altered to monitor the influence of these different factors.

Briefly, the driving force for swelling of cross-linked polymer network is made up of influences by the entropic and enthalpic changes associated with the interaction process between solvent and solute molecules.^{8,25} When the sum of these contributions imposes a negative variation on Gibbs's free energy values, expansion of the resin network occurs, reflected by the swelling degree values. This effect is therefore dependent on the polymer characteristic itself and mainly on its interaction with the solvent molecule. This implies that in the correlation approach herein applied, each type of polymer should be characterized by a maximum solvation region in a swelling versus solvent polarity plot.

The overall findings of the previous study⁴ are in close accordance with this assumption. The different solvation behavior of each resin can be summarized as follows: those containing predominantly a hydrophobic character such as the PS–DB and PS–TTEGDA (or the 6% peptide-content ING–BHAR and the 1.4 mmol/g deprotonated amine group BHAR–NH₂ described earlier⁴), swell better in more apolar solvents characterized by (AN+DN) values lower than 30. This effect is certainly due to the dominant influence of hydrophobic polystyrene matrices of these resins. For polymers such as Bu(DADP)₄–BHAR or [Bz(DADP)₄–BHAR⁴], they are in an intermediary polarity position due to the contribution of a larger amount of polar C=O and N–H dipole moieties of peptide bonds attached to their structure (near 70% peptide-content in both resins). In this case improved solvation region is shifted to solvents characterized by (AN+DN) values in the 30–40 range.

The class of more polar solutes includes the cationic 2.4 mmol/g BHAR–NH₃⁺, the entirely hydrophilic SPAR-50 and the examples of two highly peptide-loaded peptide-resins but attaching either a very polar [(NANP)₄–BHAR, 68% peptide-content] or a polar and well-known²⁶ aggregating (ING–BHAR, 47% peptide-content⁴) sequence, respectively. These polymers display improved solvation in single or mixed solvents characterized by having

(AN+DN) values about 50 or higher. Finally the characteristic solvation behavior observed with the PEG-attaching PS-based TG resin (TG), of very common use in peptide synthesis seems to emphasize the relevance of the special characteristic of the polymer backbone. This resin presents optimized solvation in practically all type of solvents with the maximum solvation region in those having (AN+DN) values around 40. But irrespective of the solvent, no swelling degree lower than 80% is observed in the contour solvation curve. As this swelling behavior is not simply expected from its polarity character, one may infer that the TG-resin might be characterized by special physico-chemical features given by a well-established copolymerization design.

In this regard a certain degree of structural inhomogeneity is known to occur when the copolymers of styrene and divinylbenzene are prepared.²⁷ This is basically due to the lack of commercial availability of pure isomers of the latter cross-linking compound and alternative examples of its replacement with other groups have been reported. In addition to the PS–TTEGDA herein examined and which contains tetraethyleneglycol-diacrylate as the cross-linking group¹⁷ there are others such as the JandaJel²⁸ containing, instead, a 1,4-bis(vinylphenoxy)-butane group. Due to the improved homogeneity some of this class of cross-linked polystyrene resins are already available commercially as is the case of the latter polymer. This structural characteristic has been taken into account, for instance,²¹ to compare through spectroscopic experiments, different characteristics of resins with potentiality for application in polymer supported synthesis methodologies.

As examples of practical relevance deriving from polymer solvation studies, the enhanced swelling of the cationic 2.4 mmol/g BHAR–NH₃⁺ observed in more polar solvents led us promptly to speculate that it might also swell reasonably in aqueous solution. It was further confirmed and this aminated resin, introduced²⁹ to be used exclusively in organic solvents as the solid support for peptide synthesis, was also employed as an alternative anion exchanger resin in column chromatography for the successful purification of negatively charged biological compounds.³⁰ Also based upon the measured lack of swelling in apolar solvents, of this highly amine-loaded BHAR (protonated form), we recently have demonstrated³¹ the need for modification in the solvent system of the traditional picric acid method³² for quantification of resin-bound amine groups.

Finally, the mixed solvents (open circles—Figs. 1–4) 21 and 22 (TFE/DMF and TFE/DMSO, respectively) are unique in terms of physicochemical characteristics. They consist of strong electron acceptor (TFE) and strong donor (DMF or DMSO) components (heterogeneous solvent) thus presenting the inherent tendency to self-associate rather than to solvate solute molecules.³³ This implies that their solvation strength is clearly dependent upon the amount of interactions to be disrupted between solute molecules or in the present case, throughout the polymer network. The stronger the association effect inside the bead, the more difficult it is for these two heterogeneous solvent systems to solvate the polymer. In this case a lower solvation degree of resin than that predicted by their polarity values is

observed. Accordingly, this shrinking effect of resin beads with solvents 21 and 22 is more clearly seen only in the highly peptide-loaded (NANP)₄-BHAR (68%, Fig. 4(A)) which contains a very folded peptide structure³⁴ or in the aggregating sequence-containing ING-BHAR (47%).⁴ To emphasize the relevance of the peptide loading effect, this latter peptide-resin, when attaching a smaller amount of peptide chains (6% peptide content) did not suffer from the shrinking effect in these two heterogeneous solvents.⁴ Thus, the comparatively smaller shrinking effect observed with Bu(DADP)₄-BHAR (Fig. 3(A)) and Bz(DADP)₄-BHAR⁴ in solvents 21 and 22 is indicative of a lower tendency of both peptide segments to self-association if compared for instance, with the (NANP)₄ sequence (all these resins contain the same peptide loading values).

Of note, this bead shrinking effect does not occur in all other resins, thus indicating the absence of any significant interaction process spread throughout their matrices. We deem the type of contour solvation curve here designed might be therefore a unique and valuable strategy for monitoring and quantifying the degree of aggregation occurring in the polymer network. Moreover an additional advantage in scaling solvent effects with the two-parameter polarity scales, as is the case of the (AN+DN) term, must be reminded. The interpretation for the unusual swelling of polymers towards heterogeneous solvents such as TFE/DMF or TFE/DMSO is not feasible when considering for instance, one-parameter solvent parameters such as δ , ϵ or $E_T(30)$. These findings make more clear the need for knowing the nature and the effect of the mixed solvent to be used (heterogeneous or homogeneous) for any types of solute-solvent interactions, unfortunately still not usually taken into account, mainly in non-chemistry areas.

The factors which may control resin solvation have been intensively investigated through a great set of experimental strategies in the last decades. The explosive trend in polymer-dependent methodologies has led to the appearance of a large number of alternative resins as well as successful spectroscopic investigations of this phenomenon. Amongst these attempts, relevant data have been collected from NMR,³⁵ CD,³⁶ IR³⁷ and EPR³⁸ studies. In our case, in complement to the beginning of the mentioned solvation study of resins through microscopic measurements of beads,⁴ we have also applied the EPR procedure but innovating as concerns the paramagnetic probe to be used. The paramagnetic amino acid TOAC (2,2,6,6-tetramethyl-piperidine-*N*-oxyl-4-amino-4-carboxylic acid)³⁹ which we have derived for peptide labeling⁴⁰ and structure-activity⁴¹ approaches, was also applied to labeling resins and peptide-resins to monitor dynamics of swollen polymer backbones.⁴²

All together these efforts seem to be of great value for improving methodologies that depend markedly on the pioneering and revolutionary concept of performing chemical reactions on heterogeneous and insoluble supports, started in the literature almost four decades ago.⁴³ The set of findings obtained in the present resin solvation-solvent polarity investigation which has encompassed a large amount of different polymeric materials and solvent systems may therefore, in association with those deriving

from spectroscopic techniques, provide new insights for optimization of many polymer-dependent chemical or biological methodologies.

4. Experimental

N^α-*tert*-Butyloxycarbonyl (Boc)- β -benzyl- or *N*^α-9-fluorenylmethyloxycarbonyl (Fmoc)-*tert*-butyl-Asp and other side chain deprotected Boc- or Fmoc-amino acids were purchased from Bachem, Torrance, CA. SPAR-50 and TentaGel-SNH₂ or TG resins were acquired from Advanced ChemTech and PS-DVB copolymer from Bio Rad Laboratories. Batches of BHAR (0.2, 1.4 and 2.4 mmol/g) were synthesized in this laboratory, following earlier reports.^{15,29} The PS-TTEGDA resin was synthesized as published elsewhere.¹⁷ Solvents and reagents were purchased from Fluka, Aldrich or Sigma Co. and those used for swelling studies were HPLC grade.

4.1. Peptide synthesis

The peptides were synthesized manually accordingly to the standard Boc^{12a,b}- or Fmoc^{12c,d}-protocols. In the Boc-chemistry, after the coupling of the C-terminal amino acid to the resin, the successive α -amino group deprotection and neutralization steps were performed in 30% TFA/DCM (30 min) and 10% DIEA/DCM (10 min). Conversely, a single 20 min piperidine/DMF treatment was needed to deprotect and neutralize the amine function of the growing sequence in the Fmoc-strategy. The amino acids were coupled with TBTU in the presence of HOBT and DIEA using DMF or 20% DMSO/NMP as solvent system. After a two-hour coupling time, the qualitative ninhydrin test was performed to estimate the completeness of the reaction. To check the purity of the synthesized peptide sequence attached to the resin cleavage reactions with small aliquots of resin were carried out with the low-high HF procedure (Boc-chemistry) or K^{12d} reagent (Fmoc-chemistry). Analytical HPLC (Waters), LC/MS (electrospray)-mass spectrometry (Micromass) and amino acid analysis (Beckman 6300 analyzer) were used to check the homogeneity of each synthesized resin-bound peptide sequences.

4.2. Swelling measurement of beads

Before use in peptide synthesis and/or in microscopic measurement of bead sizes, most resin batches were sized by sifting in porous metal sieves to lower the standard deviations of resin diameters to about 4%. Swelling studies of these narrowly sized populations of beads were performed and published previously.⁴ Briefly, 150–200 dry and swollen beads of each resin, allowed to solvate overnight, were spread over a microscope slide and measured directly with an Olympus, model SZ11 microscope coupled with a Image-Pro Plus, 3.0.01.00 version software. Since the sizes in a sample of beads are not normally but log-normally distributed, the central value and the distribution of the particle diameters were estimated by the more accurate geometric mean values and geometric standard deviations. With the exception of the ionized resin **1** and the copolymer PS-DVB (resin **2**), the others were measured with their amino groups in the deprotonated form, obtained by

3×5 min TEA/DCM/DMF (1:4.5:4.5, v/v/v) washings followed by 5×2 min DCM/DMF (1:1, v/v) and 5×2 min DCM washings. Resins were dried in vacuum using an Abderhalden-type apparatus with MeOH reflux.

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Anexo 5

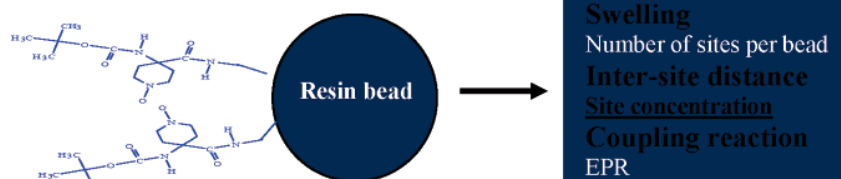
Determination of Site–Site Distance and Site Concentration within Polymer Beads: A Combined Swelling-Electron Paramagnetic Resonance Study

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This work proposes a combined swelling-electron paramagnetic resonance (EPR) approach aiming at determining some unusual polymer solvation parameters relevant for chemical processes occurring inside beads. Batches of benzhydrylamine-resin (BHAR), a copolymer of styrene–1% divinylbenzene containing phenylmethylamine groups were, labeled with the paramagnetic amino acid 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amine-4-carboxylic acid (TOAC), and their swelling properties and EPR spectra were examined in DCM and DMF. By taking into account the BHARs labeling degrees, the corresponding swelling values, and some polymer structural characteristics, it was possible to calculate polymer swelling parameters, among them, the volume and the number of sites per bead, site–site distances and site concentration. The latter values ranged from 17 to 170 Å and from 0.4 to 550 mM, respectively. EPR spectroscopy was applied to validate the multistep calculation strategy of these swelling parameters. Spin–spin interaction was detected in the labeled resins at site–site distances less than approximately 60 Å or probe concentrations higher than approximately 1×10^{-2} M, in close agreement with the values obtained for the spin probe free in solution. Complementarily, the yield of coupling reactions in different resins indicated that the greater the inter-site distance or the lower the site concentration, the faster the reaction. The results suggested that the model and the experimental measurements developed for the determination of solvation parameters represent a relevant step forward for the deeper understanding and improvement of polymer-related processes.

Introduction

The innovation of performing chemical processes within an insoluble polymer matrix came into use about four

decades ago with the development of the solid-phase peptide synthesis method^{1,2} and has been successfully expanded to create efficient synthetic methodologies for other macromolecules.³ More recently, progressively greater knowledge of solid-phase supported processes has been crucial in successfully launching the unique com-

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binatorial chemistry approach⁴ that has allowed the generation of peptide libraries and has had a remarkable impact on the development of new therapeutic agents.⁵

With the scope of using polymers for an ever-widening array of purposes, a large number of different resins has been formulated.⁶ In addition, spectroscopic techniques have been applied with the aim of reaching a deeper understanding of polymer-based processes. Among these, investigations based on nuclear magnetic resonance,⁷ infrared,⁸ fluorescence,^{9,10} Raman,¹⁰ and electron paramagnetic resonance (EPR)¹¹ have been of great value since they provide relevant information about the solvated polymeric network. Properties such as diffusion, adsorption, and distribution of sites within beads were all examined in these reports. We and other groups have described and exploited the use of the paramagnetic amino acid 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) to investigate the conformation and dynamics of labeled peptides.^{12,13} The use of TOAC has been extended to structural investigations of solvated resins and peptide resins.¹⁴

The interaction between the solvent system and any type of solute is of utmost relevance when examining

chemical or physicochemical processes.¹⁵ In the case of polymers or peptide-polymers, the solvent system affects polymer swelling, the average distance between chains (and, as a result, the degree of chain association), controls the rate of motion of components, and regulates reaction kinetics. For this reason, polymer solvation has been investigated with a variety of experimental procedures, among them the microscope measurement of beads.¹⁶

This latter experimental approach has been of value in the examination of the solvation characteristics of a great number of polymeric materials, adopted as solute models in a large amount of solvents. This approach proved very fruitful since it allowed us to obtain consistent results regarding polymer solvation and to propose a novel amphoteric solvent parameter (AN + DN),¹⁷ which is the sum of Gutmann's solvent electron acceptor (AN) and electron donor (DN) numbers¹⁸ at a ratio of 1:1. The use of the AN and DN concepts was recently extended to predict the potential of a given solvent to dissociate peptide chains not only when bound to a polymer matrix but also when free in solution.¹⁹

When making use of polymeric matrixes to perform chemical processes it is desirable to understand as much as possible, at a molecular level, the properties of the environment created by these matrixes. In this context, many studies have assessed properties such as dynamics of the polymer backbone, site-site aggregation, and swelling capacity in each solvent system.^{7–11,14} To continue these polymer-focused studies, the strategy described in the present work relied primarily on a novel approach to calculate some structural characteristics of

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TABLE 1. Swelling Parameters of Differently Labeled Boc-TOAC-BHAR in DCM

BHAR ^a (mmol/g)	sample									
	col 1	col 2	col 3	col 4	col 5	col 6	col 7	col 8	col 9	col 10
	diam dry bead (μm)	diam swollen bead (μm)	vol solvent/ bead (10 ³ μm ³)	vol dry sample/g copol ^b (mL)	wt dry sample/g copol (g)	vol dry sample/g sample (mL)	no. of beads/g sample (10 ⁷)	no. of sites/bead (10 ¹²)	site-site distance (Å)	site concn (mM)
0.003 ^c	57	98	4.0	1.8	1.17	1.54	1.59	0.1	169.8	0.4
0.019 ^c	57	98	4.0	1.8	1.18	1.53	1.58	0.7	88.9	2.9
0.035 ^c	57	98	4.0	1.8	1.18	1.53	1.58	1.3	72.2	5.4
0.050 ^c	57	98	4.0	1.8	1.18	1.53	1.58	1.9	63.7	7.9
0.065 ^d	57	98	4.0	1.8	1.19	1.51	1.56	2.5	58.1	10.4
0.134 ^d	57	98	4.0	1.8	1.22	1.48	1.53	5.2	45.5	21.7
0.646 ^e	57	98	4.0	1.8	1.45	1.24	1.28	30.3	25.3	126.3
0.988 ^f	58	99	4.1	1.9	1.66	1.14	1.12	52.9	21.2	215.0

^a Degree of Boc-TOAC-OH labeling. ^b Copolymer of styrene-1% divinylbenzene: $d = 0.99$ g/mL; average diameter of dry beads = 47 μm. ^c Obtained from 0.050 mmol/g of BHAR. ^d Obtained from 0.14 mmol/g of BHAR. ^e Obtained from 0.80 mmol/g of BHAR. ^f Obtained from 1.40 mmol/g of BHAR.

polymers in solvated conditions. Making use of TOAC labeling, EPR spectroscopy was applied to check the validity of the calculation protocol. The calculation strategy involved the initial microscopic measurement of dry and swollen beads of several spin-labeled batches of the benzhydrylamine resin (BHAR) used to synthesize α-carboxamide peptides.²⁰ Subsequently, data such as solvent volume inside the bead, number of sites per bead, site-site distance, and site concentration within beads were estimated through a sequential calculation strategy.

EPR spectra of BHAR batches, labeled with *tert*-butyloxycarbonyl-TOAC derivative (Boc-TOAC-OH),^{12a} were obtained in order to check the proposed calculation approach. The occurrence of spin-spin interaction was used as a criterion to assess site-site distances and site concentrations inside the beads. The strategy was to verify if this spectral effect is observed at similar probe-probe distances or probe concentrations within the resin beads and when the probe was free in solution.

In addition, experiments were performed to evaluate the relationship between the calculated polymer swelling data, such as site-site distance, site concentration, and the rate of amino acid coupling reactions in model resins or peptide-resins. The results demonstrate that the practical-conceptual approach presented in this work for quantifying resin swelling properties can be applied to other polymeric materials.

Results

Swelling Studies. BHAR batches, with phenylmeth-ylamine group loading ranging from 0.05 to 1.4 mmol/g at the polystyrene-1% divinylbenzene backbone, were synthesized according to a previously described protocol.²⁰ Boc-TOAC-OH was coupled to BHAR batches under controlled conditions in order to produce resins spin-labeled to different extents.

Approximately 0.5 g of BHAR with substitution degrees of 0.05, 0.14, 0.80, and 1.40 mmol/g were used to couple the Boc-TOAC-OH residue using the conventional Boc-peptide synthesis strategy.^{2a,b} If necessary, a 3-fold molar excess was applied to guarantee quantitative spin

label incorporation using the diisopropylcarbodiimide/*N*-hydroxybenzotriazole (DIC/HOBt) coupling protocol in DCM. In all BHAR batches, the coupling was complete in about 2 h. When partial incorporation was desired, the coupling step was deliberately carried out with less than equimolar amounts of Boc-TOAC-OH. The exact degree of incorporation was determined by quantitative picric acid method.²¹ Using this experimental protocol, eight batches of Boc-TOAC-BHAR with paramagnetic labeling degrees ranging from 0.003 to 0.988 mmol/g were synthesized.

Calculation Strategy To Determine Polymer Bead Swelling Parameters. By starting from simple swelling parameters such as diameters of dry and swollen beads as reference points, a sequence of calculations was designed to determine the resin parameters given in Tables 1 and 2. The tables summarize the data calculated for eight batches of Boc-TOAC-BHAR in DCM and in DMF, respectively.

To illustrate how the various resin parameters were sequentially calculated, the detailed procedure will be described below for the 0.134 mmol/g of Boc-TOAC-BHAR batch in DCM (Table 1, row 6).

Example: 0.134 mmol/g of Labeled Boc-TOAC-BHAR Swollen in DCM (Table 1). Columns 1 and 2 list the average diameters of the dry (57 μm) and DCM swollen (98 μm) beads, respectively, measured under the light microscope. The volumes of the dry ($0.97 \times 10^5 \mu\text{m}^3$) and swollen ($4.93 \times 10^5 \mu\text{m}^3$) beads were calculated, and the volume of solvent/bead ($4 \times 10^5 \mu\text{m}^3$, column 3) was obtained by subtracting the dry bead volume from the swollen bead volume.

Column 4: Volume of Dry Sample/Gram of Copolymer (1.8 mL/g of Copolymer). The ratio (diameter dry sample/diameter dry copolymer)³ represents the relationship between the volume of the dry macroscopic working sample (0.134 mmol/g of Boc-TOAC-BHAR) and that of the dry copolymer used to synthesize the sample. In the example, since the average diameters of beads of dry sample and dry copolymer are 57 and 47 μm, respectively, the ratio between the dry volumes of both resins is $(57/47)^3 = 1.78$. Considering that the number of beads in 1 g of copolymer is the same as in the sample synthesized from this amount of copolymer and taking

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TABLE 2. Swelling Parameters of Differently Labeled Boc-TOAC-BHAR in DMF

BHAR ^a (mmol/g)	sample									
	col 1	col 2	col 3	col 4	col 5	col 6	col 7	col 8	col 9	col 10
	diam dry bead (μm)	diam swollen bead (μm)	vol solvent/ bead (10 ³ μm ³)	vol dry sample/g copol ^b (mL)	wt dry sample/g copol (g)	vol dry sample/g sample (mL)	no. of beads/g sample (10 ⁷)	no. of sites/bead (10 ¹²)	site–site distance (Å)	site concn (mM)
0.003 ^c	57	81	1.8	1.8	1.17	1.54	1.59	0.1	140.9	0.9
0.019 ^c	57	81	1.8	1.8	1.18	1.53	1.58	0.7	73.7	6.5
0.035 ^c	57	81	1.8	1.8	1.18	1.53	1.58	1.3	60.0	12.0
0.050 ^c	57	81	1.8	1.8	1.18	1.53	1.58	1.9	52.8	17.6
0.065 ^d	57	79	1.6	1.8	1.19	1.51	1.56	2.5	47.0	26.0
0.134 ^d	57	79	1.6	1.8	1.22	1.48	1.53	5.2	36.8	54.2
0.646 ^e	57	80	1.7	1.8	1.45	1.24	1.28	30.3	20.5	297.1
0.988 ^f	58	79	1.6	1.9	1.66	1.14	1.12	52.9	17.0	551.0

^a Degree of Boc-TOAC-OH labeling. ^b Copolymer of styrene–1% divinylbenzene: $d = 0.99$ g/mL; average diameter of dry beads = 47 μm. ^c Obtained from 0.050 mmol/g of BHAR. ^d Obtained from 0.14 mmol/g of BHAR. ^e Obtained from 0.80 mmol/g of BHAR. ^f Obtained from 1.40 mmol/g of BHAR.

into account that the volume of 1 g of copolymer is 1.01 mL ($d = 0.99$ g/mL),^{16a} the total volume of dry sample containing 1 g of copolymer is therefore 1.78×1.01 mL, or 1.80 mL.

Column 5: Weight of Dry Sample/Gram of Copolymer (1.22 g/g of Copolymer). The 0.134 mmol Boc-TOAC-BHAR sample was synthesized by quantitative incorporation of the Boc-TOAC-OH in the 0.140 mmol/g of BHAR batch. This resin originated from partial phenylmethylamino incorporation into a heavily substituted 1.4 mmol/g benzoyl group-containing copolymer. This copolymer derivative is synthesized in the first step (Friedel–Crafts acylation) necessary to obtain BHAR.²⁰ Thus, the Boc-TOAC-BHAR sample under consideration still contains $(1.4 - 0.14)$ mmol = 1.26 mmol/g of remaining benzoyl groups attached to its backbone. Considering the total weight of groups added in all the synthetic steps, one can calculate that the sum of Boc-TOAC-OH and benzoyl groups attached to the initial copolymer corresponds to 0.182 g. Therefore, in 1 g of sample, the mass of copolymer is $1 - 0.182$ g = 0.818 g. Thus, for 1 g of starting copolymer, the weight of the 0.134 mmol Boc-TOAC-BHAR is 1.22 g.

Column 6: Volume of Dry Sample/Gram of Sample (1.48 mL/g). This parameter is calculated by dividing the value of (volume of dry sample/gram of copolymer), column 4, by (weight of dry sample/gram of copolymer), column 5. The value obtained (1.48 mL/g) represents the ratio between the volume of the dry sample (1.8 mL) and its total weight (1.22 g) and corresponds to the volume occupied by 1 g of sample in the dry form.

Column 7: Number of Beads/Gram of Sample (1.53×10^7 Beads/g of Sample). This value is calculated by dividing the volume of 1 g of dry sample (1.48 mL, column 6) by the average volume of one dry bead, which is calculated from its diameter (57 μm, column 1). Thus, the volume of one dry bead is 9.7×10^{-8} mL. The ratio $1.48 \text{ mL} / 9.7 \times 10^{-8} \text{ mL}$ yields 1.53×10^7 beads in 1 g of sample.

Column 8: Number of Sites/Bead (5.2×10^{12}). The number of sites per bead is calculated by dividing the number of sites/gram of sample by the number of beads/gram of sample (column 7). The former value corresponds to $0.134 \times 6.02 \times 10^{20}$ sites/g. Dividing this number by the number of beads in 1 g of sample (1.53×10^7 , column 7) gives 5.2×10^{12} sites/bead.

Column 9: Site–Site Distance (45.5 Å). To evaluate this important parameter, we first calculate the average volume per site. This is done by dividing the volume of one swollen bead ($4.9 \times 10^{-5} \mu\text{m}^3$, calculated from the measured diameter of one swollen bead, 98 μm, column 2) by the number of sites/bead (5.2×10^{12} , column 8). Thus, the average volume per site is $9.4 \times 10^4 \text{ Å}^3$. By assuming a uniformly distributed cubic lattice for the sites within the bead, the site–site distance corresponds to the side of a cube and is given by the cubic root of the volume occupied by one site, i.e., $(9.4 \times 10^4 \text{ Å}^3)^{1/3} = 45.5 \text{ Å}$.

Column 10: Site Concentration Inside the Bead (21.7 mM). Finally, making use of the parameters calculated in the previous steps, it is possible to determine the site concentration within the bead. Thus, for the 0.134 mmol/g of substituted Boc-TOAC-BHAR, the site concentration is obtained by dividing the number of sites/bead (5.2×10^{12} , column 8) by the volume of solvent/bead ($4 \times 10^5 \mu\text{m}^3$, column 3), that is, 1.3×10^7 sites/ μm^3 , or 1.3×10^{19} sites/mL. Considering that 6.02×10^{20} sites/mL correspond to 1 M concentration, we find that the site concentration corresponds to an effective Boc-TOAC-OH concentration of 21.7 mM.

By examining the parameters calculated in Tables 1 and 2, it can be seen that the average inter-site distance ranged from a maximum of about 170 Å (for 0.003 mmol/g of Boc-TOAC-BHAR in DCM, Table 1) to a minimum of 17 Å (for 0.988 mmol/g of Boc-TOAC-BHAR in DMF, Table 2). Furthermore, the site concentration within the beads varied from a minimum of approximately 0.4 mM to a maximum of approximately 550 mM. Under very highly loaded conditions (0.988 mmol/g of Boc-TOAC-BHAR in DMF), the concentrations were as high as those typically used during the solution peptide synthesis method.²²

Some of the parameters calculated in Tables 1 and 2, such as the number of beads per gram of the resin or the number of sites per bead, are relevant for application in polymer studies. Interestingly, values of this latter parameter ranged from approximately 0.3×10^{12} to 50×10^{12} (0.003 mmol/g and 0.988 mmol/g of Boc-TOAC-labeled resins, respectively). A linear correlation between

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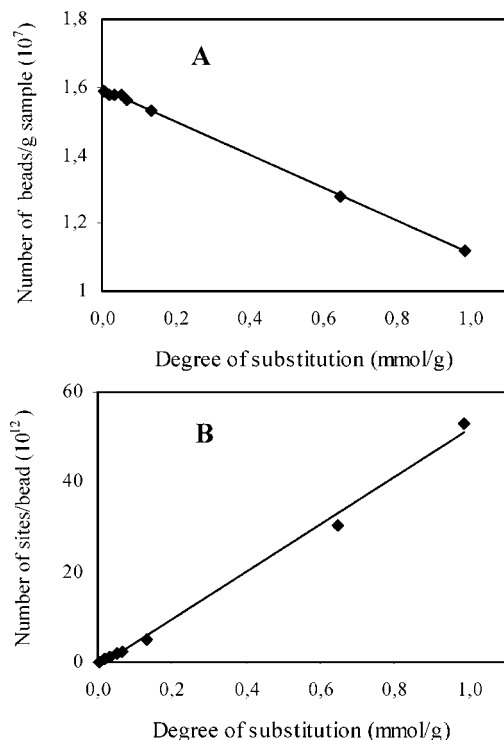


FIGURE 1. Correlation between number of beads per gram of sample (A) and number of sites per bead (B) and the degree of substitution of Boc-TOAC-BHARs.

these two parameters (columns 7 and 8) and the degree of resin labeling is observed in Figure 1. As expected, while the number of beads per gram of resin decreased, the number of sites per bead increased with the increasing degree of substitution.

It should be pointed out that this calculation strategy can be extended to resins containing other chemical groups, including peptides. Besides the dry and swollen bead diameters, it is only necessary to know the density of the starting resin (and therefore the volume occupied by 1 g of resin) and to calculate the overall weight variation due to the incorporation of the desired groups in the composite derivative.

EPR Studies. Figure 2 shows the EPR spectra of eight labeled resins in DCM and DMF (panels A and B, respectively). Spectral line broadening due to spin–spin interaction was observed in both solvents and increased with increasing Boc-TOAC-OH substitution.

The dependence of probe–probe interaction on resin calculated parameters, such as site–site distance and site concentration, was compared with data obtained for Boc-TOAC-OH in solution. Figure 3 shows the EPR spectra of the spin probe in DMF as a function of concentration. Spectral line broadening due to spin–spin interaction can be clearly observed as the probe concentration increases.

Table 3 correlates the Boc-TOAC-OH concentration with the line width of the mid-field line (ΔH) measured for the spectra in Figure 3 and with the average distance between probe molecules free in solution. Similarly to the analysis for the labeled resins (column 9 in Tables 1 and 2), a static model of probe distribution in solution was assumed, namely, the probes are uniformly distributed within a cubic lattice. The average distance between

adjacent Boc-TOAC-OH molecules in solution was estimated by calculating the amount of probe molecules at each concentration combined with the average volume occupied by each molecule. Assuming a cubic lattice distribution of probe molecules, the average intermolecular distance between adjacent molecules corresponds to the side of a cube and is given by the cubic root of the volume occupied by the probe. As an example, for 10^{-3} M Boc-TOAC-OH, the average volume occupied by each molecule is $1.7 \times 10^6 \text{ \AA}^3$ and the average distance between adjacent molecules is 118.6 $\text{\AA}$ (Table 3).

Figure 4 displays the dependence of the mid-field spectral line width of both labeled resins and of the spin label in DMF solution on site–site distance (panel A) and site concentration (panel B). Line broadening did not occur for site–site distances larger than approximately 60 $\text{\AA}$ (probe concentration of ca. 1×10^{-2} M). Similar results were obtained for the probe free in solution and spread throughout resin backbone, strongly suggesting that the sequential calculation designed for quantitative determination of the swelling parameters in Tables 1 and 2 is a valid approach.

Correlation between Resin-Swelling Parameters and the Rate of Coupling Reactions. To emphasize the usefulness of the strategy developed to determine resin swelling parameters, the relationship between site–site distance and site concentration and the rate of coupling reactions throughout a polymer network was examined. Table 4 shows the coupling yield of Boc-Pro-OH in DCM to the 1.4 mmol/g of BHAR batch. One can observe that the greater the site–site distance and lower the site concentration, the faster the coupling reaction. The rate of coupling follows the order: DCM > DMF > DMSO. Complementary swelling data of the 1.4 mmol/g of BHAR batch are given in the Supporting Information.

PSA Method in Equimolar Conditions (1 mM Reactants). Table 5 displays another example of the application of this approach, this time extended to peptidyl-resins. Four batches of the aggregating Ile-Asn-Gly sequence²³ bound to BHAR in varying amounts (up to 1.59 mmol/g) were compared under equivalent conditions with respect to the frequency of Boc-(2BrZ)-Tyr-OH coupling. Other swelling parameters of these four peptide resins are also available in the Supporting Information Section. Closely paralleling the results outlined in the previous paragraph, faster coupling reactions occurred systematically with resins that presented larger site–site distance and lower site concentration.

Discussion

The main objective of the present work was to design a novel swelling-EPR approach aimed at multiple goals. First, we intended to develop a sequential calculation that would allow the estimation of polymer swelling parameters for further practical application. Second, we applied EPR spectroscopy to paramagnetically labeled resins in order to determine at what site–site distance or site concentration values significant spin–spin interactions begin to occur. Finally, we verified the correlation between the calculated properties of solvated polymer

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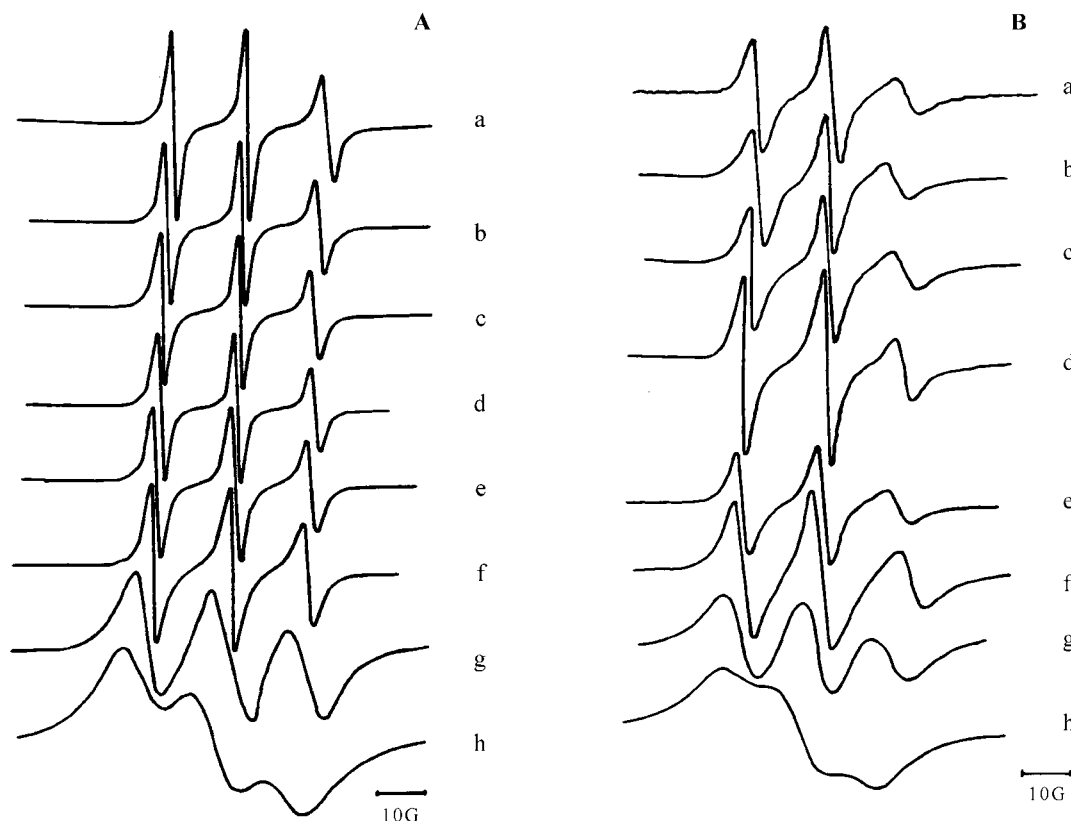


FIGURE 2. Effect of Boc-TOAC-OH loading on the EPR spectra of Boc-TOAC-BHAR in DCM (**A**) and DMF (**B**). Probe loading (mmol/g): a = 0.003; b = 0.019; c = 0.035; d = 0.050; e = 0.065; f = 0.134; g = 0.646; h = 0.988.

and the efficiency of chemical processes taking place in the polymer matrix.

The initial step in this study comprised synthesis and microscopic measurement of the swelling properties of several BHAR batches labeled with the Boc-TOAC-OH spin probe for further EPR studies. By combining swelling and structural data of these resins, a sequential calculation strategy was designed to estimate resin swelling parameters that can be useful in the polymer field (Tables 1 and 2). Among these parameters, the number of sites per bead, average volume occupied by each site, site–site distance, site concentration within beads, should be mentioned.

The combination of EPR spectroscopy with the swelling data of TOAC-labeled resins aimed at testing the validity of the calculation strategy for gauging bead-swelling information. By comparing EPR data for the probe in DMF solution with those for the paramagnetic probe attached to the polymer core at variable degrees in the same solvent, it was possible to estimate the maximum site–site distance (~ 60 Å) or minimum site concentration ($\sim 1 \times 10^{-2}$ M) for the onset of spin–spin interaction. Similar values were found for the paramagnetic probe attached to the polymer core when the solvent was DCM.

The good agreement between the results obtained with this approach and those obtained making use of the sequential protocol for calculation of swelling parameters (Figure 4) strongly suggest that the proposed protocol is correct. Several geometrical models could be adopted to estimate the average site distribution—spherical, cylindrical, etc. We chose to use a cubic distribution of sites

or probes in solution or bound to the resin, based on the classical report by Barany and Merrifield designed to estimate site–site distance within beads.²⁴ In this context, it should be recalled that more than 99% of sites are located inside the bead structure and not at its surface.²⁴

In previous EPR-solvation studies, we¹⁴ and other groups¹¹ have monitored the dynamics of resins and peptide-resins with the aim of improving the peptide synthesis methodology. In these studies the mobility of the labeled sites was analyzed by calculating rotational correlation times from measurement of line widths and line heights in the absence of spin–spin interactions, that is, at low label concentrations. Here we took advantage of the occurrence of spin–spin interactions to assess site–site distances and site concentrations inside the polymeric matrixes.

The spectral line broadening obtained with increased labeling of the resins and increased probe concentration in solution provided a clear evidence of the occurrence of increasing spin–spin interaction. The interaction could be due to exchange and/or dipolar mechanisms. The dipolar interaction has been found to be the predominant broadening mechanism in the case of doubly labeled enzymes.²⁵ In these cases the two paramagnetic centers are located at a fixed distance from each other. Moreover,

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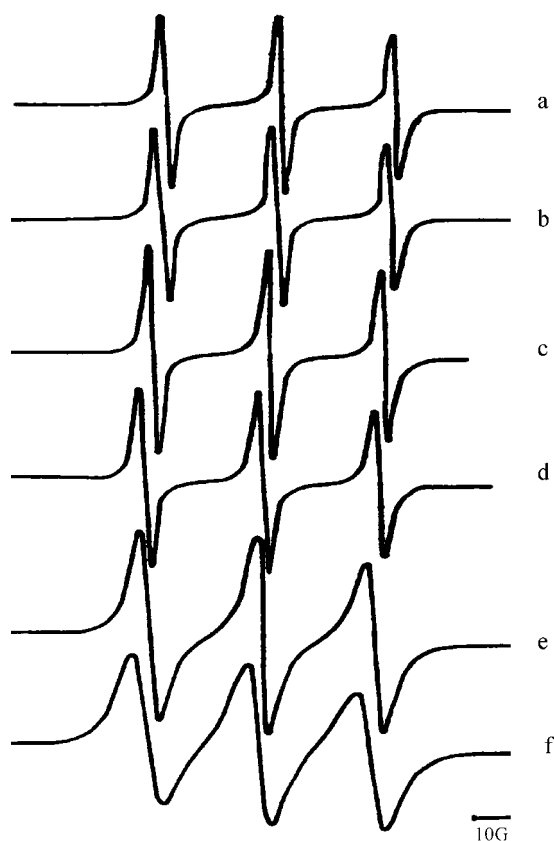


FIGURE 3. EPR spectra of Boc-TOAC-OH in DMF. Concentration (M): a = 0.0001; b = 0.001; c = 0.005; d = 0.01; e = 0.05; f = 0.1.

TABLE 3. Correlation between Concentration of Boc-TOAC-OH in DMF and the EPR Spectra's Central Peak Linewidth and the Site-Site Distance Values

concn (M)	ΔH (G)	site-site distance (Å)
1×10^{-4}	1.40	255.4
1×10^{-3}	1.40	118.6
5×10^{-3}	1.45	69.3
1×10^{-2}	1.70	55.0
5×10^{-2}	2.40	32.2
1×10^{-1}	3.65	25.5

the dipolar interaction has been shown to be detectable up to about 25 Å.^{25b} Although the resin beads are formed by a polymeric matrix, the characteristics of these polymers are quite different from those of a protein. In the polystyrene-divinylbenzene branched copolymer, the $-NH_2$ reactive groups are distributed at random, that is, the distances between pairs of attached nitroxides vary. Moreover, these polymers, immersed in solvent, are flexible (as indicated by the spectra at low resin loading, spectra a in Figure 3, panels A and B) and undergo intramolecular motions that lead to variation in the distances separating individual nitroxide groups. In addition, Boc-TOAC-OH bound to one polymer chain can encounter Boc-TOAC moieties bound to other polymer chains in the same bead.

Taking these facts into account, it is seen that the polymeric matrix is a highly dynamic structure and that the distances between Boc-TOAC-OH groups vary in space and time. Thus, the values obtained in the calcula-

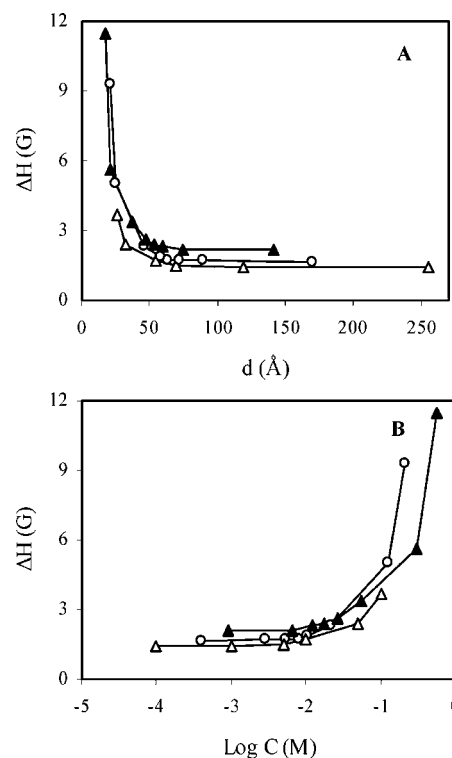


FIGURE 4. Effect of site-site distance (A) and site concentration (B) on the central peak line width of EPR spectra of Boc-TOAC-OH in DMF solution (Δ) and Boc-TOAC-BHAR in DCM (○) and in DMF (▲).

TABLE 4. Correlation between Yield^a of Boc-Pro-OH Coupling to BHAR (1.40 mmol/g) and Site Concentration and Site-Site Distance Values

solvent	site concn (M)	site-site distance (Å)	coupling (%)
DCM	0.21	21.7	90
DMF	0.55	17.0	67
DMSO	1.76	14.2	25

^a Yield of Boc-Pro-OH coupling after 30 min, at 25 °C with PSA method in equimolar conditions (1 mM of reactants).

TABLE 5. Correlation between Boc-(2BrZ)-Tyr-OH Coupling^a Yield to ING-BHAR in DCM and Site Concentration and Site-Site Distance Values

ING-BHAR ^a (mmol/g)	site concn (mM)	site-site distance (Å)	coupling yield ^b (%)
0.19	39	38	86
0.54	89	33	63
1.16	625	17	21
1.59	1963	14	1

^a Degree of ING substitution. ^b Yield of Boc-(2BrZ)-Tyr-OH coupling after 15 min, at 25 °C with PSA method in equimolar condition (10 mM concentration of reactants).

tions are average values. In this context, it seems plausible to draw an analogy with the situation found in membranes, where their constituent lipid molecules undergo lateral diffusion. Studies at high label concentrations have shown that in model²⁶ and biological²⁷ membranes the main contribution to spin-spin interaction is provided by the exchange mechanism. Although in fluid systems the exchange interaction is modulated

by the collision frequency between molecules, calculations were performed using a static model to estimate critical distances for the onset of spin–spin interaction.^{26b–d} These calculations yielded results in good agreement with the site–site distances found in the present study.

The data regarding the swelling parameters shown in Tables 1 and 2, when compared to those generated in previous swelling approaches to peptide resins,^{16a} represent a step forward in the gauging of differentiated physicochemical factors that govern polymer solvation. In this context, the various swelling parameters evaluated in this study could be of great help in deepening the understanding of the resin solvation phenomenon at the microenvironment level. Accordingly, a clear relationship between site–site distance and site concentration values and the rate of the acylation reaction is demonstrated for the first time in the present report (Tables 4 and 5).

Modern science has progressively broadened the scope of the use of polymeric materials for a variety of purposes. Resin applications range from simple use as a solid support for liquid chromatography to complex methods for synthesizing macromolecules such as peptides² and oligonucleotides,^{3a,b} as well as in combinatorial chemistry⁴ conducted for the development of new drugs. The present report describes an alternative method for the determination of polymer swelling properties in combination with the investigation of model resins by means of EPR spectroscopy. We believe that this dual approach can be of great applicability in many areas involving the use of polymeric matrixes.

Experimental Section

Materials. Reagents and solvents were of analytical grade, were collected from recently opened containers and were not further purified. Boc-TOAC-OH was synthesized according to previous reports^{12a}

Methods. Peptide Synthesis. The Ile-Asn-Gly sequence was synthesized manually by standard Boc chemistry^{2a,b} on about 0.5 g of 0.22, 0.62, 1.62, and 2.62 mmol/g of BHAR. Coupling was performed using a 2.5 excess of Boc-amino acid/DIC/HOBt (1:1:1) in DCM/DMF for approximately 2 h. All couplings were monitored by qualitative ninhydrin test, and when positive, acetylation was performed with 50% acetic anhydride in DCM for 15 min. A small portion of the peptide-resin was cleaved in anhydrous HF and the crude peptide was characterized with regard to identity and homogeneity using mass spectrometry, amino acid analysis, and analytical HPLC.

Measurement of Peptide-Resin Swelling. Before swelling measurements of Boc-TOAC-OH-labeled resins, all batches

of synthesized BHAR were sized by suspension in ethanol and fine material was decanted. The suspension was allowed to stand until approximately 90–95% had settled before decanting the supernatant. This procedure was repeated five times and was followed by suspending the beads in DCM. Solvent containing fine particles was withdrawn; this latter procedure was also repeated five times. To develop the swelling study with as narrowly sized population of beads as possible, the last resin purification step involved repeated sifting of dry beads through several 44–88 μm pore metal sieves. This sieving procedure lowered the standard deviation of the resin diameter to about 4%.

Swelling studies of the small-diameter bead populations were performed as published elsewhere^{16a,17} after the resins were dried in a vacuum using an Abderhalden-type apparatus. Subsequently, about 200 dry and swollen (allowed to solvate overnight) beads from each resin were spread over a microscope slide and measured directly with a microscope coupled with Image-Pro Plus software. The values of bead diameter distribution were estimated by the geometric means and geometric standard deviations, as published elsewhere.²⁸

EPR Studies. EPR measurements were carried out at 9.5 GHz on a Bruker ER 200D-SRC spectrometer at room temperature (22 ± 2 °C) using flat quartz cells from Wilmad Glass Co. (Buena, NJ). The magnetic field was modulated with amplitudes less than one-fifth of the line widths, and the microwave power was 5 mW to avoid saturation effects. Details of the procedure for TOAC-labeling of resins have been reported.¹⁴ Labeled peptide resins were pre-swollen overnight in the solvent under study.

Yield of the Coupling Reaction. In a reaction vessel thermostated at 25 °C, 50–100 μmol of BHAR or ING-BHAR was equilibrated with the desired solvent. Preformed symmetrical anhydride (PSA) of the Boc-Pro-OH and Boc-2-Br-carbobenzoxyl (2BrZ)-Tyr-OH residues, respectively, were produced by mixing with DCC in equimolar conditions (for 1 h, at 0 °C). The white precipitate was removed by filtration and the solution was evaporated for further dissolution with the desired solvent for comparative coupling experiments. The PSA method was deliberately chosen for these experiments as it is less susceptible to the effect of solvent polarity.^{2a,b} The rate of rotation of the reaction flask was 20 rpm. The acylating reagents were dissolved in the solvent under investigation and added in equimolar condition (at 10^{-2} M concentration of reactants) to the reaction vessel containing peptide resin pre-swollen in the same solvent. The coupling yield was monitored by the picric acid method,²¹ and each experiment was performed in duplicate.

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Supporting Information Available: Tables with swelling parameters for the 1.4 mmol/g of BHAR and ING-BHAR (four batches) are available. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Anexo 6

Study of the effect of the peptide loading and solvent system in SPPS by HRMAS-NMR

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Abstract: The SPPS methodology has continuously been investigated as a valuable model to monitor the solvation properties of polymeric materials. In this connection, the present work applied HRMAS-NMR spectroscopy to examine the dynamics of an aggregating peptide sequence attached to a resin core with varying peptide loading (up to 80%) and solvent system. Low and high substituted BHAR were used for assembling the VQAAIDYING sequence and some of its minor fragments. The HRMAS-NMR results were in agreement with the swelling of each resin, i.e. there was an improved resolution of resonance peaks in the better solvated conditions. Moreover, the peptide loading and the attached peptide sequence also affected the spectra. Strong peptide chain aggregation was observed mainly in highly peptide loaded resins when solvated in CDCl₃. Conversely, due to the better swelling of these highly loaded resins in DMSO, improved NMR spectra were acquired in this polar aprotic solvent, thus enabling the detection of relevant sequence-dependent conformational alterations. The more prominent aggregation was displayed by the VQAAIDYING segment and not by any of its intermediary fragments and these findings were also corroborated by EPR studies of these peptide-resins labelled properly with an amino acid-type spin probe. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: NMR; high-resolution magic angle spinning; solid-phase synthesis; resin; peptide

INTRODUCTION

Many spectroscopic methods have been used intensively to acquire relevant knowledge about the physicochemical factors that govern the complex solvation process of a polymer structure. Presently, focus has been given to the development of new drugs through the resin-supported combinatorial chemistry approach [1,2]. In this respect, solid phase peptide synthesis (SPPS) [3] has also continuously been selected as a target methodology for spectroscopic investigations, including CD [4], FTIR [5–7] and EPR [8–12]. In addition, NMR spectroscopy has been employed in this area [13–15] with emphasis on the high-resolution magic angle spinning (HRMAS-NMR) [16–19] technique applicable to the broad field of solid phase organic synthesis. The ability of this versatile spectroscopy has been valuable to obtain high quality NMR data on resin bound molecules including peptides [20–25] or even to compare the structural features of different polymeric materials [21,22,26]. Of these studies, only one [27] has dealt to date with the influence of the amount of peptide chain per resin bead.

The present investigation intended to extend the application of the HRMAS-NMR methodology to the physicochemical evaluation of the assembly of a

very strongly aggregating peptide sequence attached to a polymeric support, but deliberately in a very high peptide loaded condition. To achieve this goal, batches of benzhydrylamine-resin (BHAR) [28] containing 0.3–3.0 mmol/g amine functions and synthesized in strictly controlled conditions [29] were used for assembling the aggregating VQAAIDYING sequence, corresponding to the (65–74) acyl carrier protein aggregating segment [30]. Variable peptide contents ranging from less than 10% to about 80% were obtained with these peptide-resins and the influence of the solvent system were also examined by comparing the solvating characteristics of the resin beads in CDCl₃ and DMSO-d₆ – different polar organic solvents.

Finally, EPR spectroscopy was also applied to help to monitor the dynamics of peptide chains inside the resin beads [8–10,12]. The paramagnetic labelling of peptide-resins was carried out with the paramagnetic amino acid-type spin probe TOAC (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid) properly derived with the Fmoc-N^α-protecting group, which allows its covalent binding to any position in the peptide sequence [31].

EXPERIMENTAL

Materials

N^α-*tert*-butyloxycarbonyl (Boc)-amino acids were purchased from Bachem, Torrance, CA. Benzhydrylamine-resins (BHAR)

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were synthesized as previously reported [29] to obtain highly substituted resin batches. Solvents and reagents were purchased from Aldrich or Sigma Chemical Co. DMF was distilled (over P_2O_5 and ninhydrin under reduced pressure) before use. All solvents used for the swelling studies were HPLC grade and the chemicals met ACS standards.

Peptide Synthesis

The VQAAIDYING sequence was synthesized manually starting from different BHAR batches following the standard Merrifield Boc/Bzl strategy [32–34]. Cyclohexyl-(OcHex) and 2-Brcarbobenzyloxyl- (2-BrZ) side chain protecting groups were used for Asp and Tyr residues, respectively. The α -amino group deprotection and neutralization steps were performed in 30% TFA/DCM (30 min) and in 10% DIEA/DCM (10 min). The synthesis scale was 0.4 mmol and all Boc-amino acids were coupled with TBTU (2-(1-H-benzotriazole-1-yl)1,1,3,3-tetramethyl-uroniumtetrafluoroborate) in the presence of HOBt (hydroxybenzotriazole) and DIEA (diisopropylethylamine) at a 3, 3 and 4 fold excess over the amino component in the resin, respectively, using DMF or 20% DMSO/NMP. After a 2 h coupling time, the qualitative ninhydrin test was performed to estimate the completeness of the reaction and the re-coupling procedure was done when the ninhydrin test was positive. Cleavage reactions were carried out with the low-high HF procedure. The resin was rinsed with ethyl acetate and the peptide extracted in 5% acetic acid aqueous solution and lyophilized. In addition to the expected theoretical yield, the purity of the crude peptides was determined by HPLC. The HPLC conditions were: 0.1 M NaH_2PO_4 , pH 7.0 (solvent A) and acetonitrile:H₂O (9 : 1, v/v, as solvent B); a linear gradient from 5% to 50% of B in 45 min, flow rate of 1.5 ml/min, UV detection at 220 nm.

Measurement of Bead Swelling

Before being used in the peptide synthesis and the bead size microscopic measurement, all the amino protonated BHARs batches (Cl^- form) were sized by suspending in DCM, EtOH, and sifting in porous metal sieves to lower the standard deviations of the resin diameters to about 4%. Briefly, 150–200 dry and swollen beads of each resin, allowed to solvate overnight, were spread over a microscope slide and measured directly at low magnification. Since the sizes in a sample of beads are not normally, but log-normally, distributed, the central value and the distribution of the particle diameters were estimated by the more accurate geometric mean values and geometric standard deviations. All resins were measured with the amino groups in the unprotonated form obtained by 3 $\times$ 5 min TEA/DCM/DMF (1 : 4.5 : 4.5, v/v/v), followed by 5 $\times$ 2 min DCM/DMF (1 : 1, v/v) and 5 $\times$ 2 min DCM washing. After this treatment the resins were dried under vacuum until constant weight.

NMR Spectroscopy

All spectra were recorded on a Bruker DRX 400 MHz spectrometer equipped with a 4 mm $^1H/^{13}C/^{15}N$ triple resonance MAS probe head. The experiments were done without lock. Resin containing rotors were rotated at 5000 Hz.

The chemical shifts for ^1H HRMAS NMR are reported relative to water external standard.

EPR Studies

EPR measurements were carried out at 9.5 GHz in a Bruker ER 200 SRC spectrometer at room temperature ($22^\circ \pm 2^\circ C$) using flat quartz cells. Labelled peptidyl-resins were pre-swollen overnight in the solvent under study before running the spectra. The magnetic field was modulated with amplitudes less than one-fifth of the line widths, and the microwave power was 5 mW to avoid saturation effects.

RESULTS AND DISCUSSION

BHAR batches with 0.3, 1.6 and 2.6 mmol/g amine group substitution degrees were used to synthesize the VQAAIDYING sequence. A portion of peptide-resin was removed at intermediary positions ING, DYING, AIDYING for further swelling and spectroscopic studies. Peptide contents ranging from 6% to about 80% (weight/weight) were determined by amino acid analysis of the peptide-resins. To ascertain the homogeneity of each sequence synthesized, a small portion of the corresponding peptide-resin was cleaved and the purity of the crude peptides, estimated by analytical HPLC, was ca. 85%. The results from amino acid analysis and mass spectra were also consistent with the expected peptide sequences.

Swelling of Resins

In order to facilitate the interpretation of the resin HRMAS-NMR spectral findings, comparative swelling data of BHAR or peptide-BHAR in DCM and in the polar aprotic solvent DMSO were initially determined and are displayed in Table 1. As a replacement for DCM, more commonly used in peptide synthesis methodology, $CDCl_3$ was considered as a solvent in this study, assuming that both solvents will solvate equally the resin beads. This prediction was based on the similarity of the polarity values of both solvents regardless of the scale chosen. Values of 40.7 and 39.1 or 21.4 and 27.1 for DCM and $CHCl_3$, respectively, were found in the literature when the Dimroth-Reichardt ET30 [35] or the (AN + DN) amphoteric polarity [36,37] scales were considered, respectively.

In close accordance with these earlier polymer solvation studies [36,37], both BHAR batches displayed enhanced solvation in the less polar DCM as a consequence of the dominant influence of polystyrene backbone. Conversely, when attaching a significant amount of the more polar peptide sequences the very polar DMSO turns out to be the more appropriate solvent for solvating this peptide-resin (see swelling degrees of VQAAIDYING-BHARs in Table 1). It is also possible to verify that this alteration in the swelling

Table 1 Swelling Degree of Resins

Resin	Diam. dry bead (μm)	DCM		DMSO	
		Diam. swollen bead (μm)	Percentage of solvent within bead (%) ^a	Diam. swollen bead (μm)	Percentage of solvent within bead (%) ^a
BHAR 0.3 mmol/g	61	119	87	78	53
ING	56	111	87	82	68
DYING	63	99	74	100	75
AIDYING	67	79	39	98	69
VQAAIDYING	68	89	56	101	70
BHAR 3.0 mmol/g	79	144	84	109	62
ING	82	99	43	142	80
DYING	97	129	57	148	72
AIDYING	109	135	47	156	66
VQAAIDYING	116	145	49	173	70

^a [(swollen volume – dry volume)/swollen volume] $\times$ 100.

behaviour of the resin beads is typically dependent on the degree of peptide loading.

Peptide-free Resins

HRMAS-NMR spectra were initially acquired solely for the 0.3 and 3.0 mmol/g BHAR batches not attaching the peptide sequences. Figure 1 displays the results determined for these two resins swollen in CDCl_3 and in DMSO-d^6 . 1D and T2 filtered spectra after 20 ms of CPMG [38] are shown in panels A and B (Figure 1), respectively, for both BHAR batches. Due certainly to the lack of swelling of both BHAR batches in DMSO (Table 1), typical signals at 6–7 ppm assigned to polystyrene moieties were more clearly visible. These results confirm, as previously described in the literature [14,24,39], the importance of good solvation to obtain better quality HRMAS-NMR spectra. Worthy of note, was the direct relationship observed between the intensity of a peak appearing at about 7.2 ppm, usually attributed to amine functions, and the amount of this basic group of each resin. This finding suggests that this procedure might be considered as an alternative analytical protocol to estimate the substitution degree of BHAR-type resins by means of NMR experiments.

The T2 filtered spectra after 20 ms of CPMG usually shows only the most flexible components of the spectra [38]. Accordingly, the comparative spectra displayed in panels B (Figure 1) reveal more prominent and sharp peaks in the 6–7 ppm region only when in the improved solvating condition of the polymer matrices (both BHAR in CDCl_3 , panels B1 and B2). In comparison, a less pronounced peak was observed with the 3.0 mmol/g BHAR batch in DMSO-d^6 (panel B4). On the other hand, owing to the lack of solvation observed for 0.3 mmol/g BHAR in DMSO (53% swelling degree – Table 1), no significant resonance signal at 6–7 ppm was detected in panel B3.

Peptide-Resins

In the following steps, an investigation of the influence of the peptide sequence and loading was initiated. As a model, the intermediary ING sequence assembled in 0.3, 1.6 and 3.0 mmol/g BHAR (6%, 30% and 50% peptide content, respectively) was examined by ^1H -NMR. Figure 2 displays the comparative spectra of these peptidyl-resins in CDCl_3 and in DMSO-d^6 . In agreement with the variation of swelling degrees of these peptide-resins in both solvating conditions (Table 1), a clear spectral change was detected as the amount of polar peptide chains increased in the solid support. The higher the peptide content of the resin, the better was the ^1H -NMR peak resolution in the more polar medium (DMSO). As already emphasized, the dominant influence of the apolar polystyrene groups in the low peptide loaded resin (0.3 mmol/g BHAR) was strongly altered when higher amounts of polar peptide chains were attached to the polymer backbone (1.6 and 3.0 mmol/g BHAR). Accordingly, the greater chain mobility indicated by the appearance of well-defined peaks in the 6–8 ppm range (Figure 2) was only observed for the low peptide loaded ING-BHAR (0.3 mmol/g) when in CDCl_3 , or in DMSO-d^6 , for the two more heavily loaded resins (30% and 50% peptide contents). The inadequacy of DCM or CHCl_3 solvents to disrupt the strongly associated peptide chains inside the resin beads (maintained by a large amount of inter-chain hydrogen bonding) is well documented and is clearly visible in the immobilized spectra shown in panels B₁ and C₁ of Figure 2.

Alternatively, the difference in the NMR spectral quality of these peptide-resins can be examined by making use of the quality spectrum. In this case, the number of peaks, line shape and peak intensity of each spectrum were examined by looking at both the peptide and polymer resonances. Emphasizing again

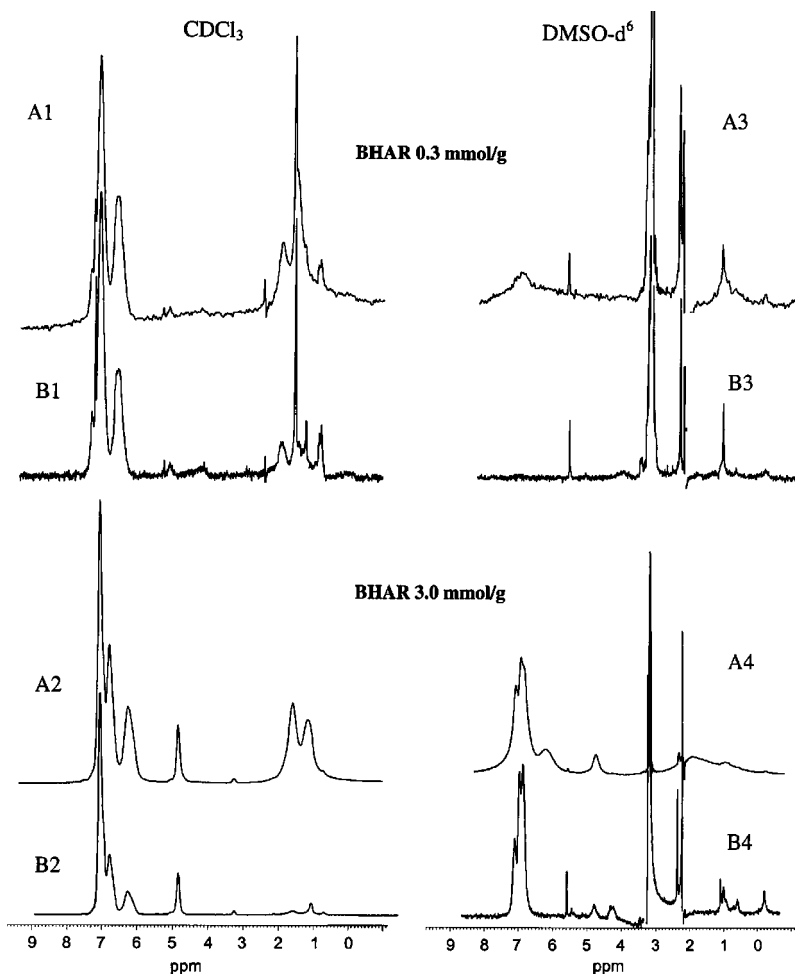


Figure 1 HRMAS NMR ^1H spectra (A) and T2 filtered spectra after 20 ms of CPMG (B): A1 and B1, BHAR with 0.3 mmol/g in CDCl_3 ; A2 and B2, BHAR with 3.0 mmol/g in CDCl_3 ; A3 and B3, BHAR with 0.3 mmol/g in DMSO-d_6 ; A4 and B4, BHAR with 3.0 mmol/g in DMSO-d_6 . Spectra were recorded at 400 MHz in a 4 mm rotor with ≈ 5 mg of resin spinning at the magic angle at 5 KHz.

the relevance of the solvation degree of the beads to the NMR evaluation of resins, a better quality of NMR spectra was only observed in CDCl_3 and in DMSO-d_6 for lower and heavier peptide loaded resins, respectively.

In addition, a significant decrease in the quality degree was detected when the peptide content of the ING aggregating sequence increased from 30% to 50% (1.6 and 3.0 mmol/g BHAR, respectively), thus suggesting the existence of a limit for the capacity of disruption of peptide chain aggregates, even when using a very strong electron donor solvent such as DMSO. Certainly, this limit will depend upon the peptide sequence and loading, the resin structure and the solvent system.

Protonated Peptide Resin

Relevant peptide conformational features may be obtained by altering the α -amine group ionization state, even when bound to a polymeric matrix as occurs during the solid phase peptide synthesis method [32,33]. These changes carry to the introduction of

in situ neutralization protocols, mainly in Boc chemistry [40,41]. This method gives a significant increase in the efficiency of chain assembly, especially for 'difficult' sequences arising from sequence-dependent peptide chain aggregation, in comparison with standard protocols (neutralization prior to coupling).

To check the influence of the *N*-terminal form (unprotonated or protonated with trifluoroacetate) in the aggregation of the peptide, the ING peptide sequence bound to BHAR of 3.0 mmol/g was examined by ^1H -NMR HRMAS.

Figure 3 displays the spectra of ^1H -NMR (A) and T2 filtered HRMAS NMR 1D (B) of protonated ING-BHAR of 3.0 mmol/g in DMSO-d_6 . When the tripeptide ING was in the protonated form it displayed a better quality spectral degree, mainly in the amide proton region, corresponding to the three amino acids, when compared with the unprotonated form (Figure 2C2). On the other hand, the swelling and EPR mobility did not follow the spectral quality obtained by NMR. In this resin, the swelling was 82% in the unprotonated and 83%

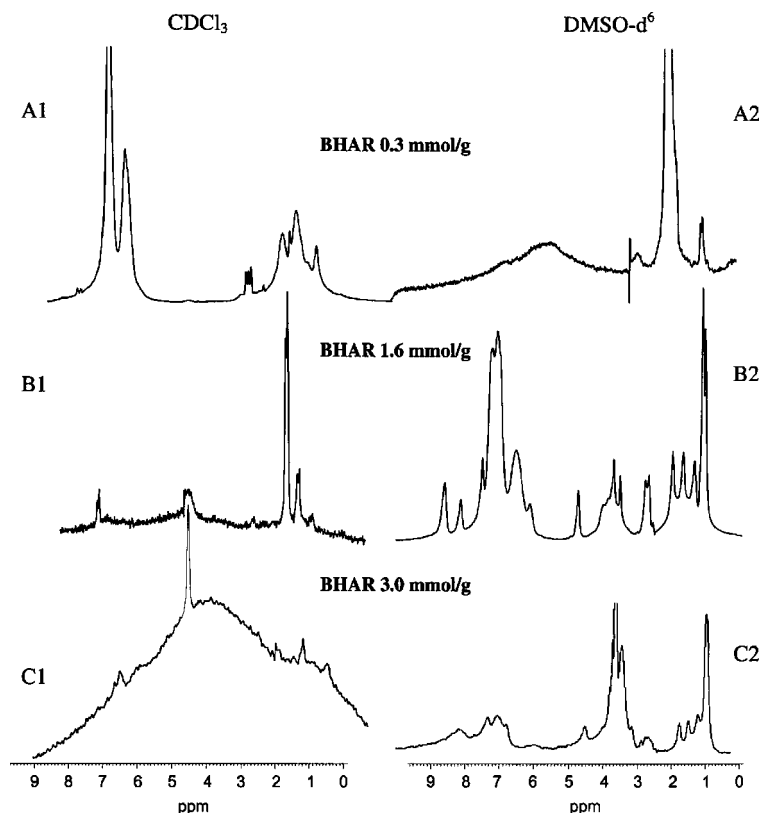


Figure 2 HRMAS NMR ^1H spectra of ING-BHAR with 0.3 (A), 1.6 (B) and 3.0 (C) mmol/g in CDCl_3 (1) and DMSO-d_6 (2). Spectra were recorded at 400 MHz in a 4 mm rotor with ≈ 5 mg of resin spinning at the magic angle at 5 KHz.

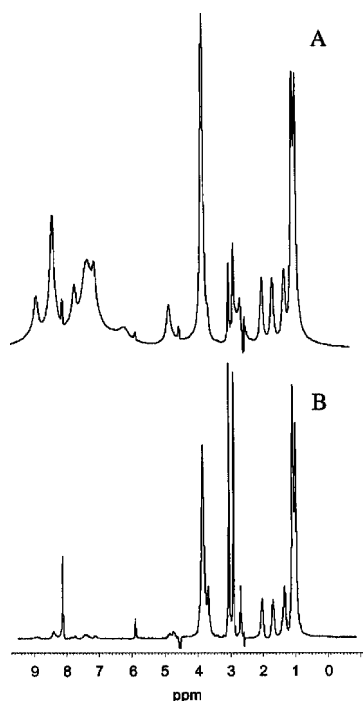


Figure 3 HRMAS NMR ^1H spectra (A) and T2 filtered spectra after 20 ms of CPMG (B) of ING-BHAR of 3.0 mmol/g in DMSO-d_6 (amine groups in protonated form). Spectra were recorded at 400 MHz in a 4 mm rotor with ≈ 5 mg of resin spinning at the magic angle at 5 KHz.

in the trifluoroacetate form. For the EPR studies, the mobility estimated by the central peak line width (W_0), which reflects the degree of motion where the probe is located, was the same (3.0 G). This failure is attributed to the greater sensitivity of the NMR technique to find change conformations in the whole peptide, while the EPR shows only the mobility, in this sequence, of the *N*-terminal group.

Monitoring the synthesis of VQAADYING in highly loaded conditions

To analyse the viability of peptide syntheses in highly substituted conditions (3.0 mmol/g), the solvation study was extended specifically in DMSO to several minor fragments. Emphasis was given to this challenging protocol, because the synthesis in heavily peptide loaded condition can achieve less consumption of solvents and in time to obtain larger amount of peptide. Even in terms of application for other approaches such as for a peptide library, a greater number of peptide chains per bead would be essential for helping to characterize the amino acid sequences of assembled peptides or for detecting more easily the interaction processes of enzymes with peptide segments.

NMR study. The ^1H NMR spectra of ING, DYING, AIDY-ING and VQAADYING bound to highly substituted

3.0 mmol/g BHAR and solvated in DMSO are represented in Figure 4. Due to the occurrence of strong shrinking of the beads in apolar solvents (Table 1), the NMR experiments were not carried out in this type of solvent system (ex. CDCl_3). Despite the similarity of swelling of the four peptide-resins in DMSO (Table 1), the ^1H -NMR or T2 filtered HRMAS NMR 1D spectra (columns I or II, Figure 4) showed a significant difference between these highly peptide loaded resins. The degree of mobility of the peptide chains inside the resin matrix seems to be clearly sequence dependent and certainly aggravated by the high peptide content inside the resin bead. The stronger immobilization was observed in the complete VQAAIDYING segment (Figure 4D). The greater number of well-resolved background signals in the aromatic and aliphatic regions of the spectra, besides peaks in the 7–9 ppm region, could be attributed to chemical shifts of different hydrogen from the secondary structure under investigation [27]. The smallest sequence (ING) has numerous background

signals in the aromatic and aliphatic regions of the spectra and provides good-quality spectra. Of note, a greater motion degree of the peptide-resin backbone was detected more clearly for the intermediary AIDYING sequence, thus stressing again the sensitivity of the NMR spectra to detect the sequence-dependent occurrence of peptide chain associations throughout the polymer matrix.

EPR study. Paralleling the NMR investigation, EPR spectroscopy was also applied specifically to the challenging condition of synthesized aggregating sequence in highly substituted resins. Thus, ING, AIDYING and VQAAIDYING segments assembled in the 3.0 mmol/g BHAR were labelled at their *N*-terminal portion with the Fmoc-TOAC amino acid-type probe [31], as previously described in the literature [8,9]. In order to avoid a spin-spin exchange interaction which broadens the EPR lines [42], the paramagnetic labelling was kept as low as possible [8,9] and quantified by the modified picric acid methodology [43] before

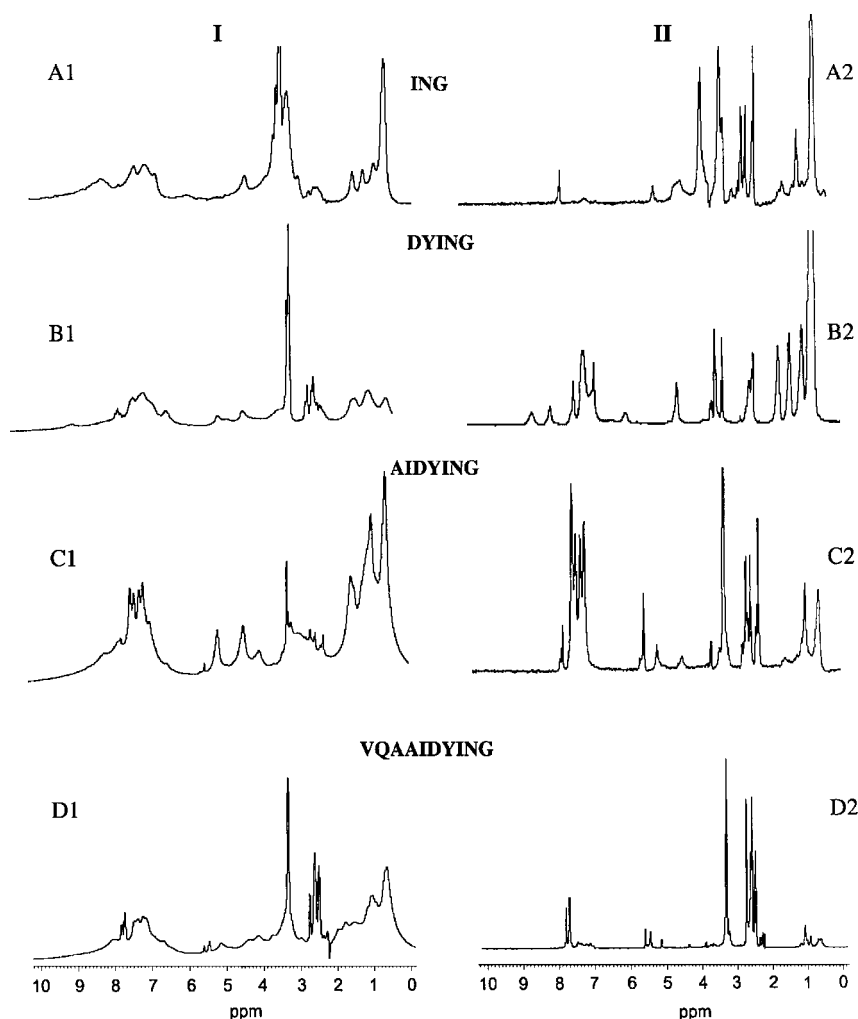


Figure 4 HRMAS NMR ^1H spectra (column I) and T2 filtered spectra after 20 ms of CPMG (column II) of ING, DYING, AIDYING and VQAAIDYING fragments bound to 3.0 mmol BHAR and swollen in DMSO-d_6 . Spectra were recorded at 400 MHz in a 4 mm rotor with ≈ 5 mg of resin spinning at the magic angle at 5 KHz.

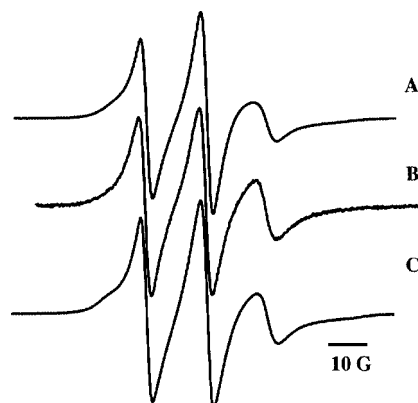


Figure 5 EPR spectra of A (ING), B (AIDYING) and C (VQAADYING) bound to 3.0 mmol/g BHAR in DMSO.

and after coupling the Fmoc-TOAC spin label. Moreover, the low labelling protocol allows the physicochemical and steric perturbations due to the introduction of the spin probe to be kept to a minimum, decreasing their influence on the solvation characteristics of the peptidyl-resin under investigation.

Figure 5 displays the EPR spectra of the three peptide-resins. By measuring the mid-field line width (W_0) of the spectra – the greater the W_0 values, the more immobilized is the position where the spin probe is attached – values of 3.0, 3.0 and 3.1 G were measured for ING-, AIDYING- and VQAADYING-BHAR (3.0 mmol/g) resins, respectively. These results depicted that a more aggregated condition is detected with the octapeptide sequence, in agreement with previous NMR investigation.

CONCLUSION

These findings reinforced the validity of examining the dynamics of solvated polymeric structures using spectroscopic approaches, mainly by the sensitive HRMAS-NMR technique. The combination of this method with EPR seems to be an efficient approach to investigate clusters of microenvironments spread throughout the peptide-polymer backbone, thus helping to overcome the severe synthesis conditions such as those deliberately employed in the present work (synthesis of strong aggregating sequence in very high peptide loading condition).

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Anexo 7

EPR investigation of the influence of side chain protecting groups on peptide–resin solvation of the Asx and Glx model containing peptides

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Abstract—In spite of all progressive efforts aiming to optimize SPPS, serious problems mainly affecting the assembly of aggregating sequences have persisted. Following the study intended to unravel the complex solvation phenomenon of peptide–resin beads, the XING and XAAAA model aggregating segments were labeled with a paramagnetic probe and studied via EPR spectroscopy. Low and high substituted resins were also comparatively used, with the X residue being Asx or Glx containing the main protecting groups used in the SPPS. Notably, the *cyclo*-hexyl group used for Asp and Glu residues in Boc-chemistry induced greater chain immobilization than its *tert*-butyl partner-protecting group of the Fmoc strategy. Otherwise, the most impressive peptide chain immobilization occurred when the large trytil group was used for Asn and Gln protection in Fmoc-chemistry. These surprising results thus seem to stress the possibility of the relevant influence of the amino-acid side chain protecting groups in the overall peptide synthesis yield.

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After the seminar proposition of the solid-phase peptide synthesis (SPPS) method in the literature,¹ this innovative approach still suffers from some drawbacks related mainly to the difficulty in achieving complete incorporation of amino acid residues in peptide chain elongation.² This usually occurs during the synthesis of particular sequences characterized by presenting a tendency of strong chain association. To overcome this type of problem, which is in most cases sequence-dependent, innumerable studies have appeared in the last few decades aiming at improving the SPPS methodology. These efforts comprised the use of more reactive coupling reagents,^{3,4} elevated temperature,⁵ alternative solid supports^{6,7} and microwave radiation to optimize amino-acid coupling reactions.^{8,9} Nevertheless, a significant part of these efforts have failed to give us a better understanding of the physicochemical features of the complex peptide–resins in solvated state. Neither have they

helped us discover approaches or strategies that might help overcome specific chain growth difficulties. In this context, many reports have applied the classical procedure of measuring the swelling volume of resins in different solvent systems.^{10–12} However, the majority of works have indeed used spectroscopic methods such as IR,^{13,14} NMR,^{15–17} fluorescence and EPR.¹⁸ In our case, the peptide resin swelling determination was also the first approach tested to better understand the peptide–resin solvation. But in this context, this process is considered a type of solvent effect investigation and the solute is a collection of model peptide–resins. Thus, by handling a dozen peptide–resins and solvent systems differentiated by parameters such as their polarity or acid/base properties, it has been possible to propose some rules that seem to govern peptide–polymer solvation.^{19,20} In addition, this investigation also allowed the proposition of a novel and dimensionless solvent polarity scale, which proved to be more practical and sensitive than all those existing to date in the literature.²⁰ As a continuation of this effort, we started a different strategy where the EPR method was conjugated with the use of the stable free radical TOAC (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid)²¹ derived from

Keywords: Solid-phase peptide synthesis; Difficult sequences; Aggregation; EPR; Swelling.

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its amino group with the *tert*-butyloxycarbonyl (Boc)²² or 9-fluorenylmethyloxycarbonyl (Fmoc).²³ These groups temporarily protect the TOAC, thus allowing its coupling to peptide sequences in solution or attached to polymers and even to other active group-containing macromolecules or systems. The goal in this case was to determine the mobility of peptide chains in different conditions and make a correlation to the efficiency of coupling reaction in the synthesis.

The latter strategy fortunately has led us to obtain some important information in respect to peptide–resin characteristics, comprising some rules of peptide chain solvation and detection of strong aggregated sequences^{24,25} and the effect of an increase in temperature.²⁶ More recently, a real-time monitoring of the coupling reaction in the resin²⁷ or calculation strategy of unusual polymer parameters was also demonstrated. Examples of this are the number of sites per bead, the concentration and the distance existing between these sites when in the solvated state.²⁸ Lastly and of great relevance to the present Letter, it was possible to confirm a direct relationship existing between solvation degree of the bead and chain mobility with the rate of coupling reactions.^{25,28,29}

Thus, taking into account all the progress achieved to date in the understanding of the solvation phenomenon during the peptide chain assembly, we now decided to start evaluating the influence of the amino-acid side chain protecting groups in the overall solvation degree of peptide–resins. This effect has not yet been evaluated and we decided to combine for this purpose, the use of the EPR method and the TOAC labeling strategy. To maximize possible problems for appropriate solvation of peptide–resins, two well-known aggregating peptide sequences were selected. For the EPR experiments, the strategy already applied²⁵ was selected and, the higher the central field peak line-width (W_0) values, the greater the immobilization of the labeled peptide chains.

The ING (65–74) fragment of the acyl carrier protein³⁰ and polyalanine AAAA¹⁷ sequence, both known as aggregating segment, were synthesized via the conventional Fmoc/*t*-Bu-solid phase method using methylbenzhydrylamine-resin (MBHAR), either in low (0.5 mmol/g) or in highly substituted conditions (2.3 mmol/g). The reason for the use of the latter resin lies in the idea of promoting deliberately stronger chain associations inside the beads. After the assembly of both sequences separately in these two MBHAR batches, guest amino acids (Asx or Glx) were introduced at the N-terminal extremity of their sequence. The Trt (trityl) group was selected to protect the Asn and Gln residues; and the *t*-Bu (*t*-butyl) was selected for Asp and Glu (both used in Fmoc/*t*-Bu chemistry).³¹ In the case of Boc chemistry,³² Asn and Gln were evaluated either in free form or protected with the Xan (xanthenyl) group, whereas the *c*-HxO (*cyclo*-hexyl) or Bzl (benzyl) groups were studied as protecting groups for the Asp and Glu residues. The integrity of the synthesized peptide resins was verified by cleaving a small portion of the sample and the crude peptides were characterized by analytical HPLC, amino acid analysis and mass spectrometry. For

the EPR studies, the peptidyl-resins were thus labeled with the Fmoc–TOAC derivative and in order to avoid spin–spin exchange interactions, which may broaden the EPR lines, and to minimize possible physicochemical and steric perturbations, the extent of labeling was kept as low as possible. It was also assumed that the TOAC-labeled peptide chains are dispersed homogeneously throughout the resin matrix and behave similarly to the unlabeled chains in all solvent systems tested. Samples were placed in flat quartz cells and EPR measurements were carried out at 9.5 GHz in a Bruker ER 200 spectrometer using 298 K as the temperature. The magnetic field was modulated with amplitudes less than one-fifth of the line widths and the microwave power was 5 mW to avoid saturation effects.

For example, Figure 1 displays the EPR spectra of low peptide-loaded (0.5 mmol/g) DAAAA–MBHAR swollen in DMF, NMP and DMSO solvents. In DMSO, which is a polar and strong nucleophilic solvent, the spectrum displays two components, one with broad lines and the other with narrow lines, corresponding to strongly and weakly immobilized spin label populations, respectively. This second component was found in MBHARs in most of the low-substitution peptide–MBHARs. This is in accordance with the dominant influence of the 1% polystyrene–styrene apolar matrix of the solid support over the more polar attached peptide chains. Table 1 summarizes the peptide chain mobility degrees estimated by W_0 values of all these peptide–resins in DMF, NMP and DMSO. In these peptides only the N-terminal residue (Asx or Glx) and the corresponding protecting groups were changed, as necessary.

The evaluation of the W_0 values of this table allows the following conclusions: (i) the ING sequence is more aggregated than the AAAA segment. This conclusion was obtained by the greater average W_0 values of the former sequence, regardless of the substitution degree of the resin or the solvent used; (ii) using these same W_0 solvation data, DMSO seems to be the less appropriate solvent for solvating peptide–resins in a low peptide-content condition (this solvent presented greater W_0

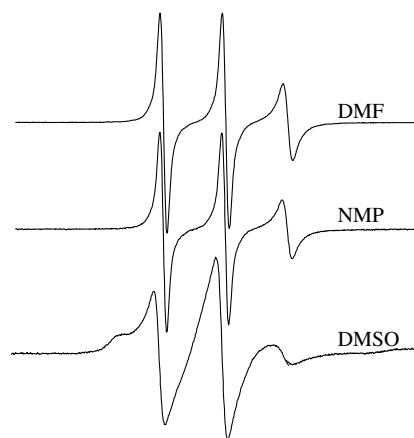


Figure 1. Effect of solvent on EPR spectra of TOAC-labeled low load DAAAA–MBHAR.

Table 1. Effect of side chain protector on the EPR spectra of low and highly loaded TOAC–XAAAA and XING–MBHAR swollen in DMF, NMP and DMSO

X residue/protector	W_0 (G)					
	DMF ^a	NMP ^a	DMSO ^a	DMF ^b	NMP ^b	DMSO ^b
<i>XAAAA</i>						
Asp (<i>t</i> -Bu)	1.74	1.67	2.93	1.80	1.83	1.77
Asp (Bzl)	1.87	1.73	2.95	1.95	2.03	2.24
Asp (<i>c</i> -HxO)	2.51	1.99	4.82	1.95	1.94	2.12
Glu (<i>t</i> -Bu)	1.79	1.84	3.04	1.85	1.92	2.06
Glu (Bzl)	1.84	1.99	3.20	1.91	2.02	1.92
Glu (<i>c</i> -HxO)	1.81	1.88	3.20	1.99	2.04	2.05
Asn (NH ₂)	1.79	1.81	2.05	1.90	2.03	1.84
Asn (Xan)	1.77	1.81	1.97	1.95	2.11	1.82
Asn (Trt)	1.91	1.93	2.80	2.10	2.19	2.12
Gln (NH ₂)	1.80	1.84	1.99	1.96	1.96	1.82
Gln (Xan)	1.84	1.83	1.96	1.87	1.96	1.99
Gln (Trt)	1.90	1.97	3.25	2.01	2.16	2.10
<i>XING</i>						
Asp (<i>t</i> -Bu)	1.88	1.87	1.89	1.93	2.09	1.92
Asp (Bzl)	1.82	2.17	2.12	2.13	2.60	2.17
Asp (<i>c</i> -HxO)	2.30	2.60	2.26	1.99	2.18	2.07
Glu (<i>t</i> -Bu)	1.95	1.76	1.86	1.91	2.00	1.94
Glu (Bzl)	1.88	1.95	1.88	2.01	2.26	1.97
Glu (<i>c</i> -HxO)	1.91	1.86	1.96	1.98	2.20	2.03
Asn (NH ₂)	1.95	2.06	2.02	2.05	2.20	2.10
Asn (Xan)	2.17	2.70	2.08	2.14	2.32	2.01
Asn (Trt)	2.37	1.91	Powder spectra	2.59	2.57	2.67
Gln (NH ₂)	2.11	1.79	2.37	2.00	2.30	1.71
Gln (Xan)	1.99	2.01	2.27	2.00	2.34	1.80
Gln (Trt)	2.25	2.21	3.91	2.12	2.35	2.47

^a 0.5 mmol/g.^b 2.3 mmol/g.

values in comparison with DMF or NMP); (iii) in contrast with the aforementioned, no significant difference in the solvation degree was verified among these three solvents when heavily substituted MBHAR is used; (iv) in terms of Boc-chemistry, no significant difference is observed when Asn or Gln residues contain their side chains in free form or attached to the Xan group; (v) otherwise in Fmoc chemistry, the Trt protection induced the more pronounced immobilization of peptide chains listed in Table 1; (vi) when the EPR mobility of Asp and Glu residues attached to the peptides are examined, one can conclude that the *c*-HxO protecting group induces greater chain immobilization than Bzl (in Boc chemistry) and also than *t*-Bu (in Fmoc chemistry).

Collectively, these data strongly point out that the type of single-protecting group significantly affects the overall peptide chain mobility during the peptide synthesis. An increase of about 0.1 G or higher for the W_0 parameter has been previously correlated with a significant decrease in the rate of coupling reaction.^{24,25} These findings thus strongly suggest that depending on the side chain protecting groups used for the synthesis, pronounced influence in the overall solvation of the peptide resin can occur with significant consequences to the synthesis. The controversy that still exists in comparing the efficiency of Boc and Fmoc-synthesis strategies^{33–35} must be therefore discussed in the light of the solvation influence over the side chain protecting-group mobility of each chemical strategy. This possibility has currently

been investigated making different combinations of the presence of these protecting groups in peptide sequence models. This has been done to further the application of the EPR/TOAC strategy aiming at verifying the corresponding influence for the solid-phase peptide synthesis methodology.

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Anexo 8

Anexo 9

Evaluation of the Trifluoromethanesulfonic Acid/Trifluoroacetic Acid/Thioanisole Cleavage Procedure for Application in Solid-Phase Peptide Synthesis^{1,2)}

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As an extension of our investigation of peptidyl-resin linkage stability towards different cleavage procedures used in the solid-phase peptide synthesis (SPPS) technique, the present paper evaluated the trifluoromethanesulfonic acid (TFMSA)/trifluoroacetic acid (TFA)/thioanisole method, varying the type of resin (benzhydrylamine-resin, BHAR; methylbenzhydrylamine-resin, MBHAR and 4-(oxymethyl)-phenylacetamidomethyl-resin, PAMR) and peptide resin-bound residue (Gly and Phe). The vasoactive angiotensin II (AII, DRVYIHPF) and its [Gly⁸]-AII analogue linked to those resins used routinely in *tert*-butyloxycarbonyl (Boc)-SPPS chemistry were submitted comparatively to a time course study towards TFMSA/TFA cleavage. At 0 °C, [Gly⁸]-AII was completely removed from all resins in less than 6 h, but the hydrophobic Phe⁸ moiety-containing AII sequence was only partially cleaved (not more than 15%) from BHAR or MBHAR in this period. At 25 °C, [Gly⁸]-AII cleavage time decreased to less than 2 h irrespective of the solid support, and quantitative removal of AII from PAMR and MBHAR occurred in less than 3 h. However, about 10–15 h seemed to be necessary for cleavage of AII from BHAR, and in this extended cleavage reaction a significant increase in peptide degradation rate was observed. Regardless of the cleavage temperature used, the decreasing order of acid stability measured for resins was BHAR>MBHAR>PAMR. Collectively, these findings demonstrated the feasibility of applying TFMSA/TFA solution as a substitute for anhydrous HF at the cleavage step in Boc-SPPS methodology. Care should be taken however, as the cleavage efficacy depends on multiple factors including the resin, peptide sequence, the time and temperature of reaction.

Key words peptide synthesis; peptidyl-resin cleavage; trifluoromethanesulfonic acid; 4-(oxymethyl)-phenylacetamidomethyl-resin; benzhydrylamine-resin; methylbenzhydrylamine-resin

We recently reported³⁾ a rule for resin selection routinely used in *tert*-butyloxycarbonyl (Boc)-peptide synthesis chemistry.^{4,5)} This study was based upon the quantitative determination of the lability of model peptidyl-resin linkages towards both TFA amine group deprotection and HF final peptide cleavage steps. These effects depend basically upon the lability of the peptide-resin linkage which, in turn, is affected by the resin itself, the C-terminal amino acid and how often this chemical bond is submitted to TFA treatment (possibility of premature chain loss) during peptide growth. A rule for the choice of the best resin to be used depending on the peptide sequence to be synthesized was proposed. However, due to well-known practical and safety problems concerning use of the HF method, other acidolytic strategies have been proposed in the literature.^{6–8)} The present work alternatively evaluates the potentiality of the TFMSA/TFA/thioanisole cleavage method^{9–11)} for Boc-solid phase synthesis.

Thus, the present investigation reports data found in the time-course study of TFMSA/TFA/thioanisole treatment of peptidyl-resins using solid supports routinely applied for Boc-peptide synthesis chemistry. Amongst these, the benzhydrylamine-resin (BHAR)¹²⁾ and methylbenzhydrylamine-resin (MBHAR)¹³⁾ are both used for the synthesis of peptide-amides and 4-(oxymethyl)-phenylacetamidomethyl-resin (PAMR)¹⁴⁾ for peptide-acids. The vasoactive angiotensin II (AII, DRVYIHPF)¹⁵⁾ and its [Gly⁸]-AII analogue were selected. Although this cleavage procedure is used routinely at low temperature in solution synthesis,^{16,17)} we decided to compare the rate of peptide chain removal from a total of six

peptidyl-resins at both 0 °C and 25 °C. Therefore, the main goal was to investigate the influence of different factors upon the efficacy of the TFMSA/TFA method. These findings would further facilitate the resin selection to be used and the correct protocol for application of the acid cleavage method which may depend on the type of peptide sequence to be assembled.

Table 1 shows the TFMSA/TFA/thioanisole comparative cleavage yields at 0 °C of Phe or Gly-bearing AII at its C-terminal position and bound to BHAR, MBHAR and PAMR. As expected [Gly⁸]-AII was much more easily removed from the resin than the hydrophobic or larger Phe residue-containing AII sequence. If the lability dependence to the type of resin was considered, the following decreasing order of peptidyl-resin stability was observed: BHAR>MBHAR>PAMR. Intriguingly, the higher lability of peptide linkage to

Table 1. Percentage of TFMSA/TFA/Thioanisole Cleavage (at 0 °C) of AA⁸-AII-Resins

Peptidyl-resin	Cleavage time (h)				
	0.5	1	2	4	6
[Gly ⁸]AII-BHAR	15	39	62	83	100
[Gly ⁸]AII-MBHAR	37	64	100	—	—
[Gly ⁸]AII-PAMR	78	91	100	—	—
[Phe ⁸]AII-BHAR	0	0	0	1	5
[Phe ⁸]AII-MBHAR	0	1	6	12	13
[Phe ⁸]AII-PAMR	38	82	100	—	—

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PAMR than to MBAR is not in agreement with previous results obtained during peptidyl-resin HCl/propionic acid hydrolysis¹⁸⁾ or time course HF and TFA³⁾ investigations.

The [Gly⁸]-AII sequence was completely removed in approximately 2 h from PAMR and MBHAR and in 6 h from BHAR at 0 °C, while the more stable [Phe⁸]-AII-resin linkage was cleaved quantitatively in 2 h from PAMR but only partially from MBHAR or BHAR. At this low temperature, the yield of [Phe⁸]-AII cleavage bound to these two amineresins reached values as low as 13 and 5%, respectively, after extended 6 h reaction. By comparing results earlier reported for these two peptidyl-resins at 0 °C in HF,³⁾ the apparent rate of cleavage seems to be higher in this than in the TFMSA/TFA-containing mixed solution.

In an advantage over the HF method where use of a low temperature is mandatory,¹⁹⁾ the TFMSA/TFA strategy might allow an increase in temperature during the cleavage process. Table 2 displays the time course data obtained at 25 °C. As expected, [Gly⁸]-AII removal from MBHAR and BHAR was accelerated (around 2 and 3 h, respectively) but for the more acid stable [Phe⁸]-AII-resin linkage, the peptide sequence was only quantitatively cleaved after approximately 3 h and 10–20 h when bound to MBHAR and BHAR, respectively.

At this latter forceful cleavage condition, a decrease in the peptide purity was monitored through analytical RP-HPLC. Figure 1 shows the corresponding chromatogram profiles of crude AII cleaved from BHAR in 1, 6 and 24 h with

TFMSA/TFA at 25 °C. After a cleavage time of nearly 6 h, it is possible to see the appearance of a contaminant overlapped with the AII main peak (at around 6 min) which increased significantly in the 24 h-cleavage time HPLC profile. By quantifying the main peak area in each displayed chromatogram, it is possible to conclude that peptide degradation yield reached values around 20–25% in 24 h. Certainly this degradation rate might be more related to AII-type sequence. Further experimental investigations are needed with other sequences to better evaluate the validity in associating high temperature with extended cleavage reaction time.

Lastly, to better evaluate the rule for TFMSA/TFA application depending upon the resin, peptide length and the nature of its C-terminal residue, Table 3 presents the theoretical syntheses yield estimated when, for instance, cleavage of a short and long (16 and 40-mer) peptide sequence is to be carried out at 0 °C and 25 °C. For this estimation, the most common 2 h-cleavage time was employed for quantitative estimation of the overall decrease in synthesis yield. These values were obtained by considering simultaneously the already quantified yield of chain loss of these peptides during 8 and 20 h-TFA deprotection³⁾ (corresponding to 30 min treatment of both 16 and 40 amino acid-long peptides) and the incomplete TFMSA/TFA cleavage values displayed in Tables 1 and 2. This was done simply by multiplying the yield of both acid treatments, in percentage. It must be stressed that no other source of side reactions or chemical problems during the synthesis was considered, other than that derived from the TFA and final cleavage steps.

The analysis of data displayed in Table 3, in association with those of Tables 1 and 2 provided the following conclusions/comments:

(i) Despite the resin or peptide sequence, PAMR is always adequate for TFMSA/TFA cleavage regardless of temperature. The lowest yield when using this resin (88%, Table 3) can be estimated, when a 40-mer long sequence is going to be synthesized. The 12% decrease in the yield is essentially due to the chain loss during the prolonged (20 h) TFA treat-

Table 2. Percentage of TFMSA/TFA/Thioanisole Cleavage of AA⁸-AII-Resins at 25 °C

Peptidyl-resin	Cleavage time (h)						
	0.5	1	2	3	4	6	24
[Gly ⁸]AII-BHAR	75	88	97	100	—	—	—
[Gly ⁸]AII-MBHAR	87	96	100	—	—	—	—
[Phe ⁸]AII-BHAR	—	20	70	73	84	87	100
[Phe ⁸]AII-MBHAR	—	49	89	100	—	—	—

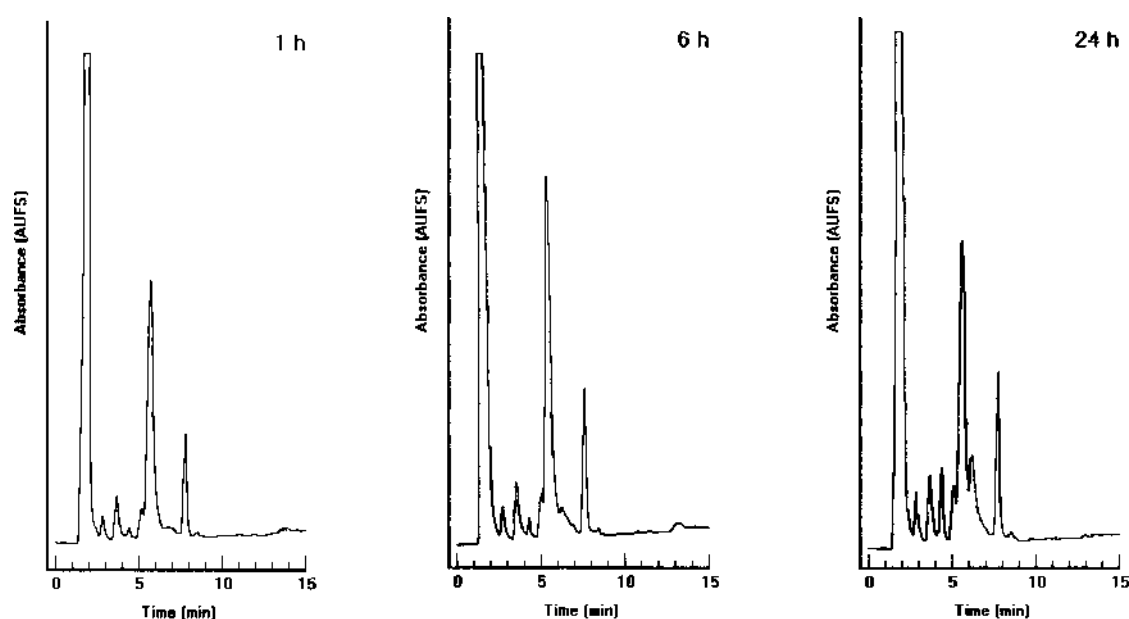


Fig. 1. RP-HPLC Profiles of the [Phe⁸]-AII-BHAR after 1, 6 and 24 h TFMSA/TFA/Thioanisole Treatment at 25 °C

Table 3. Theoretical Synthesis Yield of Model Peptidyl-Resins Considering the TFA and 2 h TFMSA/TFA/Thioanisole Treatments at 0 °C and 25 °C^{a)}

Resins	Theoretical yield (%)					
	BHAR		MBHAR		PAMR	
Number of residues	16	40	16	40	16	40
C-terminal residue	0 °C					
Gly	60	57	96	86	96	88
Phe	0	0	6	5	96	91
	25 °C					
Gly	94	89	96	86	96	88
Phe	68	67	86	81	96	91

a) No other sources of error or side reactions were considered in this study other than those derived from partial TFMSA/TFA/thioanisole or TFA peptide chain cleavages.

ment. No decrease in the yield is due to the cleavage step as it is complete in the 2 h-reaction.

(ii) Regarding MBHAR solid support, the feasibility of the use of TFMSA/TFA cleavage procedure is highly dependent on the nature of the C-terminal residue. For a hydrophilic and small amino acid such as Gly, it is possible to obtain over 85% synthesis yield, regardless of the temperature used in the reaction. However, its use is limited in the case of peptides with hydrophobic C-terminal residues such as Phe. Synthesis yield of about 80% (even at 25 °C) and not higher than 6% (at 0 °C) are expected when cleaving Phe-MBHAR-type compound for 2 h.

(iii) As expected, more severe restriction for use of the TFMSA/TFA method is observed when BHAR is employed. Acceptable yield is only obtained at higher temperature (25 °C) and when the peptide contains Gly-type hydrophilic residues at its carboxy-terminal portion. For sequences containing hydrophobic moiety at this position, the synthesis yield using BHAR does not reach 70% even at 25 °C, in the standardized 2 h cleavage protocol. This finding is relevant as we have already demonstrated that depending upon the peptide length and the nature of its C-terminal residue, BHAR is more appropriate than MBHAR for peptide synthesis.³⁾ This is basically due to higher acid stability of peptide-BHAR linkage which avoids premature peptide chain detachment from the resin during the prolonged TFA treatment.

(iv) To protect the peptide integrity when solution synthesis is used, most chemists carry out TFMSA/TFA cleavage at 0 °C. Our results emphasized that neither MBHAR nor BHAR can be employed at this low-temperature condition when attaching peptides containing hydrophobic residue at their C-terminal position. In this case, syntheses yields around 6% and zero are estimated for these two resins, respectively. In the more severe case (BHAR), even if a peptide sequence containing hydrophilic moiety at its C-terminal portion is synthesized, the final yield did not achieve values higher than 60% (Table 3).

In conclusion, the feasibility of using the TFMSA/TFA/thioanisole cleavage procedure for Boc-SPPS was demonstrated but with some restrictions. Higher cleavage temperature combined with extended reaction time might be a valid alternative to overcome some of these problems. Care should

be taken in this case, mainly concerning possible degradation of the peptide sequence containing acid-labile residues. Despite this shortcoming the most relevant advantage of the TFMSA/TFA method lies in its simplicity and safety compared with the HF procedure which requires a special perflon-type apparatus and the handling of a very dangerous vapor acid.

Since its inception, the SPPS methodology has been progressively improved through a great variety of experimental investigations aiming for instance, to overcome incomplete α -amine group deprotection,²⁰⁾ difficulties in coupling reactions with the use of efficient acylating reagent,^{21,22)} elevated temperature^{23,24)} or by enhancing the knowledge of the peptidyl-resin solvation phenomenon.^{25,26)} Spectroscopic techniques of NMR,^{27,28)} IR^{29,30)} or EPR^{31,32)} with the use of a paramagnetic amino acid³³⁾ or associated with fluorescence³⁴⁾ have been intensively applied to achieve the same objective. In this context, the decrease in overall synthesis yield due to premature chain detachment in the TFA step combined with possible incomplete peptide cleavage from the resin has been routinely neglected. The present work emphasized that depending upon the chemical strategy to be used, this decrease in synthesis yield as a consequence of these two acid steps of the synthesis cycle can be comparatively relevant.

Experimental

Most of the solvents and reagents were purchased from Fluka or Aldrich and all met ACS standards. Trifluoroacetic acid was acquired from Fluka and the anhydrous hydrogen fluoride (5 l capacity cylinder) was from Merck Co.

Peptide Synthesis Stepwise build-up of the peptides was done manually by Boc-chemistry solid phase methodology. Benzhydrylamine resin (BHAR), methylbenzhydrylamine resin (MBHAR) and 4-(oxymethyl)-phenylacetamidomethyl-linker-containing resins (PAMR) with substitution degree ranging from 0.2 to 0.6 mmol/g were used. These resins were acquired from different companies (Advanced Chemtech, Novabiochem, Peninsula and Bachem). The *N*^α-*tert*-butyloxycarbonyl (Boc) protecting group was removed with 30% trifluoroacetic acid (TFA) in dichloromethane (DCM) in the presence of 2% anisole for 30 min. The following side chain protecting groups were used: mesitylene-2-sulfonyl (Mts) for Arg and benzyloxymethyl (Bom) for His, 2-bromobenzyloxycarbonyl (2-BrZ) for Tyr and cyclohexyl group (cHex) for Asp. Amine group neutralization was performed for 1×1 min and 1×10 min with 10% triethylamine (TEA). Coupling reactions were done using 2.5 excess of Boc-amino acid/2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/diisopropylethylamine (DIEA) (1 : 1 : 2) in DCM or in 1 : 1 (v/v) DCM/dimethylformamide (DMF) mixture. All couplings were monitored by qualitative ninhydrin test. Before TFMSA and TFA time-course investigations, a small amount of each sample was cleaved by HF. The purity of the crude peptide was assessed by analytical reverse-phase high performance liquid chromatography (RP-HPLC) and the structure was confirmed by the LC/MS and amino acid analysis.

Amino Acid Analysis Prior to the TFMSA and TFA time-course studies, all peptidyl-resins were hydrolyzed with 12*N* HCl/propionic acid mixture for 100 h at 130 °C to guarantee quantitative removal of peptide chains from the resin as recently proposed.¹⁸⁾ Pyrex tubes with plastic Teflon-coated screw caps (13×1 cm) were used for the hydrolyses and the amino acid analyses were performed in a Beckman System 6300 amino acid analyzer to determine the amount of peptide attached to the resin.

TFMSA/TFA/Thioanisole Time Course Cleavage Study In several small round-bottom flasks equipped with a micro stirring bar, 50 μ l of *m*-cresol and 124 μ l of thioanisole were added to isolated portions of protected peptide-resins (approximately 50 mg each). After stirring for approximately 2 min, 736 μ l of TFA and 90 μ l of TFMSA were added to each flask at 0 °C or 25 °C. Each resin underwent for cleavage reaction for different periods of time and was submitted to exhaustive washings with TFA/DCM, DCM, ethyl acetate, DMF, water, 10% AcOH/water and MeOH to guarantee the removal of all cleaved peptides and other side products of the reaction. After this treatment, small aliquots of each dried resin were hydrolyzed according to

the previous report¹⁸⁾ for further amino acid analysis. The calculated peptide content of the cleaved resin was compared to the value of the initial peptidyl-resin, taken as 100%. Otherwise when the purity of removed peptide was to be evaluated, the cleaved peptide was isolated by precipitation with cold ethyl ether in the resin, further extracted with 10% AcOH/water and lyophilized.

Analytical RP-HPLC RP-HPLC analyses were achieved in TFA/acetonitrile gradient using a Waters Associates HPLC system consisting of two 510 HPLC pumps, automated gradient controller, Rheodyne manual injector, 486 UV detector and 746 data module. Solvent A: 0.1% TFA/H₂O and Solvent B: 60% acetonitrile/0.1% TFA/H₂O with a gradient of 5—95% of B in 30 min, at a flow rate of 1.5 ml/min were used. The column employed was a Vydac C₁₈ column (0.46×25 cm, 5 μm particle size, 300 Å pore size), and detection at λ=210 nm.

Liquid Chromatography/Mass Spectrometry The crude lyophilized peptides were analyzed on a system composed of a Micromass Platform LCZ Spectrometer, a Waters Alliance HPLC, a Waters 996 photodiode array detector, and a Compaq Workstation. The peptides were loaded on a reversed-phase HPLC column Waters Nova-Pak C₁₈ (2.1×150 mm—3.5 μm particle size, and 60 Å pore size), Solvent A, 0.1% TFA/H₂O, and B, 0.1% TFA in CH₃CN/H₂O at a flow rate of 0.4 ml/min, detection at 210 nm in a mass range of 500—3930 daltons.

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References and Notes

- 1) Preliminary aspects of the present investigation were recently presented at the 26th European Peptide Symposium, in Montpellier, France (September 2000).
- 2) Abbreviations used in this report for amino acids, peptides and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485—2489 (1966); **6**, 362—369 (1967); **11**, 1726—1732 (1972). The symbols represent the L-isomer unless otherwise specified. The following additional abbreviations are used: AcOH, acetic acid; 2-Br-Z, 2-bromobenzyloxy-carbonyl; BHAR, benzhydrylamine-resin; Bom, benzyloxymethyl; Boc, *tert*-butoxycarbonyl; Mts, mesitylene-2-sulfonyl for Arg, CMR, chloromethyl-resin; cHex, cyclohexyl; C₁₈, octadecyl; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; EPR, electron paramagnetic resonance; HOBt, hydroxybenzotriazole; IR, infra-red; LC/MS, liquid chromatography/mass spectrometry; MBHAR, 4-methylbenzhydrylamine-resin; NMR, nuclear magnetic resonance; PAMR, 4-(oxymethyl)-phenylacetamidomethyl-linker-containing resin; RP-HPLC, reversed phase high performance liquid chromatography; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEA, triethylamine; TFA, trifluoroacetic acid; Tos, *p*-toluenesulfonyl.
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Anexo 10

First synthesis of a fully active spin-labeled peptide hormone

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Abstract For the first time in the electron spin resonance (ESR) and peptide synthesis fields, a fully active spin-labeled peptide hormone was reported. The ESR spectra of this α -melanocyte stimulating hormone (α -MSH) analogue (acetyl-Toac⁰- α -MSH) where Toac is the paramagnetic amino acid probe 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid, suggested a pH-independent conformation and a more restricted movement comparatively to the free Toac. Owing to its equivalent biological potency in a skin pigmentation assay as compared to the native α -MSH and its unique characteristic (paramagnetic, naturally fluorescent and fully active), this analogue is of great potential for investigation of relevant physiological roles reported for α -MSH.

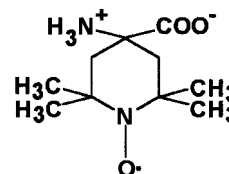
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Key words: Spin-labeled peptide; α -Melanocyte stimulating hormone; Electron spin resonance; Skin pigmentation

1. Introduction

The potentials of the ESR method in peptide chemistry and biology have been the subject of our studies for many years. The initial challenge was to find out a strategy to bind through a peptide bond, a stable and paramagnetic compound (spin label) into a peptide sequence. Thus, our introduction into the realm of ESR application in peptide synthesis method [1–3] initiated almost two decades ago [4] with the synthesis of the 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic

acid (Toac) spin probe [5], see below, but protected in its amino group with the acid labile tert-butyloxycarbonyl (Boc) temporary



protecting group necessary for peptide chain assembly (Boc-Toac). Unlike most spin-labeling strategies applied so far, where long and flexible nitroxide-containing probes have been routinely used [6,7], the Toac labeling is clearly more advantageous. It binds more rigidly to the target molecule as a consequence of its C α -tetrasubstituted cyclic structure, where the rotation about side-chain bonds is hampered by incorporation of the nitroxide nitrogen and C α , C β and C γ atoms into the same heterocyclic moiety. Due to these characteristics the probe is highly sensitive to conformational states of the peptide backbone under study.

Hence, the Boc-Toac derivative was initially used for the syntheses of two vasoactive peptide angiotensin II (AII) analogues (Toac⁰- and Toac¹-AII) but considerable AII activity was lost after the structural alterations [8]. At that time, there was no chemical strategy proposed to introduce the Toac probe at internal positions of the peptide sequence. This shortcoming was due to the irreversible degradation of the nitroxide moiety of the Boc-Toac during the repeated trifluoroacetic acid treatments necessary for removal of Boc group from Toac and from other incoming amino acid residues for further peptide chain elongation with the Boc strategy [1]. Only one decade later [9], we were able to propose an alternative procedure using the base labile 9-fluorenylmethyloxycarbonyl (Fmoc) group [2,3] for the Toac amino group protection and conjugating both Fmoc (for peptide chain elongation) and Boc (for peptide cleavage from the resin) chemistries. Thus, the first synthesis of an internally containing spin probe peptide sequence was achieved with the Toac⁷-AII [9], and more recently other vasoactive peptide (bradykinin, BK) was also internally labeled [10]. However, both Toac³-BK and Toac⁷-AII analogues were totally devoid of biological activity, whereas the BK analogue labeled at the N-terminus (Toac⁰-BK) maintained only a partial activity [10]. Additionally, Toac has also been used to label: (i) a seven transmembrane helix AII receptor fragment to investigate its binding to lipid bilayers and micelles [11], (ii) model peptide-resins to evaluate the peptide chain aggregation inside beads during the synthesis [12]. Moreover, the special bend-inducing property of the Toac molecule [13] has permitted in

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Abbreviations: Boc, tert-butyloxycarbonyl; Bzl, benzyl; C₁₈, octadecyl; 2-BrZ, 2-bromobenzyloxycarbonyl; 2-ClZ, 2-chlorobenzyloxycarbonyl; OcHex, cyclohexyl; For, formyl; Tos, *p*-toluenesulfonyl; Ac, acetyl; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMS, dimethylsulphide; EDT, ethanedithiol; Fmoc, 9-fluorenylmethyloxycarbonyl; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MeCN, acetonitrile; MBHAR, methylbenzohydrylamine-resin; NMP, 1-methyl-2-pyrrolidinone; TFA, trifluoroacetic acid; Toac, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid

Abbreviations for amino acids and nomenclature of peptide structure follow the recommendations of the IUPAC-IUB, J. Biol. Chem. 264 (1989) 668–673.

recent years the examination of helix-type conformational properties of single and double Toac-labeled model peptide sequences [14–16].

The next challenge concerning this spin-labeling approach was therefore to demonstrate the feasibility of synthesizing a Toac-containing active peptide that might retain its full biological potency. The tridecapeptide α -melanocyte stimulating hormone, α -MSH [17] containing the sequence acetyl-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH₂ was chosen for the present labeling investigation. This important hormone synthesized in the pituitary gland and skin of vertebrates is involved, among a great variety of physiological processes, in the skin darkening of most vertebrates, including humans [18], erectile function [19] and satiety [20]. Several modifications of the α -MSH primary structure have yielded reasonable elucidation of its structure-activity relationship and the synthesis of a potent and long lasting analogue [21] has been already reported. Owing mainly to its higher stability, most of α -MSH-labeling studies with several markers have been focused on this more potent analogue, with no loss of biological activity [22,23]. Differently, the present report describes the first synthesis of a native α -MSH analogue, labeled with the Toac spin probe (Ac-Toac⁰- α -MSH) and that still retained its entire potency.

2. Materials and methods

2.1. Peptide synthesis [1–3]

The native α -MSH was synthesized in 0.1 mmol scale according to the Boc chemistry and using methylbenzohydramine-resin (MBHAR) and the following Boc amino acid derivatives: Bzl for Ser, OcHex for Glu, 2-Br-Z for Lys, Tos for His and Arg. Couplings were performed with Boc amino acid/TBTU/HOBt (1:1:1) components in the presence of DIEA in NMP. The synthesized α -MSH was cleaved from the resin with HF:*o*-cresol:DMS:EDT (8.5:0.5:0.5:0.5, v/v) solution at 0°C for 90 min. After evaporation, the resin was washed with ethyl acetate, dried, and the peptide was extracted into 5% acetic acid in water and lyophilized.

The Ac-Toac⁰- α -MSH synthesis followed almost the same protocol applied for the native α -MSH but with some alterations as already reported [9]. The introduction of the Toac probe was performed with its Fmoc derivative, which was removed with the alkaline piperidine/DMF (20%, v/v) treatment for 30 min. The acetylation of the Toac amine group was done with acetic anhydride/DMF (1:4, v/v) solution containing 0.1 ml of pyridine for 30 min. After HF cleavage, the crude spin-labeled peptide was submitted to alkaline treatment (pH 10, for 6 h at 25°C) for complete reversion (monitored by analytical HPLC) of the N-O protonation that occurs during HF treatment.

Both peptides were purified in preparative HPLC (C₁₈-column) using aqueous 0.02 M ammonium acetate (pH 5.0) and 60% MeCN solutions as solvents A and B, respectively (linear gradient of 30–70% B in 2 h, flow rate of 10 ml/min). The homogeneity of both peptides was confirmed through analytical HPLC, matrix-assisted laser desorption ionization-mass spectrometry and amino acid analysis. In this latter case, the Toac residue is not determined as it decomposes during the acid hydrolysis.

2.2. Biological assays [24]

The thigh and dorsal skin of the frog was excised, cut in square (2×2 cm²) pieces, which were placed between two PVC rings and kept for 1 h in Ringer's solution. After this period, the melanin granules were aggregated in the perinuclear region of the melanocytes, which assumed a punctuate state, and the skins become light. Upon addition of α -MSH or the analogue to the medium, the pigment disperses out into the dendritic processes of the cell, resulting in skin darkening. The changes in skin color (decrease in skin reflectance) were monitored with a Photovolt reflectometer and expressed as percent change of the initial value. Dose-response curves and the EC₅₀ (the concentration eliciting 50% of the maximal darkening, confidence interval of 95%) were determined for both peptides.

3. Results and discussion

Knowing some of the structural requirements for α -MSH biological activity, the Toac was inserted between the acetyl group and the Ser¹ residue out of the important 4–12 core of its sequence [25]. The chemical strategy to obtain the Ac-Toac⁰- α -MSH analogue involved a conjugation of Boc and Fmoc chemistries as already mentioned [9]. The selection of the solvent for the critical coupling reaction step amino acid residues was based upon our recent peptidyl-resin solvation strategy [26] where a novel solvent polarity parameter was also proposed. The polar aprotic 1-methyl-2-pyrrolidinone (NMP) swelled almost 80% of peptide-resin bead volume throughout all chain assembly and was therefore used with success for the synthesis of both peptides. After alkaline reversion of the nitroxide moiety protonation that occurs during HF cleavage of peptide from the resin and HPLC purification, 47 mg and 33 mg of pure α -MSH and Ac-Toac⁰- α -MSH were obtained, respectively.

The frog (*Rana catesbeiana*) skin bioassay was performed in vitro as previously described [24]. Ac-Toac⁰- α -MSH was a full agonist on the frog melanocyte, promoting a dose-dependent skin darkening (Fig. 1) with the same potency as the native α -MSH. The concentration eliciting 50% of the maximal darkening (EC₅₀) and the confidence interval of 95% (CI) were for the labeled analogue and the native hormone, respectively 2.70×10^{-10} M (1.02–7.10) and 1.96×10^{-10} M (0.72–5.34). In addition, after removal of the agonist, and several Ringer's rinses, the reversal of the maximal response to Ac-Toac⁰- α -MSH was achieved after 180 min, at the same rate exhibited by α -MSH (Fig. 2). These results show, for the first time, that the spin labeling of the native sequence of α -MSH was successfully performed, full agonism being retained, with no loss of potency as compared to the native hormone. It also means that, in some circumstances, the most serious criticism of the ESR method, i.e. the lack of meaning in studying a spin-la-

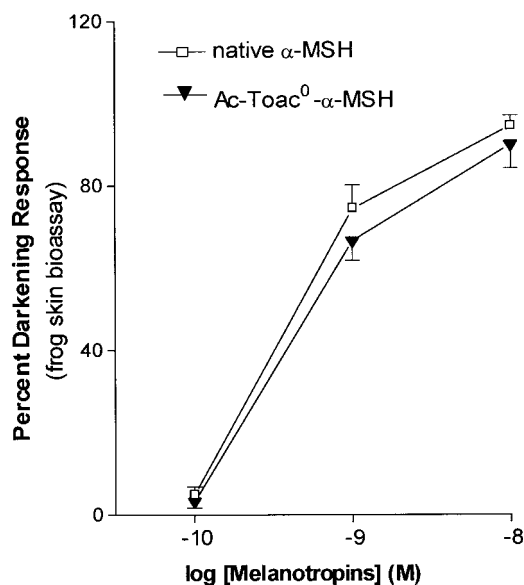


Fig. 1. Dose-response curves to Ac-Toac⁰- α -MSH as compared to the native α -MSH, in the frog *Rana catesbeiana* skin bioassay. Each point represents the mean ($n=10$) $\pm$ S.E.M. (standard error of the mean) darkening response at the concentrations noted.

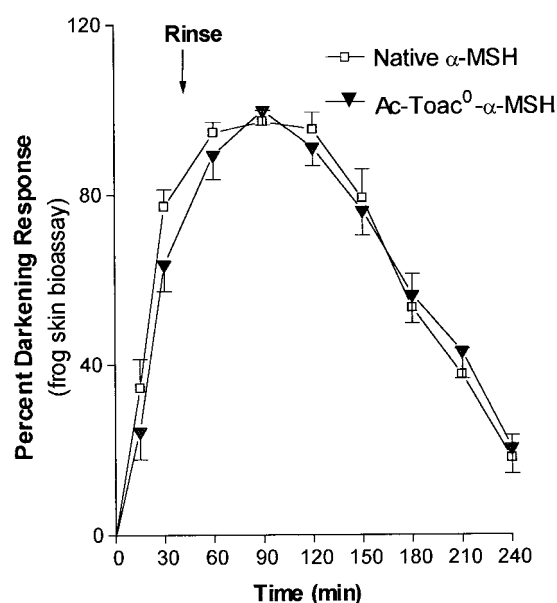


Fig. 2. Reversal of the maximal responses to the native hormone and to Ac-Toac⁰-α-MSH (10^{-8} M) after removal of the peptides and rinsing of the preparation (arrow). Each point is the mean ($n=10$) $\pm$ S.E.M., *Rana catesbeiana* skin darkening at the times noted.

beled system due to the introduction of a non-natural component in its structure, may be not acceptable.

The ESR spectra demonstrate the different mobility of the free and peptide-bound Toac (Fig. 3). Both compounds display narrow lines, as expected for small molecules tumbling in a non-viscous solvent. However, the correlation times (τ) for α-MSH-bound Toac are one order of magnitude higher than those obtained for free Toac (Table 1). The different rotational correlation time τ_B and τ_C values obtained for the labeled peptide and not for Toac indicate an anisotropic movement [27] for the former. Preliminary results with Ac-Toac⁰-α-MSH also suggest that its conformation does not depend on the pH of the media, as its ESR parameters have not changed in acid or alkaline solutions (Table 1). Moreover, equivalent isotropic

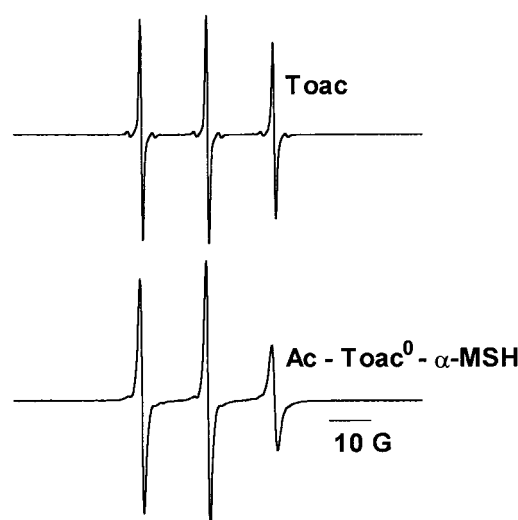


Fig. 3. ESR spectra of 10^{-4} M Toac and Ac-Toac⁰-α-MSH in aqueous solutions (pH 5.0).

hyperfine parameter (a_0) values indicate similar microenvironment for Toac either free or bound to the peptide, at all pH values.

In conclusion we herein described the first synthesis of a fully active spin-labeled peptide hormone. The availability of such unique analogue (Ac-Toac⁰-α-MSH) that is paramagnetic, naturally fluorescent due to the tryptophan residue of its structure and fully active, is clearly of great potential. Besides the expected more complete ESR studies in solution, the use of this paramagnetic peptide may allow for instance, the inversion of the more common strategy used up to now for peptide binding studies in membrane-mimetic systems. In the place of spin labeling the lipid bilayer and evaluating the binding of the agonist, as already described for the α-MSH itself [28], the labeling site is now located within the native hormone structure as already reported with external fragments of some seven transmembrane helix proteins [11,29]. The same approach would be also further extended for the approach [30] which evaluates interaction features of transmembrane helix segments with model membrane. Moreover, one can also simultaneously spin label both the agonist and the system under study, for structure and binding investigation through the assessment of spin-spin interactions phenomenon [31] that may occur depending upon the average distance between probes. The α-MSH binding assay to lipid bilayers has already been examined by fluorescence, monitoring the native Trp⁹ residue of the hormone [32]. Complementarily, the use of the paramagnetic hormone may provide an alternative route to that investigation due to the well-known fluorescence quenching effect [33] of the nitroxide function. The secondary structure of nicotinic acetylcholine receptor inside the membrane was for instance investigated through the quenching phenomenon [34]. Therefore, the use of this special quenching agonist may be also valuable for further imaging and quantification studies of melanotropin binding to α-MSH receptors present in normal melanocytes or melanoma cell lines [35,36].

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Table 1
ESR data for Toac and Ac-Toac⁰-α-MSH

System	a_0 (G)	τ_B (ns)	τ_C (ns)
Toac			
pH 5.0	16.32	0.036	0.036
Ac-Toac ⁰ -α-MSH			
pH 3.0	16.29	0.324	0.396
pH 5.0	16.29	0.313	0.382
pH 9.0	16.26	0.305	0.372

The estimated errors in the values of a_0 and τ are around ± 0.03 G and 0.005 ns, respectively. ESR measurements were performed at 25°C with a Bruker EMX spectrometer. A field-modulation amplitude of 0.5 G and microwave power of 5 mW was used. The temperature was controlled to about 0.2°C with a Bruker BVT-2000 variable temperature device. The spectral parameters were found by fitting each line to a Gaussian-Lorentzian sum function taking advantage of the fact that the sum function is an accurate representation of a Gaussian-Lorentzian convolution, the Voigt function [31]. The hyperfine splitting, a_0 , was taken to be one-half the difference in the resonance fields of the high- and low-field lines. The intrinsic (Lorentzian) line widths and the line heights were determined from the fits and rotational correlation times, τ_B and τ_C were calculated [27].

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Anexo 11

Comparative EPR and fluorescence conformational studies of fully active spin-labeled melanotropic peptides

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Abstract Similar to melanocyte stimulating hormone (α -MSH), its potent and long-acting analogue, [Nle⁴, D-Phe⁷] α -MSH, when labeled with the paramagnetic amino acid probe 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid (Toac), maintains its full biological potency, thus validating any comparative structural investigations between the two labeled peptides. Correlation times, calculated from the electron paramagnetic resonance signal of Toac bound to the peptides, and Toac–Trp distances, estimated from the Toac fluorescence quenching of the Trp residue present in the peptides, indicate a more rigid and folded structure for the potent analogue as compared to the hormone, in aqueous medium. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Peptide; α -Melanocyte stimulating hormone; Electron paramagnetic resonance; 2,2,6,6-Tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid; Fluorescence; Spin label

1. Introduction

In most vertebrates, the linear tridecapeptide α -melanocyte stimulating hormone (α -MSH; acetyl-SYSMEHFRWGKPV-amide) is known as the most relevant physiological hormone regulating skin darkening [1]. This peptide is also involved in a variety of other physiological and neurological processes, such as fetal growth, thermoregulation, food intake, learning, memory and attention [2]. The minimal sequence required to stimulate melanocytes was determined to be the core 6-9 fragment, generally referred to as the primary active site or 'message sequence' [3], due to its conservation in several species. Among the several α -MSH analogues, [Nle⁴, D-Phe⁷] α -MSH (hereafter referred to as NDP-MSH) has been identified as very potent and long-acting analogue in various vertebrate

species [4,5]. Due to its high activity and enzymatic resistance, this analogue has been used in the characterization of melanocortin receptors, mainly by radioligand binding analysis [5,6].

Although the important question about the hormone structure–activity relationship has been largely addressed in the melanotropic peptides literature, there is not a consensus about the conformations acquired by both α -MSH and NDP-MSH, either in solution or during the membrane–receptor binding. Several studies have indicated that the peptides assume a folded conformation in aqueous medium (a β -structure or a hairpin loop), comprising the critical 6-9 fragment, and it was postulated that the increased potency of the NDP-MSH derivative was due to a reverse turn-type structure stabilized by the D-Phe⁷ substitution [7–9]. Otherwise, there are investigations pointing to a rather flexible backbone for α -MSH in solution, whereas the presence of the D-Phe⁷ residue favors a more folded structure for the NDP-MSH analogue [10–12]. It is noteworthy that in the presence of either anionic sodium dodecylsulfate micelles or dimyristoyl phosphatidylglycerol vesicles, a small percentage of the proposed biological relevant structure (a β -like conformation) could be observed in the circular dichroism spectra of both peptides [11]. That result should be regarded in the light of the possible catalytic role of the membrane bilayer phase, inducing a particular peptide conformation appropriate for its interaction with the receptor [13].

To initiate an alternative conformational approach for melanotropic peptides studies, we have recently reported [14] the synthesis of a paramagnetic α -MSH analogue labeled with Toac, an amino acid-type spin probe (2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid) earlier introduced [15,16] in the peptide chemistry field. As emphasized in those works, due to the special rigidity of its covalent binding to the peptide molecule, the Toac spin label is highly sensitive to the peptide backbone conformation and dynamics. Despite the insertion of this non-natural probe in its structure, the synthesized Ac-Toac⁰- α -MSH analogue maintained its original potency in the frog *Rana catesbeiana* skin bioassay thus becoming [14] the first example of fully active spin-labeled peptide hormone.

By pursuing the synthesis of alternative fully active paramagnetic melanotropic derivatives for further comparative physiological studies, the present work describes the successful

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Abbreviations: Abbreviations for amino acids and nomenclature of peptide structure follow the recommendations of the IUPAC-IUB, (1989) J. Biol. Chem. 264, 668–673; EPR, electron paramagnetic resonance; Toac, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid

Toac-labeling of the more potent and long-acting NDP-MSH derivative. The findings here detailed for the synthesized Ac-Toac⁰-NDP-MSH analogue demonstrate that the NDP-MSH original activity was entirely maintained after this structural modification, thus validating any further conformational studies of this class of peptides. In addition to the electron paramagnetic resonance (EPR) analysis of the Toac signal of the labeled melanotropic peptides, the presence of the naturally fluorescent Trp residue in their core sequences has also allowed structural comparisons between labeled α -MSH and NDP-MSH analogues via the Trp fluorescence quenching due to energy transfer to the Toac molecule.

2. Materials and methods

2.1. Peptide synthesis

The NDP-MSH and Ac-Toac⁰-NDP-MSH analogues were synthesized in the same manner as already detailed for the native α -MSH and its Ac-Toac⁰- α -MSH derivative [14]. The base labile 9-(fluorenylmethoxycarbonyl) group (Fmoc) was used for temporary protection of the Toac α -amine group which allows its insertion at any position in the peptide sequence without the danger in decomposing its nitroxide function during the synthesis cycle. After HF cleavage, the extracted spin-labeled analogue was submitted to alkaline treatment for complete reversion (monitored by analytical high performance liquid chromatography (HPLC)) of the N–O protonation that occurs during this anhydrous acid treatment. After purification in preparative HPLC (C₁₈ (octadecyl) column) using aqueous 0.02 M ammonium acetate (pH 5.0) and 60% acetonitrile solutions as solvents A and B, the purified NDP-MSH and Ac-Toac⁰-NDP-MSH peptides were characterized by analytical HPLC, mass spectrometry and amino acid analysis.

2.2. Biological assay [17,18]

The thigh and dorsal skin of the frog, *R. catesbeiana*, was excised, cut in square (2×2 cm) pieces, which were placed between two PVC rings and kept for 1 h in Ringer's solution. After this period, the melanin granules were aggregated in the perinuclear region of the melanocytes, which assumed a punctate state, leading to skin lightening. Upon addition of Ac-NDP-MSH or Toac⁰-NDP-MSH to the medium, the pigment disperses out into the dendritic processes of the cell, resulting in skin darkening. The changes in skin color (decrease in skin reflectance) were monitored with a Photovolt reflectometer and expressed as percent change of the initial value. Dose–response curves and the EC₅₀s (the concentration eliciting 50% of the maximal darkening, confidence interval (CI) of 95%) were determined for the peptides. The data were compared employing the SNK test ($P \leq 0.05$).

2.3. Sample preparation

For the spectroscopic measurements, stock solutions of the peptides (10^{−3} M) were prepared in water and diluted to the final desired concentrations. The values of pH were adjusted with HCl or NaOH.

2.4. EPR spectroscopy

EPR measurements were performed at 25°C with a Bruker EMX spectrometer. A field-modulation amplitude of 0.5 G and microwave power of 5 mW were used. The temperature was controlled to about

0.2°C with a Bruker BVT-2000 variable temperature device. The spectral parameters were found by fitting each line to a Gaussian–Lorentzian sum function taking advantage of the fact that the sum function is an accurate representation of a Voigt function [19]. The corrected B and C parameters were calculated as described by Bales [19], as a function of the three line heights, making the corrections for the contribution of non-resolved hyperfine splittings. Rotational correlation times were calculated with both parameters, and called τ_B and τ_C , using the principal components of the g and hyperfine tensors of doxyl-propane, and 3300 G for the magnetic field [20]. Considering that those two correlation times should be identical for rapid, isotropic movement, and due to the difficulty in postulating a preferential rotational axis, the movement asymmetry will be estimated by the ratio between τ_C and τ_B . The hyperfine splitting, a_o , was taken to be one-half the difference in the resonance fields of the high- and low-field lines (fit by the computer program).

2.5. Optical and fluorescence spectroscopies

Optical absorption measurements were performed with a HP 8452 A diode array spectrophotometer. For steady state fluorescence experiments a Fluorolog 3 Jobin Yvon-Spex spectrometer was used, with excitation and emission slits of 1 mm or 2 mm, depending on the fluorescence intensity of the sample. The excitation wavelength was set to 295 nm and the quantum yields were estimated using the Trp value 0.14 as a reference. Time-resolved experiments were performed using an apparatus based on the time-correlated single photon counting method. The excitation source was a Tsunami 3950 Spectra Physics titanium–sapphire laser, pumped by a 2060 Spectra Physics argon laser. The repetition rate of the 5 ps pulses was set to 400 kHz with the pulse picker 3980 Spectra Physics. The laser was tuned to give an output at 897 nm, and a third harmonic generator BBO crystal (GWN-23PL Spectra Physics) gave the 297 nm excitation pulse that was directed to an Edinburgh FL900 spectrometer. The emission wavelength was selected by a monochromator, and emitted photons were detected at right angle from excitation by a refrigerated Hamamatsu R3809U microchannel plate photomultiplier. The FWHM (full width at half maximum) of the instrument response function was typically 45 ps and the time resolution 12 ps per channel. A software provided by the Edinburgh Instruments was used to analyze the decay curves and the adequacy of the fitting was judged by the inspection of the weighted residual plots and by the analysis of statistical parameters such as reduced chi-square.

3. Results and discussion

The in vitro frog (*R. catesbeiana*) skin bioassay was performed as previously described [17,18]. NDP-MSH from Sigma, NDP-MSH and Ac-Toac⁰-NDP-MSH (this paper) are full agonists on the frog melanocyte, promoting a dose-dependent skin darkening (Fig. 1), and are about five to eight times more potent than α -MSH in this bioassay (1.96×10^{-10} M [14]). The EC₅₀s and the CIs of 95% were 2.4×10^{-11} M (0.38–8.57), 3.8×10^{-11} M (0.59–13.56) and 3.7×10^{-11} M (0.58–13.21), respectively, and were not statistically different. After the removal of these compounds, and several rinses in Ringer's solution, the reversal of the maximal responses was not achieved, at least until 90 min (data not shown). It had

Table 1

EPR parameters for free Toac and Toac bound to the peptides: nitrogen isotropic hyperfine splitting, a_o , and rotational correlation times, τ_B and τ_C

	System	a_o (G)	τ_B (ns)	τ_C (ns)	τ_C/τ_B
pH 3.0	Toac (pH 5.0)	16.330	0.034	0.036	1.06
	Ac-Toac ⁰ - α -MSH	16.238	0.314	0.370	1.18
	Ac-Toac ⁰ -NDP-MSH	16.231	0.333	0.408	1.22
pH 5.0	Ac-Toac ⁰ - α -MSH	16.249	0.306	0.359	1.17
	Ac-Toac ⁰ -NDP-MSH	16.241	0.321	0.391	1.22
pH 9.0	Ac-Toac ⁰ - α -MSH	16.252	0.301	0.353	1.17
	Ac-Toac ⁰ -NDP-MSH	16.242	0.335	0.407	1.22

The estimated errors in the values of a_o and τ_B or τ_C are 0.005 G and 0.005 ns, respectively.

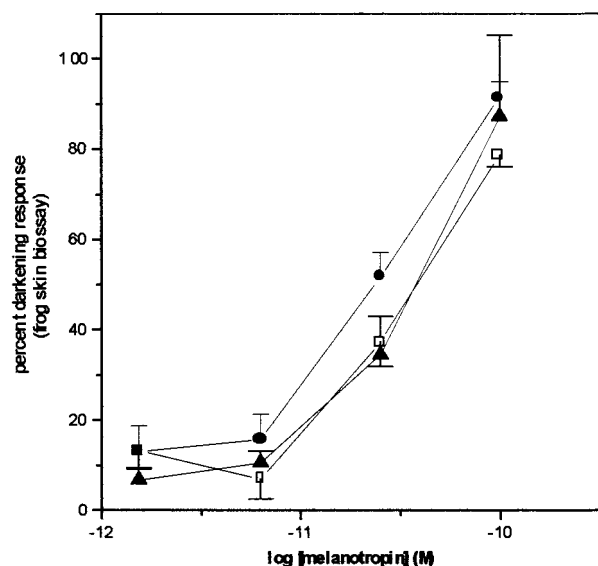


Fig. 1. Dose-response curves to Ac-Toac⁰-NDP-MSH (▲) as compared to NDP-MSH (□, from Sigma; ●, this paper) in the frog *R. catesbeiana* skin bioassay. Each point represents the mean ($n=8$) $\pm$ S.E.M. darkening response at the concentration noted.

already been demonstrated that NDP-MSH is a potent and long-acting agonist [4], and these properties were not changed with the insertion of the spin label, Toac.

In aqueous solution, the paramagnetic Ac-Toac⁰-NDP-MSH analogue displays an EPR spectrum similar to that obtained with Ac-Toac⁰- α -MSH already reported [14]. The parameters yielded by the EPR spectra of the two Toac-bound peptides at different pH values are comparatively summarized in Table 1. The EPR parameters (rotational correlation times, τ_B and τ_C , and nitrogen isotropic hyperfine splitting, a_0) are also compared to those of the free Toac molecule. Considering that the free and the peptide-bound Toac EPR spectra are typical of the motional narrowing region (between 10^{-11} and 10^{-9} s), the τ_B and τ_C values were calculated according to Schreier et al. [20] using the corrections for non-resolved hyperfine splittings proposed by Bales [19] (see Section 2). As expected, the τ values of both Toac-bound melanotropic peptides are one order of magnitude higher than those of the free Toac molecule in solution, indicating that the Toac peptide EPR spectrum reflects the mobility of the

peptide backbone. This is in close accordance with data already reported for other small paramagnetic labeled active peptides [14–16]. Moreover, considering that the ratio between the two correlation times, τ_C/τ_B , can be used as an estimation of the anisotropy of the spin-labeled systems [20], it is evident that the binding to the peptides also turns the Toac movement less isotropic (higher τ_C/τ_B ratio). It is interesting to note that the correlation times are slightly higher for the Toac-containing NDP-MSH as compared to Ac-Toac⁰- α -MSH: τ_C values are around 10%, 9% and 15% higher at pH 3, 5 and 9, respectively. Besides displaying a slower rate of movement, the former peptide also shows a more anisotropic rotation than when bound to the latter ($(\tau_C/\tau_B)=1.22$ and 1.17 , respectively), at the three pH values. Those results suggest that the two peptides are characterized by different conformers.

The analysis of Toac nitrogen isotropic hyperfine splitting (a_0) values, which are sensitive to the polarity of the nitroxide group micro-environment [21], also reveals a significant difference between the free and the peptide-bound spin label: in the EPR spectra of the Toac-bound peptide, a_0 is 0.08–0.10 G lower than that of the free paramagnetic molecule. That difference could be attributed to a less polar environment neighboring the Toac nitroxide function, when inserted in the peptide structure, and/or to a differentiated unpaired electron distribution over the N-O moiety of the spin probe, when in the free or peptide-bound states. Otherwise, the a_0 values of the Ac-Toac⁰- α -MSH are somehow higher than those of Ac-Toac⁰-NDP-MSH, in all pH values. Considering that the covalent binding of Toac in the two peptides is identical, that difference could only be assigned to slightly different environments experienced by the paramagnetic molecule in the two peptides. Taking together, the higher correlation times, the higher anisotropy of movement, and the lower hyperfine splitting indicate a slightly more folded structure for the Toac-labeled potent analogue as compared to Ac-Toac⁰- α -MSH.

Optical absorption spectra of Toac, α -MSH and Ac-Toac⁰- α -MSH in aqueous solution, pH 7.0, are illustrated in Fig. 2.

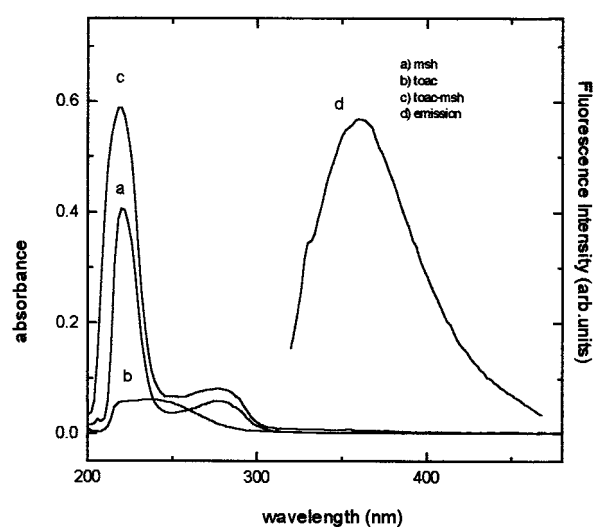


Fig. 2. Optical absorption spectra (optical path length 1.0 cm) of α -MSH (a), Toac (b) and Ac-Toac⁰- α -MSH (c), and the fluorescence emission spectra of Ac-Toac⁰- α -MSH excited at 295 nm (d). Peptides and Toac concentration 2.5×10^{-5} M, in aqueous solution, pH 7.0, 22°C.

Table 2
Mean lifetime of Trp fluorescence decay, $\langle \tau \rangle$, and estimated Trp-Toac distances in Toac-bound peptides, r

	System	$\langle \tau \rangle$ (ns)	r (Å)
pH 3.0	α -MSH	1.95	
	Ac-Toac ⁰ - α -MSH	1.89	16.7
	NDP-MSH	2.13	
	Ac-Toac ⁰ -NDP-MSH	1.75	12.1
pH 5.0	α -MSH	2.14	
	Ac-Toac ⁰ - α -MSH	2.03	15.3
	NDP-MSH	2.30	
	Ac-Toac ⁰ -NDP-MSH	1.95	12.6
pH 9.0	α -MSH	2.62	
	Ac-Toac ⁰ - α -MSH	2.32	13.1
	NDP-MSH	2.70	
	Ac-Toac ⁰ -NDP-MSH	2.17	12.1

The estimated error in the values of $\langle \tau \rangle$ is 0.05 ns.

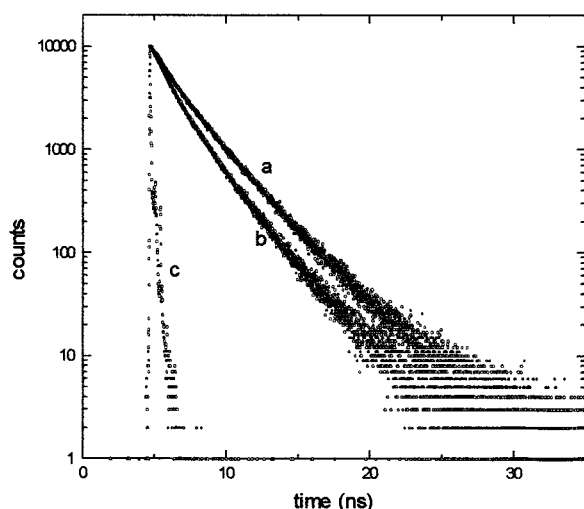


Fig. 3. Fluorescence decay of α -MSH (a), and Ac-Toac⁰- α -MSH (b) in aqueous solution, pH 7.0. Concentration 2.5×10^{-5} M, 22°C. The instrument response function at 297 nm excitation is also shown (c).

In the near-UV region, around 280 nm (extinction coefficient $\approx 5.7 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$), the spectrum of the labeled peptide is dominated by the electronic transitions characteristic of the Trp residue, superimposed to a small contribution from the Toac molecule. The Ac-Toac⁰- α -MSH also presents a very weak absorption band centered around 410 nm, with an extinction coefficient about $20 \text{ M}^{-1} \text{ cm}^{-1}$, as observed recently [22] in Toac-bound peptides (not evident in Fig. 2, due to the scale used). The same features are present in the spectrum of the analogue Ac-Toac⁰-NDP-MSH (data not shown). Pure Toac samples, excited at 295 nm, do not show any fluorescence emission. However, under excitation at that wavelength, both labeled and unlabeled peptides present an emission band centered around 355 nm, typical of Trp in aqueous environment (Fig. 1d). The values of the quantum yield of α -MSH and NDP-MSH are about 0.090, comparable to previous reports for the peptides [23]. Compared to non-labeled peptides, the fluorescence intensity of Trp in the Toac-bound peptides decreases, due to the quenching by the Toac group. In acidic pH values the quantum yields of unbound and Toac-bound peptides decrease with respect to the values at higher pH.

The excited state decay of all the compounds, labeled or not, was found to be heterogeneous, as observed before for the non-labeled peptides in neutral pH [23], and could be properly fit with three lifetime components. The α -MSH and NDP-MSH lifetime values for the high pH samples were also comparable to those previously reported for the peptides in buffer, pH 7.4. However, the Toac-labeled peptides presented faster fluorescence decay (Fig. 3), compatible with Trp fluorescence quenching by the spin label. The decrease in lifetime can be examined by calculating a mean lifetime $\langle \tau \rangle$ for the decay, according to $\langle \tau \rangle = \sum a_i \tau_i^2 / \sum a_i \tau_i$, where τ_i and a_i are the lifetime and the pre-exponential factor of the i -th component, respectively. As observed in Table 2, lifetimes of Toac-labeled peptides are systematically lower than those of original peptides, independent of the solution pH value.

Mechanisms like charge transfer or collisional quenching can contribute to the decrease of Trp fluorescence, and processes requiring close proximity between the fluorophore and nitroxide spin labels were assumed, for example, in the paral-

ax method employed to estimate the penetration depth of melanotropins into the lipid bilayer of phospholipid vesicles [24]. In the present case, as mentioned, the Toac peptides present a very weak absorption band centered around 410 nm, overlapping the red tail of the Trp emission band, allowing for fluorescence resonant energy transfer between the side chain residue as donor and the spin probe as acceptor. The Trp residue and the Toac group are far apart in the peptide chain and the Förster model was recently applied to the study of the Toac-labeled peptides [22], and a Förster distance (R_0) of 9.3 Å was calculated for the pair Trp–Toac. Likewise, an estimation of the intramolecular Toac–Trp distances in the labeled melanotropins can be done, based on the calculated mean lifetimes (Table 2). A donor–acceptor distance, $r = R_0(1/E-1)^{1/6}$, can be estimated from the efficiency of energy transfer, given by $E = 1 - \langle \tau_{da} \rangle / \langle \tau_d \rangle$, where $\langle \tau_{da} \rangle$ and $\langle \tau_d \rangle$ are the mean lifetimes for the Toac-labeled and unlabeled peptides, respectively, and using the R_0 value calculated by Pispisa et al. [22]. The estimated Toac–Trp distances given in Table 2 were obtained under the assumption of fast movement for donor and acceptor during the fluorescence lifetime. As usually reported in the literature, donor–acceptor distances are estimated within 20% error.

Our results demonstrate that, in all pH values, the Toac–Trp distance is shorter in NDP-MSH, as compared to the native peptide α -MSH. That finding taken together with the results from EPR spectroscopy as discussed above indicate a more compact conformation for the potent analogue and fit with the proposed turn in the NDP-MSH conformation [10–12]. It should also be noted that the analogue conformation does not seem to be dependent on the sample pH value, whereas the Toac–Trp distance in α -MSH increases when the pH decreases. Changes in the pH of the medium affect the protonation state of several side chain groups in the peptides. Therefore, the consequences of those modifications on the conformation of the peptides could be evaluated from a detailed study of EPR and fluorescence parameters of labeled and non-labeled melanotropins as a function of the medium pH value. We stress that the Toac–Trp distances presented here are estimates of mean distances. More detailed information about the conformation of the peptides will depend, for example, on the analysis of the decay associated spectra, effects of the protonable groups, and analysis of resonant energy transfer in the framework of donor–acceptor distance distribution models [25].

The Toac-labeling approach has been considered unique and with great potential for application in a variety of biochemical applications [26]. In this context, the relevance of the present work refers to the introduction of a second fully active Toac-labeled peptide hormone in the literature and its usefulness for structural investigation of melanotropic peptides, in aqueous solution. Otherwise, when membrane-mimetic systems are taken into account, it is already known that this class of peptides strongly interacts with anionic micelles and vesicles, partially penetrating their hydrophobic core [11,27]. We can therefore envisage that, as an expected extension of the present work, conformational changes upon insertion of Toac-containing α -MSH and/or NDP-MSH peptides into the lipid phase could be deeper investigated by simultaneous analysis of the fluorescence properties of Trp residue and the paramagnetic properties of Toac, possibly inserted in different positions of the melanotropic peptide sequence.

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Anexo 12

Synthesis and pharmacological properties of TOAC-labeled angiotensin and bradykinin analogs

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Abstract

Angiotensin II (AngII) and bradykinin (BK) derivatives containing the TOAC (2,2,6,6-tetramethylpiperidine-N-oxyl-4-amino-4-carboxylic acid) spin label were synthesized by solid phase methodology. Ammonium hydroxide (pH 10, 50°C, 1 h) was the best means for reverting nitroxide protonation occurring during peptide cleavage. EPR spectra yielded rotational correlation times for internally labeled analogs that were nearly twice as large as those of N-terminally labeled analogs. Except for TOAC¹-AngII and TOAC⁰-BK, which showed high intrinsic activities, other derivatives were inactive in smooth muscle preparations. These active paramagnetic analogs may be useful for conformational studies in solution and in the presence of model and biological membranes. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Angiotensin analogs; Bradykinin analogs; Peptide synthesis; Spin labeled peptides; TOAC spin label; Electron paramagnetic resonance

1. Introduction

The use of the amino acid spin label TOAC (2,2,6,6-tetramethylpiperidine-4-amino-4-carboxylic acid) in the peptide chemistry field was first successfully carried out almost two decades ago by the synthesis of the dipeptide TOAC-Gly [19] and of the octapeptide angiotensin II (AngII, DRVYIHPF) analogs TOAC⁰- and TOAC¹-AngII [20]. An AngII analog had been previously described [18] containing an N-terminal Cys residue labeled with nitroxide-bearing maleimide. In this case, the free rotation about several single bonds between the reporter group and the peptide backbone made it more difficult to obtain appropriate conformational information. The use of the TOAC marker represented the first approach that allowed linking of a spin label to the peptide backbone *via* a peptide bond. Such linking and TOAC's special C^α-tetrasubstituted cyclic structure render the EPR spectra of labeled peptides highly

sensitive to conformational features of the peptide molecule as a whole.

At the time [20], the existing peptide synthesis strategy [3,33] restricted the paramagnetic labeling to the N-terminus due to the chemical decomposition of the TOAC free radical. Later, a second synthesis protocol [2,10] allowed the insertion of this spin label at internal positions of the peptide backbone [16]. This finding opened the possibility of TOAC replacement for any amino acid residue in a peptide structure. As a consequence, a great variety of applications, encompassing from chemical to pharmacological fields, has been reported and recently evaluated [34]. Briefly, TOAC has been applied in conformational studies of: i) model peptide segments [12,32,35]; ii) peptides corresponding to loops of G protein-coupled receptors [26,27]; iii) fungal peptides in solution [1] or in membrane-mimetic environments [17]. The use of TOAC has also been extended to monitor peptide chain association in solution [28] or, when spread throughout resin matrix, with the aim of improving peptide synthesis methodologies [8,9]. Nevertheless, the investigation of the structure-activity relationship of physiologically relevant TOAC-labeled peptides has been very scarce. Besides the initial study of AngII analogs

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labeled with TOAC at the N-terminal position [20], only recently it was reported that a melanocyte stimulating hormone and its potent analog melanotan bearing the non-natural TOAC group in their structure maintained their full biological potencies [4,21].

In order to improve the knowledge concerning the use of TOAC for structure-activity studies, here we describe the synthesis and pharmacological properties of new TOAC-labeled derivatives of AngII, as well as of the vasoactive peptide bradykinin (BK, RPPGFSPFR). In addition to already reported TOAC¹-AngII [20], TOAC³-AngII, TOAC⁷-AngII, TOAC⁰-BK and TOAC³-BK were also synthesized and their biological potencies in isolated rabbit aorta, rat uterus, and guinea-pig ileum preparations were determined.

We also present a more elaborated investigation of the crucial alkaline deprotonation step necessary for synthesizing these special TOAC-containing peptides. This treatment is needed to revert the protonation of the nitroxide moiety which occurs under the strong acidic conditions routinely used for peptide cleavage from the resin [23,30]. Different alkaline treatments were compared in order to establish a more appropriate protocol for this step in the paramagnetic peptide synthesis.

2. Materials and methods

2.1. Peptide synthesis

TOAC-containing AngII and BK derivatives were synthesized in 0.1-mmol scale according to the 9-fluorenylmethoxycarbonyl (Fmoc)/tert-butyl (tBut) strategy [2,10], starting from a Wang-type resin. The following Fmoc-amino acid protecting groups were used: 2,2,5,7,8-pentamethylchroma-6-sulfonyl for Arg, tBut for Tyr, Asp and Ser and trityl for His. Couplings were performed with a 1:1:1 mixture of Fmoc-amino acid/O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluoro-phosphate (HATU) [6]/1-hydroxy-7-azabenzotriazole (HOAt) [7] in the presence of *N,N*-diisopropylethylamine in 1-methyl-2-pyrrolidinone. Owing to the extremely low nucleophilicity of the TOAC amine group when bound to a peptide fragment [19,20], the coupling of the next amino acid residue of the sequence was carried out in unusual conditions using a large excess of reactants (5-fold) and repeating the coupling step three times. Moreover, the low basicity of the TOAC amine group also hampers the use of the qualitative ninhydrin method [14] to monitor the coupling reaction. Therefore, the extent of the next amino acid incorporation after TOAC insertion was determined by amino acid hydrolysis of the peptidyl-resin. This process was needed before continuation of the peptide chain assembly.

The peptides were cleaved from the resin with an anhydrous solution of HF:o-cresol:dimethylsulfide:ethanedithiol (8.5:0.5:0.5:0.5, v/v) at 0°C for 2 h. HF was used to avoid destruction of the nitroxide group by the commonly used

TFA cleavage agent. After evaporation, the resin was washed with ethyl acetate, dried, and the peptide was extracted into 5% acetic acid in water and lyophilized. The crude spin labeled peptides were submitted to alkaline treatment (pH 10, 1h, 50°C) for complete reversal of the nitroxide moiety protonation that occurs during the HF reaction (see details in the following section). The peptides were purified by preparative high-performance liquid chromatography (HPLC) using a C₁₈-column and aqueous 0.02 M ammonium acetate (pH 5) and 60% acetonitrile solutions as solvents A and B, respectively (linear gradient of 30–70% B in 2 h, flow rate of 10 ml/min). The peptide's homogeneity was confirmed by analytical HPLC, amino acid analysis and liquid chromatography-electron spray mass spectrometry.

2.2. EPR studies

EPR measurements were carried out at 9.5 GHz in a Bruker ER 200D-SRC spectrometer at room temperature (22 ± 2°C) using flat quartz aqueous cells (Wilma Glass Company Inc., Buena, New Jersey). The magnetic field was modulated with amplitudes less than one-fifth of the line-widths, and the microwave power was 5 mW to avoid saturation effects. Peptides were dissolved in aqueous acetic acid, pH 3.0. Rotational correlation times (τ) were calculated according to a previous report [5].

2.3. Bioassays

For isolated smooth muscle preparations, rabbits, rats and guinea-pigs were killed by a blow to the head and exsanguinated, their organs were removed and mounted in an organ bath. After a 2-hour equilibration period isometric responses were recorded by means of force-displacement transducers (Hewlett-Packard model FTA-10) through an amplifier (Hewlett-Packard model E805) and a potentiometric recorder (ECB model RB102).

2.3.1. Rabbit aorta

Rings of 1-cm length, cut from thoracic aortas obtained from rabbits of either sex (2.5–4.0 kg body weight) were everted and the endothelium was removed as previously described [31]. The rings were mounted under a 1-g load in 6.0-ml chambers containing Krebs-bicarbonate solution (pH 7.40) of the following composition (in mM): NaCl 118; KCl 4.70; CaCl₂ 2.5; KH₂PO₄ 1.18; MgSO₄ 1.18; NaHCO₃ 25; glucose 10. The temperature was kept at 37 ± 0.5°C and the solution was bubbled with a gas mixture of 95% O₂ - 5% CO₂.

2.3.2. Rat uterus

Uterine horns from female rats (200–240 g), which received 100 mg diethylstilbestrol per 100 g body weight 24 h before the experiments, were removed and mounted in 5-ml organ baths containing De Jalon's solution of the following

composition (in mM): NaCl 137; KCl 5.36; CaCl_2 0.41; MgCl_2 0.19; Na_2HPO_4 0.36; NaHCO_3 11.9; glucose 5.5. The solution was bubbled with a gas mixture of 95% O_2 - 5% CO_2 and the temperature was kept at 30°C in order to inhibit the spontaneous contractions observed at higher temperatures.

2.3.3. Guinea-pig ileum

The lower portion of the ileum was excised from guinea-pigs (220–250 g) and washed in warm Tyrode solution of the following composition (in mM): NaCl 136.8; KCl 2.7; CaCl_2 1.36; MgCl_2 0.49; NaH_2PO_4 0.36; NaHCO_3 11.9; glucose 5.5. Segments of 4-cm length were cut and suspended in 5-ml organ baths which contained aerated Tyrode solution bubbled with a gas mixture of 95% O_2 - 5% CO_2 and the temperature was kept at $37 \pm 0.5^\circ\text{C}$.

2.3.4. Biological potency measurements

In rabbit aorta rings, concentration-response curves were obtained by cumulative addition of the peptide solution to the medium until the maximum response was reached. In rat uterus and guinea-pig ileum, the concentration-response curves were obtained by administration of successive treatments with the agonists for 90 s, at 5-min intervals. Intrinsic activities were estimated by the maximum response relative to that of the parent peptide (AngII or BK). Biological potencies were estimated by the cologarithm of the peptide concentration causing 50% of the maximum response (pD_2).

3. Results

3.1. Peptide synthesis

The following TOAC-labeled AngII and BK analogs were synthesized: $\text{TOAC}^1\text{-AngII}$, $\text{TOAC}^7\text{-AngII}$, $\text{TOAC}^3\text{-AngII}$, $\text{TOAC}^0\text{-BK}$ and $\text{TOAC}^3\text{-BK}$. The first two AngII analogs have already been reported [16,20]. As already stressed [16], unusual difficulties were found for coupling of the next amino acid following the incorporation of TOAC. This effect is likely due to the very low basicity of the TOAC amine group, whose pK_a decreased from 8 (when free) to lower than 6 when bound to peptide [19,20]. Recouplings required larger excess of reactants to assure quantitative incorporation of the subsequent amino acid residue in the sequence. More efficient acylating reagents [6,7] were also employed to enhance the coupling reaction.

3.2. Reversal of the nitroxide group protonation

An issue still not definitely evaluated in the synthesis of TOAC-containing peptides is the efficacy of the alkaline reversal step necessary for deprotonation of the nitroxide group after cleavage of the peptide from the resin in anhydrous HF. Different alkaline treatments varying the reaction time (up to 25 h), pH (between 9 and 11), and temperature

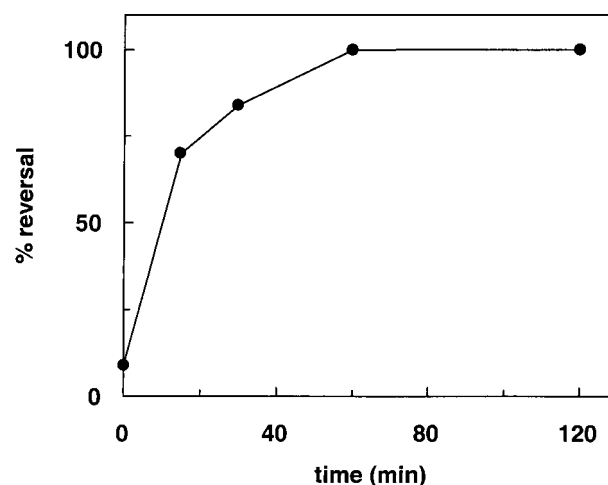


Fig. 1. Time course of $\text{TOAC}^3\text{-AngII}$ nitroxide deprotonation at pH 10 and 50°C , monitored by analytical HPLC.

(from 25 to 50°C) were compared, using $\text{TOAC}^3\text{-AngII}$ as a model. The deprotonation kinetics were monitored either by the recovery of the sample's EPR signal or by the increase in the area of the analytical HPLC peak corresponding to the deprotonated component, which progressively emerges during the alkaline treatment. In this latter approach, due to the more polar character of the positively charged protonated nitroxide group, this component eluted slightly faster in the HPLC chromatogram (ca. 1–2 min) than the parent deprotonated compound. The data revealed that the fastest and still efficient protocol was the treatment of labeled peptide at pH 10 for 1 h at 50°C (Fig. 1). After the purification step by preparative HPLC, the presence of paramagnetic TOAC-labeled analogs was assessed by EPR spectroscopy (Fig. 2).

3.3. Biological assays

The biological activity of the spin-labeled peptides was determined by analyzing concentration-response curves in three isolated smooth muscle preparations: rabbit aorta, rat uterus and guinea-pig ileum. In the case of the BK derivatives only the two last preparations were used, as the rabbit aorta is not sensitive to BK. Fig. 3 illustrates the concentration-response results for $\text{TOAC}^1\text{-AngII}$ in the rabbit aorta and for $\text{TOAC}^0\text{-BK}$ in the rat uterus, and Table 1 shows the values estimated for the potency and intrinsic affinities of the angiotensin and bradykinin analogs, obtained from their concentration-response curves. Only the spin-labeled Ang II and BK derivatives in which the TOAC residue was placed at the N-terminal end of the sequence presented significant biological potency in terms of spasmogenic activity and a high intrinsic activity. This was particularly evident in the case of $\text{TOAC}^1\text{-AngII}$, which has an intrinsic activity significantly higher than that of the parent compound, Ang II.

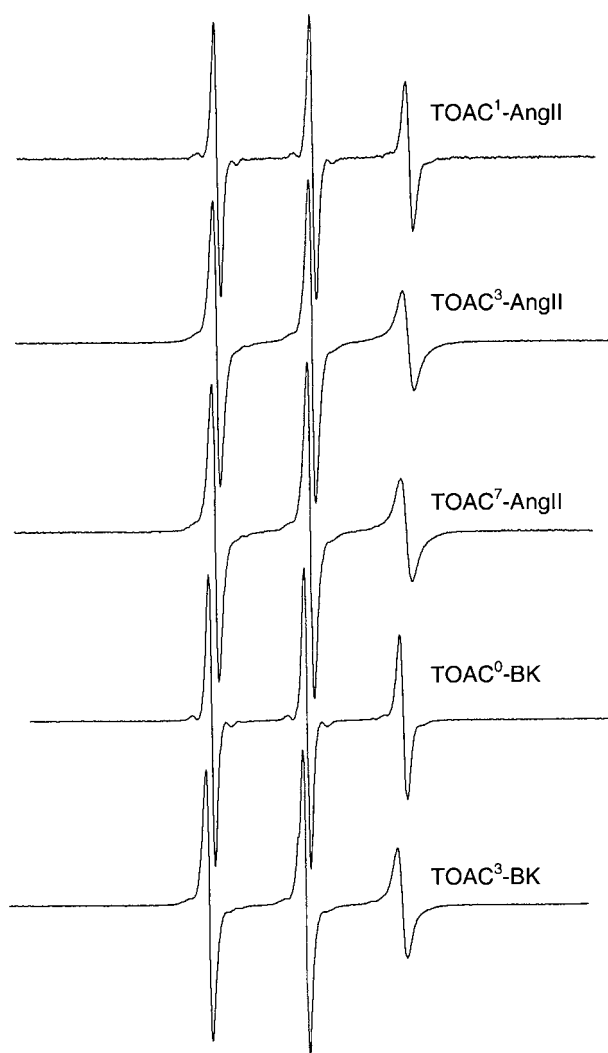


Fig. 2. EPR spectra of 5×10^{-5} TOAC-labeled AngII and BK analogs at pH 3.0.

4. Discussion

The EPR spectra of the TOAC-labeled analogs (Fig. 2) are characterized by narrow lines, as expected for small molecules tumbling freely in solution. Nevertheless, a significant difference is observed between N-terminally and internally labeled derivatives. The spectra of the latter displayed broader lines, indicative of a more restricted mobility. Calculated τ values were about 2×10^{-10} s and 5×10^{-10} s for N-terminally and internally labeled analogs, respectively. These differences probably arise from the contribution of two main factors: the greater motional restriction of TOAC when it is located in the middle of the peptide chain and different conformational properties of N-terminally and internally labeled peptides.

With regard to biological activity, TOAC¹-AngII presented a reduced, but still significant, potency on the three smooth muscle preparations, as previously reported [20]. However, the concentration-response curves evidenced a

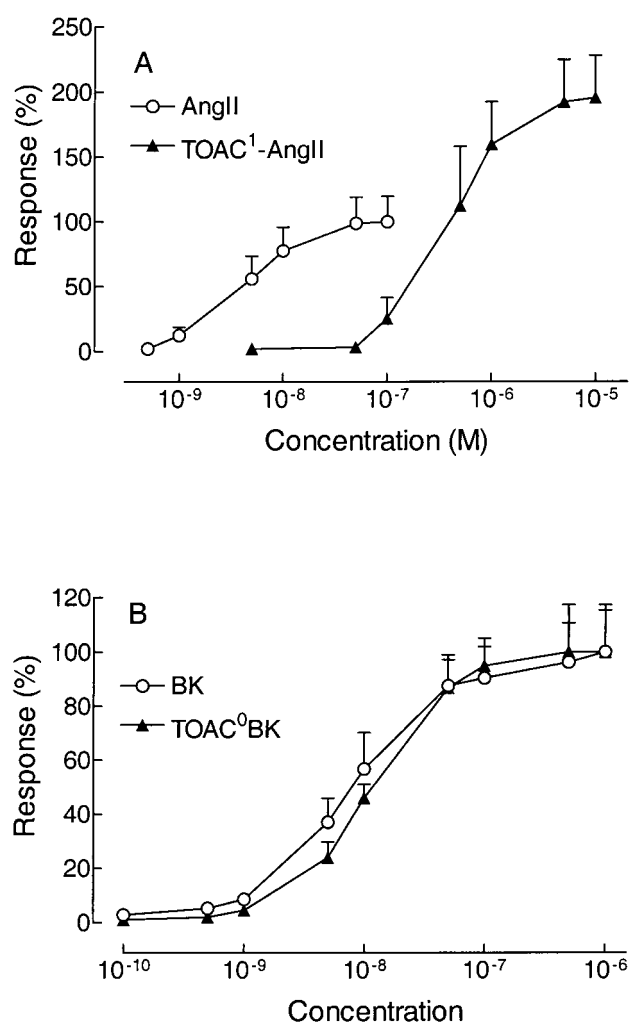


Fig. 3. Log concentration-response curves for the effects of: (A) AngII and TOAC¹-AngII on deendothelized rabbit aortic rings; (B) BK and TOAC⁰-BK on the isolated rat uterus. Each point is the average of three independent measurements and the standard deviations are indicated.

high intrinsic activity which, in the case of rabbit aorta was much higher than that of AngII itself. This indicates that, although this analog binds to the rabbit aorta receptor with lower affinity, once bound, it is more efficient than the parent compound in triggering a response. It has long been known, from structure-activity studies, that the N-terminal portion of AngII is not important for triggering the smooth muscle response [15,25], which is mainly due to the interaction of the Phe⁸ side-chain with helix VI of the AT₁ receptor [11,24]. The introduction of the TOAC moiety in position 1 of the peptide, in spite of its negative effect on the ligand's binding to the receptor, apparently places the C-terminal portion of the molecule in a more favorable position to activate the response. This hypothesis merits further investigation by studying the interaction of TOAC¹-AngII with the AT₁ receptor mutated at residues thought to be involved in the initiation of the signal for the smooth muscle cell response.

The AngII analogs in which the spin labels were located

Table 1

Biological activities of spin-labeled AngII and BK analogs in isolated smooth muscle preparations

Peptide	Rabbit aorta		Rat uterus		Guinea-pig ileum	
	pD ₂ *	Intrinsic activity [†]	pD ₂ *	Intrinsic activity [†]	pD ₂ *	Intrinsic activity [†]
AngII	8.18 ± 0.07	1.00	8.52 ± 0.33	1.00	8.72 ± 0.05	1.00
TOAC ¹ -AngII	6.41 ± 0.16 (2%)	2.01 ± 0.26	7.80 ± 0.30 (19%)	1.05 ± 0.01	7.98 ± 0.11 (18%)	1.25 ± 0.13
BK	ND	ND	8.10 ± 0.01	1.00	7.68 ± 0.11	1.00
TOAC ⁰ -BK	ND	ND	7.94 ± 0.02 (69%)	0.94 ± 0.03	6.36 ± 0.03 (5%)	1.07 ± 0.04

* pD₂ values are the negative logarithms of the concentration causing 50% of maximum response (-log ED₅₀) obtained by interpolation in concentration-response curves; values inside parentheses represent biological potency relative to AngII or BK.

[†] Relative intrinsic activities were obtained from the maximum responses. The values are averages ± S.D. from 6 experiments. ND, not determined.

at internal positions in the peptide chain (TOAC³- and TOAC⁷-AngII) had no detectable biological activity, indicating that the restricted conformation imposed by the TOAC residue in these positions is detrimental to the interaction of the peptide with its receptor. Moreover, it has been shown that the presence of Val³ is relevant, in conjunction with Ile⁵ and His⁶, to maintain the exact spatial features of the AngII residues for binding to the receptor [22]. Likewise, the lack of activity of TOAC⁷-AngII also reinforces the idea that the TOAC residue imposes its own characteristic bend in the peptide chain causing loss of AngII affinity for its receptor. This result suggests that, although TOAC is replacing another bend-inducing amino acid (proline), the conformational restriction imposed by the two amino acids is different. Further investigation focusing AngII analogs containing the cyclic TOAC probe in other positions is currently in progress aiming at better understanding the ligand's receptor binding mode and the complex cascade of steps with leads to physiological function.

The relatively high activity of the N-terminally labeled TOAC⁰-BK may be credited to the fact that the critical Arg¹ residue [29] is maintained in this analog's structure. Otherwise, the lack of activity observed for the internally labeled TOAC³-BK is in accordance with the crucial role of the Pro³ residue for BK activity [13] and reinforces the above conclusion that TOAC and Pro do not induce similar bends. It should be pointed out that TOAC⁰-BK is the first spin-labeled BK analog to retain a significant biological effect, with both high affinity for the receptor and high intrinsic activity. This should render this active spin labeled analog of great value for further BK structure-function studies.

In conclusion, we provide clear evidence for the different conformational properties imparted by labeling at different peptide positions and the consequent alterations in the hormone's biological activity. Therefore, TOAC labeling presents the possibility of obtaining inactive and partially or totally active peptide analogs, thus enhancing the potential of this approach for comparative structure-activity studies.

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Anexo 13



Conformational studies of TOAC-labeled bradykinin analogues in model membranes

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Key words: bradykinin, CD, EPR, micelle, peptide conformation, spin label, structure-activity relationship, TOAC

Summary

Spin-labeled analogues of bradykinin (BK) were synthesized containing the amino acid TOAC (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid) either before Arg¹ (TOAC⁰-BK) or replacing Pro³ (TOAC³-BK). Whereas the latter is inactive, the former retains about 70% of BK's activity in isolated rat uterus. A combined electron paramagnetic resonance (EPR)-circular dichroism (CD) approach was used to examine the conformational properties of the peptides in the presence of membrane-mimetic systems (negatively charged sodium dodecyl sulfate, SDS, and zwitterionic N-hexadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate, HPS). While the peptides bind to both monomeric and micellar SDS, no interaction occurs with HPS, evincing the contribution of electrostatic interactions. TOAC³-BK's EPR spectral lineshapes are broader than those of TOAC⁰-BK, indicating a more restricted degree of motion at position 3. Moreover, the motional freedom of both peptides decreased upon binding to SDS. BK and TOAC⁰-BK solution CD spectra indicate highly flexible conformations (possibly an equilibrium between rapidly interconverting forms), while TOAC³-BK's spectra correspond to a more ordered structure. SDS binding induces drastic changes in BK and TOAC⁰-BK spectra, indicating stabilization of similar folds. The micelle interface promotes a higher degree of secondary structure by favoring intramolecular hydrogen bonds. In contrast, TOAC³-BK spectra remain essentially unchanged. These results are interpreted as due to TOAC's ring imposing a more constrained conformation. This rigidity is very likely responsible for the inability of TOAC³-BK to acquire the correct receptor-bound conformation, leading to loss of biological activity. On the other hand, the greater flexibility of TOAC⁰-BK and the similarity between its conformational behavior and that of the native hormone are probably related to their similar biological activity.

Abbreviations: AII, angiotensin II; Aib, α -aminoisobutyric acid; BK, bradykinin; Boc, N^α-*tert*-butoxycarbonyl; CD, circular dichroism; cmc, critical micelle concentration; DMF, N,N-dimethylformamide; EPR, electron paramagnetic resonance; Fmoc, 9-fluorenylmethyloxycarbonyl; HPLC, high-performance liquid chromatography; HPS, N-hexadecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate; NMR, nuclear magnetic resonance; POAC, 2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-amino-4-carboxylic acid; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TOAC, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid.

Introduction

The use of the amino acid spin label TOAC (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid) [1] in peptide chemistry was pioneered by our group two decades ago. The strategy initially used had

the limitation of attaching the probe solely at the N-terminus of the peptide sequence [2, 3]. About one decade later [4], an approach involving the combination of two different strategies enabled the insertion of TOAC at any internal position of the peptide chain, greatly expanding its use for a multitude of object-

ives. Recently, we have described a new paramagnetic amino acid probe (POAC) for use in peptide labeling [5].

The introduction of TOAC has contributed to the study of conformational properties of synthetic and natural peptides by means of the analysis of their electron paramagnetic resonance (EPR) spectra. In several occasions EPR data have been complemented by the use of other spectroscopic techniques [6–9] and of X-ray crystallography [9, 10]. Due to its special $C^{\alpha,\alpha}$ -tetrasubstituted cyclic structure TOAC binds rigidly to target molecules or systems being extremely sensitive to their conformational states. TOAC has been found to favor the formation of bends and 3_{10} - and α -helices [10]. Reports in the literature include conformational studies of single [10–12] or doubly labeled model peptide fragments [6, 13], fungal peptides in solution [14, 15] or in the presence of membrane-mimetic environments [7, 8], peptides corresponding to loops of G protein-coupled receptors, in solution and in the presence of model membranes [16, 17], and of peptide aggregation processes in solution [9]. TOAC was also attached to polymer matrices to monitor solvation effects during peptide synthesis [18–20] or to improve solid phase analytical methods [21]. TOAC's fluorescence quenching properties have also been explored [22, 23].

In studies focusing biologically active peptides, such as hormones, structure-biological activity relationships of TOAC-labeled compounds have been examined. One early study focused on the vasoactive peptide angiotensin II (AII) [3]. More recently, research was performed on melanocyte stimulating hormone [24] and its potent analogue melanotan [23]. These represent the first TOAC-bearing compounds that maintained the full original potency of the native peptides. The vasoactive peptide hormone bradykinin (RPPGFSPFR, BK) [25, 26] was labeled at two different positions and biological assays revealed that when TOAC was bound to Arg¹ of BK (TOAC⁰-BK), this N-terminally-labeled derivative maintained about 70% of the activity of the native hormone in rat uterus, whereas internally labeled TOAC³-BK was totally inactive [27, 28]. Preliminary conformational studies of these paramagnetic peptides in solution by means of EPR and CD spectroscopies have shown that in aqueous solution the two peptides possess different conformations, TOAC⁰-BK being more flexible and displaying CD spectra that resemble very closely those of the unlabeled peptide. On the other hand, TOAC³-

BK exhibits spectra suggestive of a more constrained conformation [29].

In the present report the EPR-CD approach is extended to the investigation of the conformation and dynamics of the TOAC-containing BK derivatives in the presence of membrane mimetic systems. The conformational behavior of TOAC⁰-BK and TOAC³-BK is compared to that of the native hormone in solution and upon interaction with variable concentrations of anionic (sodium dodecylsulfate, SDS) and zwitterionic (N-hexadecyl-N,N-dimethyl-3-ammonium-1-propanesulfonate, HPS) surfactants. The results are discussed in the light of their biological activity.

Material and methods

Materials

N^α-*tert*-butyloxycarbonyl (Boc) amino acids were from Bachem, Torrance, CA; 9-fluorenylmethoxycarbonyl (Fmoc) amino acids came from Novabiochem, San Diego, CA. Solvents and reagents were from Aldrich-Sigma Co. DMF was distilled (over P₂O₅ and ninhydrin under reduced pressure) before use. All chemicals met ACS standards.

Methods

Peptide synthesis

Details of the syntheses of BK, TOAC⁰-BK and TOAC³-BK are published elsewhere [28].

Analytical HPLC

The purity of peptides was verified in a Waters Associates analytical HPLC system, consisting of two 510 HPLC pumps, an automated gradient controller, Rheodyne manual injector, 486 UV detector and 746 data module. Solvent A: 0.1% TFA/H₂O and solvent B: 60% acetonitrile/0.1% TFA/H₂O with a gradient of 5–95% of B in 30 min, at a flow rate of 1.5 ml min⁻¹.

Sample preparation

The peptide solutions, in the absence and presence of variable surfactant concentrations, were prepared in 15 mM phosphate-borate-citrate (PBC) buffer, at pH 7.0.

EPR studies

EPR measurements were carried out at 9.5 GHz in a Bruker ER 200D-SRC spectrometer at room temperature (22 ± 2 °C) using flat quartz cells (Wilma Glass Company Inc., Buena, New Jersey). The magnetic field was modulated with amplitudes less than one-fifth of the line widths, and the microwave power was 5 mW to avoid saturation effects. Rotational correlation times (τ_B , τ_C) were calculated according to the equations given in Ref. 30.

CD studies

Circular dichroism spectra were recorded either on Jobin Yvon CD6 (using a thermostated water bath to control the cell temperature) or on Jasco J-810 (at room temperature) spectropolarimeters. Samples were placed in 0.2 mm pathlength circular quartz cells and the spectra were run in the 260–195 nm wavelength range, using 0.5 nm bandwidth. The response time was 1 or 8 s, and the spectra are the result of four or eight accumulations when using the Jobin Yvon or the Jasco instruments, respectively. The data are expressed as mean residue molar ellipticity $[\theta]$ ($\text{deg cm}^2 \text{dmol}^{-1}$).

Results and discussion

EPR studies

Figure 1 shows that the EPR spectra of both TOAC⁰-BK and TOAC³-BK in aqueous solution exhibit narrow lines, as expected for molecules of molecular weight ca. 1000, which tumble fast in the time scale of the experiment. The fact that the TOAC moiety is linked to the peptide backbone via a peptide bond enables this probe to report more faithfully on the motional properties of the entire molecule. A comparison between the spectra of both peptides in solution (Figures 1Aa and 1Ba) shows that TOAC⁰-BK presents narrower lines, indicating that the paramagnetic amino acid has a larger degree of freedom when it is located at the peptide's N-terminus. This is quantitatively expressed by means of the calculation of rotational correlation times, τ_B and τ_C [30]. In aqueous solution the value of τ_C for TOAC³-BK (ca. 5×10^{-10} s) is approximately 2.5 times larger than that of TOAC⁰-BK (ca. 2×10^{-10} s) (Figure 2A). Moreover, it is possible to evaluate the anisotropy of molecular motion by calculating the τ_C/τ_B ratio (Figure 2C) [30]. It is seen that, while this ratio is ca. 1.18 for TOAC⁰-BK, it increases to ca. 1.4 for TOAC³-BK. This result again

emphasizes the greater mobility at the N-terminus, a well known fact for peptides in general. Interestingly, the value of τ_C/τ_B ($\gg 1$) for TOAC³-BK reveals a large degree of anisotropic motion and corroborates the conclusion from CD spectroscopy that this derivative already displays a more restricted conformation even in aqueous solution (see below).

Upon addition of increasing SDS concentrations, the EPR spectra of the two TOAC-labeled BK derivatives undergo progressive line broadening, indicating that the peptides interact with the surfactant (Figures 1A and B). In contrast, essentially no spectral changes occur in the presence of zwitterionic HPS (Figures 1C and D). Accordingly, the parameters calculated from the EPR spectra (τ_C , the τ_C/τ_B ratio, and the isotropic hyperfine splitting, a_N , Figure 2) also remain essentially unchanged in the presence of HPS. These results point to the role of electrostatic interactions in the binding of the positively charged peptide to the negatively charged SDS. The spectra also show that, similarly to what occurs in aqueous solution, the lines are broader for TOAC³-BK, indicating that this analog is also more strongly immobilized in the micellar environment. In agreement with the qualitative line broadening observation, τ_C values increase (by a factor of 5–6) with increasing SDS concentration, reaching maximum values of ca. 1.1×10^{-9} s for TOAC⁰-BK and 3×10^{-9} s for TOAC³-BK (Figure 2A). It is also noteworthy that the peptide-SDS interaction takes place both above and below the detergent's cmc (8 mM, Ref. 31). In addition, the τ_C/τ_B ratio for TOAC³-BK is greater below (1.8) than above (1.6) the cmc of SDS (Figure 2C), suggesting that the complex formed by the peptide and the detergent's monomers is highly organized.

In Figure 2B we monitor the polarity of the environment probed by TOAC in both peptides by means of the measurement of a_N . The a_N values are seen to decrease upon peptide binding to SDS, indicating an environment of lower polarity. The value of a_N is somewhat lower for TOAC³-BK than for TOAC⁰-BK in micellar SDS. This might be due to the fact that the peptide's third amino acid is more deeply inserted in the micelle than its N-terminus.

CD studies

CD spectra of BK and its TOAC derivatives were obtained in solution and in the presence of increasing SDS (Figure 3) and HPS concentrations (not shown). In aqueous solution the spectrum of BK closely re-

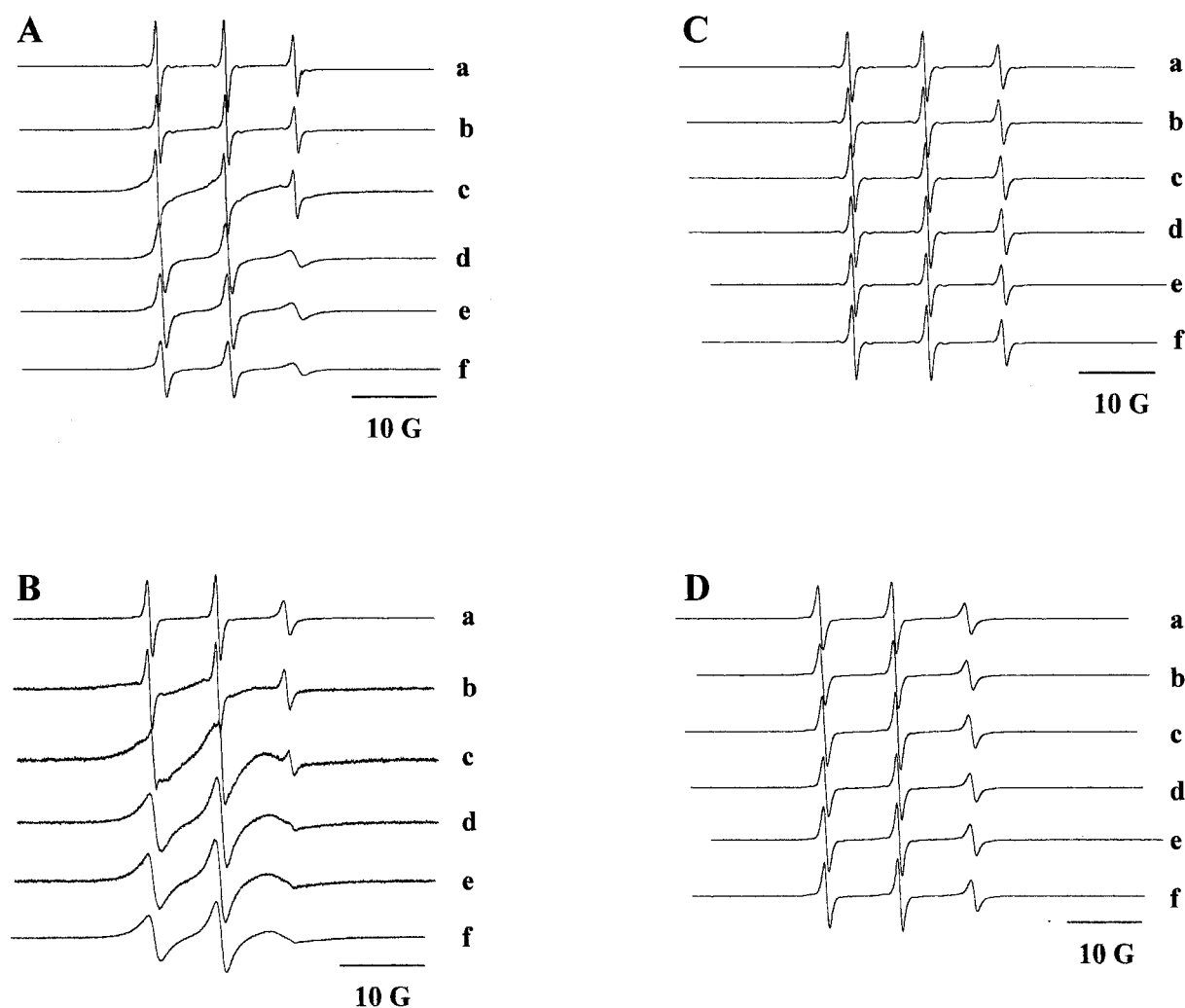


Figure 1. EPR spectra of 0.1 mM (A, C) TOAC⁰-BK and (B, D) TOAC³-BK in the presence of (a) 0, (b) 0.5, (c) 1.0, (d) 5.0, (e) 10, (f) 50 mM SDS (A, B) and (a) 0, (b) 0.05, (c) 0.5, (d) 1.0, (e) 5.0, (f) 10 mM HPS (C, D). 15 mM PBC, pH 7.0.

sembles those obtained previously by different groups [32–35]. This spectrum displays a weak negative band centered at ca. 230 nm and a strong negative band at ca. 202 nm and has been ascribed to a flexible structure (probably an equilibrium between rapidly interconverting conformations) [34, 36, 37]. Figure 3B shows that the solution conformation of TOAC⁰-BK resembles very much that of the unlabeled peptide, demonstrating that binding of TOAC to the hormone's N-terminus has little effect on its conformational properties.

In contrast, when the paramagnetic amino acid replaces Pro³, a considerable effect is noted on the peptide's conformation (Figure 3C). The CD spectrum

of TOAC³-BK indicates a more restricted conformation resembling that obtained when Pro³ was replaced by α -aminoisobutyric acid (Aib) [38]. Analysis of the CD and NMR spectra of Aib³-BK led to the conclusion this substitution stabilizes a degree of β -turn conformation in the N-terminal region of this analogue [38]. Aib imposes a different fold since this amino acid is thought to favor structures corresponding to *cis*-Pro conformers [38], while the Pro²-Pro³ bond was found to be about 90% *trans* in native BK [39]. In agreement with the lack of biological activity found for TOAC³-BK, Aib³-BK also displays a considerable loss of activity [40]. The similarity between the folds imposed by TOAC and Aib seems to indicate

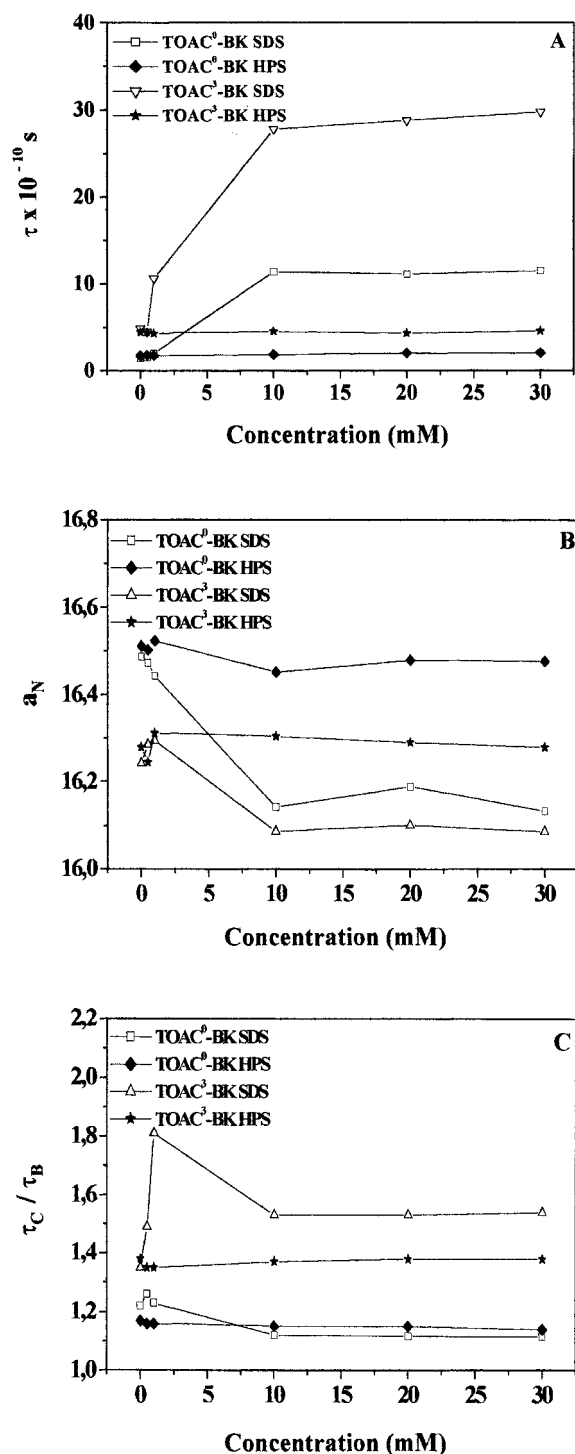


Figure 2. Effect of SDS and HPS concentration on the EPR spectral parameters τ_C (A), a_N (B) and τ_C/τ_B (C) for TOAC⁰-BK and TOAC³-BK, calculated from the spectra in Figure 1.

that both amino acids exert similar spatial constraints, preventing the substituted peptides from achieving their receptor-bound conformation. Moreover, in studies of the Aib-containing peptide antibiotic trichogin GA IV, it was found that the substitution of TOAC for Aib did not have strong effects either upon the peptide's conformation or function [7]. These data reinforce the notion that TOAC and Aib impart similar conformational characteristics on peptides.

Addition of SDS led to conformational changes in the CD spectra of BK and its N-terminal TOAC derivative (Figures 3A and B, respectively), but essentially no changes in TOAC³-BK's spectra (Figure 3C). In agreement with the EPR results, the peptides CD spectra remained essentially unaltered in the presence of HPS (not shown). The CD spectra of SDS-bound BK (Figure 3A) and TOAC⁰-BK (Figure 3B) resemble those previously reported for the parent peptide [41]. Analysis of the CD [41] and NMR [42] spectra of SDS-bound BK led to the suggestion that binding leads to the formation of a β -type turn at residues 6–9, while the molecule's N-terminal portion is quite flexible. In agreement with the EPR results, the peptides are seen to interact with SDS both below and above the detergent's cmc. The spectra show that the peptides conformation does not vary much in both conditions.

A different conformational behavior is found for TOAC³-BK upon interaction with SDS. The peptide's CD spectra undergo almost no changes as a result of binding to monomeric or micellar SDS (Figure 3C). We interpret this behavior as being due to the already constrained conformation of this derivative imposed by TOAC at position 3. Although the peptide clearly interacts with the detergent, as demonstrated by the EPR results, this interaction does not lead to significant conformational changes. It is noteworthy that the addition of increasing amounts of the secondary structure-inducing solvent trifluoroethanol (TFE) also promoted conformational changes in the TOAC-labeled BK analogues, but in this case, too, the final conformations achieved by both peptides were different [29].

It has been proposed that binding to lipid environments would modulate the accessibility of peptide hormones to their membrane-bound receptors and that this binding could induce the hormone receptor-bound conformation [43]. In the light of these arguments, the present results have shown that, similarly to native BK, upon binding to SDS, the biologically active TOAC⁰-BK derivative, is capable of having a β -turn stabilized at its C-terminal portion. In contrast, the

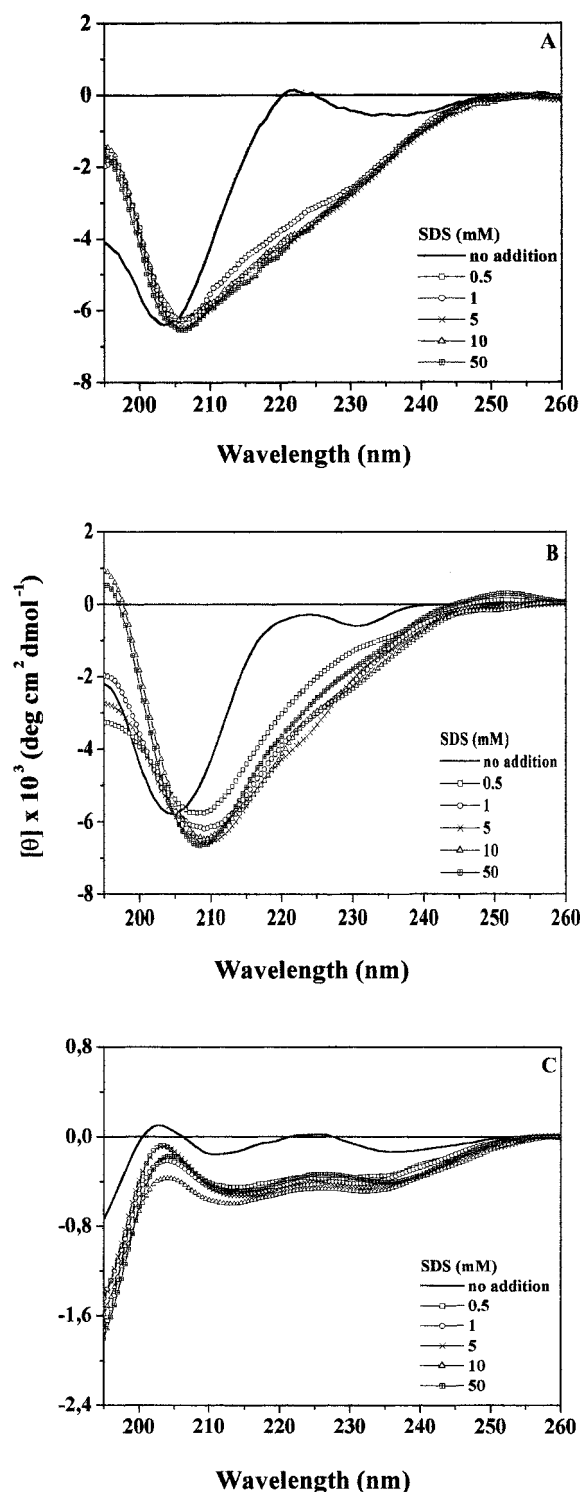


Figure 3. CD spectra of 0.1 mM BK (A), TOAC⁰-BK (B), and TOAC³-BK (C) in the absence and presence of increasing SDS concentrations. 15 mM PBC pH 7.0.

fold promoted by TOAC in TOAC³-BK is similar to that induced by Aib, resulting in conformationally constrained and biologically inactive derivatives in both cases.

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Anexo 14

Monitoring the Chemical Assembly of a Transmembrane Bradykinin Receptor Fragment: Correlation Between Resin Solvation, Peptide Chain Mobility, and Rate of Coupling

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Keywords: Bradykinin / EPR spectroscopy / Membranes / Peptides / Resins

A combined resin solvation-peptide chain motion and kinetics of coupling reaction approach was applied to monitor details of the synthesis of TM-34, a 34-residue transmembrane segment of the bradykinin receptor. The dynamics of resin-bound peptide fragments attached to a stable free radical amino acid were examined by EPR spectroscopy. In agreement with an abrupt decrease (from 83 to 43%) in peptide purity occurring in the 12–16 region when DMF was used, a much more strongly immobilized chain population was detected, especially at the 12-mer stage. Conversely, faster

couplings and improved synthesis were observed in 20% DMSO/NMP, probably due to the higher chain mobility in this mixed solvent. In addition, findings relating to solvation of peptide resins seemed to corroborate the previously advanced proposition that the 1:1 sum of electron acceptor and electron donor properties of a solvent can be considered to be an alternative and more appropriate parameter for its polarity.

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Introduction

Some decades ago, the use of beaded-form cross-linked polymers was mostly restricted to stationary phases in column chromatography. The landmark development that launched such polymeric materials towards more specialized levels of application in many different fields occurred in the early 1960s^[1] with the introduction of the solid-phase peptide synthesis (SPPS) method.^[2] The concept of performing chemical processes on an insoluble polymer matrix has been successfully extended to the development of efficient synthetic methodologies for oligonucleotides^[3] and polysaccharides.^[4] More recently, solid phase-based combinatorial chemistry methods have again proven fruitful not only in the generation of peptide libraries,^[5] but also in the development of solid-phase organic synthesis strategies, with a remarkable impact on drug development.^[6]

As a consequence of this increasing trend in the use of polymer-dependent experimental procedures, a large number of different resins have been developed,^[7] together with analytical methods aiming to clarify the potential of each polymer-based methodology. In this latter case, many efforts have been made, involving the use of NMR,^[8] IR,^[9] fluorescence,^[10] and CD^[11] spectroscopy. Although applied comparatively less often, EPR^[12] has been of great value, as it provides relevant information about the solvated polymeric network. In this context, the use of the paramagnetic amino acid probe TOAC (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid),^[13] initially used for peptide labeling^[14] and later for structural investigation of solvated or peptide-supporting polymers,^[15] has also been actively pursued.

In the spectroscopic evaluation of polymeric material, the solvent system plays a crucial role. Solvent molecules may affect the average distance between chains (and, as a result, the degree of chain-chain association), or control the rate of motion of components and the kinetics of reaction. For this reason, polymer solvation has been intensively studied by a variety of experimental procedures.^[16] In this regard, a study of the solvation characteristics of model peptide-resins in about 30 single and mixed solvents of different polarity led us to propose the (AN+DN) parameter,^[17] the sum of Gutmann's electron acceptor (AN) and electron

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donor (DN) solvent numbers^[18] in a 1:1 proportion, as an alternative polarity scale.

In this context, step-by-step monitoring of physico-chemical variations that may occur during the resin-bound elongation of a transmembrane fragment bound to a polymer structure may be a distinct and valuable strategy through which to address this issue. This is due to the inherent propensity of long, hydrophobic peptide sequences to undergo aggregation/association processes throughout the swollen resin network. Moreover, it can be of great value in the detection of minor, but crucial, microenvironmental details relevant for monitoring of peptide-resin elongation during SPPS. Thus, taking into account our previous work^[19] showing the difficulty of synthesizing and purifying a very hydrophobic 34-residue peptide (TM-34, CTVAEIYLGNLGADLILASGLPFWAITIANNFD) corresponding to a transmembrane segment (residues 69–97) of the rat bradykinin B2 receptor,^[20] this report presents an investigation of the dynamics of the association properties of shorter peptides from the TM-34 sequence. The work focuses on peptide-resin solvation (swelling measurement of bead sizes), peptide chain mobility (EPR study of TOAC-labeled peptide-resins), and the kinetics of the coupling reaction. The purpose was to obtain relevant physicochemical data regarding the swelling properties of the polymer backbone during peptide elongation and the dependence on the solvent system used for the coupling reaction. Special emphasis was placed on the TM-34 region, in which a pronounced difference in bead solvation was observed.^[19]

Results

Solvation Studies

To monitor the solvation behavior as a function of chain length, the degrees of swelling of peptide-resins containing different portions of the TM-34 sequence were determined in DCM, DMF, and 20% DMSO/NMP. Table 1 shows a sharp decrease in the peptide-resin solvation at the 12-mer stage in DMF, but not in DCM or in 20% DMSO/NMP

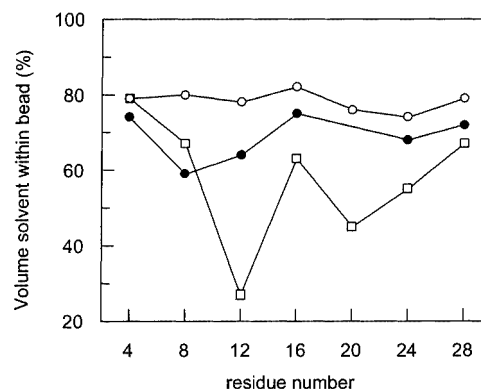


Figure 1. Degree of swelling of TM-(4, 8, 12, 16, 20, 24 and 28)-resins in DCM (●), DMF (□) and 20% DMSO/NMP (○)

(Figure 1), revealing considerable solvent-dependent peptide chain aggregation at this position. Much better swelling (about 80% of swollen bead volume occupied by the solvent) was found in 20% DMSO/NMP; a smaller improvement was seen with the less polar solvent DCM. These findings seem to be in accordance with a preliminary study of the TM-34 assembly^[19] in which a strongly solvent-dependent synthesis yield was observed, together with a critical region located at the 12–16-residue segment. A sharp decrease in the yield (from 83 to 43%) was observed for this region when DMF was used for coupling. However, the yield was considerably improved when the mixed 20% DMSO/NMP solvent system was employed instead.

The (AN+DN) solvent parameter has been proposed as a novel polarity parameter in two complementarily reports.^[17] In order to compare this scale with that based on the dielectric constant ϵ , the swelling behavior of two peptide-resins, TM-12 and TM-24, was investigated in several solvent systems (Table 2) commonly used in some steps of a cycle of the SPPS method. In close agreement with these earlier reports, which focused on other polymers,^[17] the dispersion of points in the swelling versus solvent polarity plot when the (AN+DN) parameter was employed was less for both peptide-resins than that obtained when the ϵ term was used (Figure 2).

Table 1. Degrees of swelling of TM-(4, 8, 12, 16, 20, 24 and 28) resins in different solvents

Peptide resin	DCM		DMF		20% DMSO/NMP	
	Diameter of swollen bead (μm)	Solvent within bead (%) ^[a]	Diameter of swollen bead (μm)	Solvent within bead (%) ^[a]	Diameter of swollen bead (μm)	Solvent within bead (%) ^[a]
TM-4	77	74	82	79	83	79
TM-8	70	59	75	67	91	80
TM-12	76	64	60	37	90	78
TM-16	87	75	78	63	97	82
TM-20	—	—	67	45	88	76
TM-24	82	68	73	55	88	74
TM-28	89	72	84	67	97	79

^[a] [(swollen volume – dry volume)/swollen volume] × 100 using the following values for measured diameters of dry beads: Resins: TM-4 = 49 μm, TM-8 = 52 μm, TM-12 = 54 μm, TM-16 = 55 μm, TM-20 = 55 μm, TM-24 = 56 μm, TM-28 = 58 μm.

Table 2. Degrees of swelling of TM-12 and TM-24-resins in different solvents

Entry	Solvent	Solvent parameter		Solvent within bead ^[a] (%)	
		ϵ	(AN+DN)	TM-12	TM-24
1.	TOL	2.4	3.4	66	60
2.	DCM	8.9	21.4	64	68
3.	CHCl ₃	4.7	27.1	75	72
4.	NMP	33.0	40.6	80	76
5.	DMF	36.7	42.6	37	55
6.	DMSO	46.7	49.1	52	62
7.	TFE	26.7	53.5	15	32
8.	EtOH	24.3	69.1	0	38
9.	MeOH	32.6	71.3	5	25
10.	Formamide	109.5	63.8	10	12
11.	50% TFE/TOL	14.6	28.5	59	71
12.	20% TFE/DCM	12.5	27.5	60	80
13.	50% TFE/DCM	17.8	37.5	58	72
14.	80% TFE/DCM	23.1	47.4	26	39
15.	20% DMSO/NMP	35.7	42.3	78	74
16.	50% DMSO/THF	27.1	38.6	56	63
17.	65% NMP/THF	24.1	36.1	76	73
18.	50% DCM/DMF	22.8	32.0	64	63
19.	50% DMSO/DCM	27.8	35.3	49	64
20.	50% DMSO/MeOH	39.7	60.2	28	39
21.	50% TFE/DMF	31.7	48.1	10	29
22.	50% TFE/DMSO	36.7	51.3	7	24
23.	10% TEA/DCM	8.3	25.1	76	81
24.	10% TEA/DMF	33.3	44.5	60	59
25.	10% TEA/DMSO	42.3	50.4	46	53

^[a] [(swollen volume – dry volume)/swollen volume] × 100.

The maximum solvation region occurred at low (AN+DN) values both for the TM-12 and for the TM-24 resins (see C and D in Figure 2). The fact that this region corresponds to those of more apolar solvents is in accordance with the hydrophobicity of these sequences and, more significantly, with the strong and dominant characteristic of the polystyrene structure of the methylbenzhydrylamine-resin. Figure 2 (see C) also shows the lower degree of solvation of TM-12-resin in DMF (solvent 5), as compared to other polar aprotic solvents such as DMSO (solvent 6) or NMP (solvent 4). Swelling in the mixed solvents 21 and 22 (open circles) was slightly less than predicted by their polarity values and is discussed further below in the light of the acidity and basicity of their components.

EPR Studies

EPR spectra of TOAC-labeled peptide-resins containing 8, 12, 16, 20, and 28 residues of the TM-34 sequence in DMF and in 20% DMSO/NMP are shown in Figure 3. Whereas the spectral line-shapes for all peptide-resins are similar in 20% DMSO/NMP (see A in Figure 3), indicating a relatively high mobility, the spectra reveal different mobility for the sequences in DMF (see B in Figure 3). While TM-8 and TM-16 exhibit considerable freedom of motion, the other sequences give rise to spectra indicative of two populations, one strongly (broad triplet) and one weakly (narrow triplet) immobilized. The proportion of the immobilized component is greatest for TM-12.

Table 3 presents some spectral parameters previously employed to assess the dynamics of labeled sites in the polymer network.^[15] The central peak linewidth (W_o) contains the contributions of both the weakly immobilized and the strongly immobilized chain populations and the h_{-1} term of the ratio of heights of the high- and mid-field lines (h_{-1}/h_0) corresponds essentially to the more mobile component. Thus, the lower the values of W_o , or the higher the h_{-1}/h_0 ratio, the faster the motion of the labeled resin sites. The EPR parameters in Table 3 corroborate the differentiated solvent-dependent behavior of some of the sequences, especially the TM-12-resin. While the variation of both W_o and h_{-1}/h_0 is small in 20% DMSO/NMP, these values vary considerably in DMF. The W_o value increases in the order TM-8 < TM-16 < TM-20 < TM-28 < TM-12. Accordingly, the h_{-1}/h_0 ratio decreases in the same order, except for the first two peptide-resins. It can also be seen that the W_o (h_{-1}/h_0) values for TM-28 and TM-12 in DMF are much larger (smaller) than for the other peptide-resins; these values are also larger (smaller) than those found in 20% DMSO/NMP for all peptide-resins.

Study of the Rate of Coupling

In view of the striking differences with regard to the TM-12-resin's solvation properties and chain mobility inside resin beads, the kinetics of coupling reactions of this peptide-resin in DMF and in 20% DMSO/NMP were compared. The yield of coupling of the next incoming amino

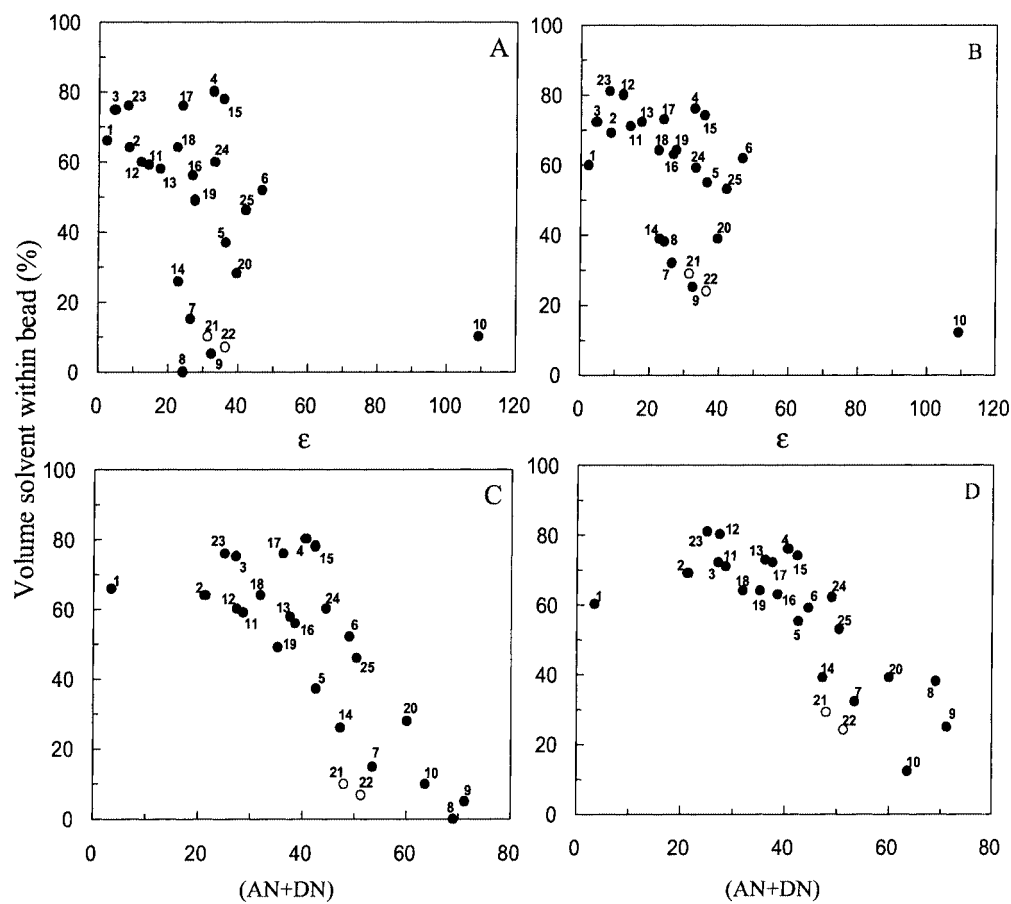


Figure 2. Degree of swelling of TM-12 [A, C] and TM-24 [B, D] resins as a function of parameters ϵ (A, B) and (AN+DN) (C, D) values, in 25 solvents

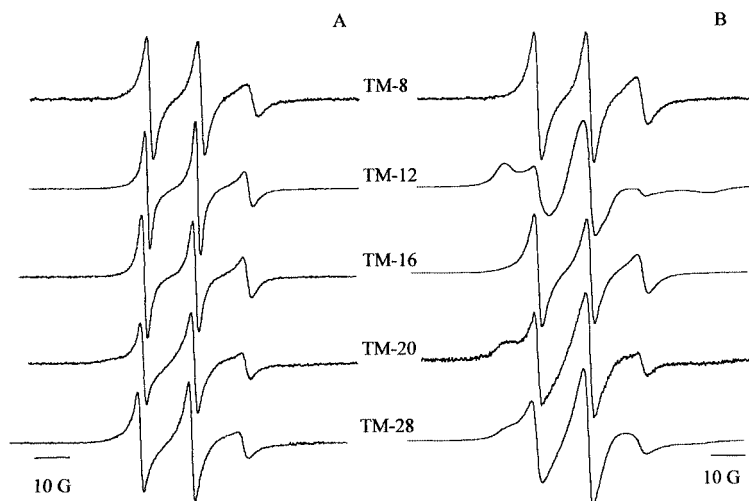


Figure 3. EPR spectra of TM-(8, 12, 16, 20 and 28)] resins in 20% DMSO/NMP (A) and DMF (B)

acid in the sequence (Leu¹³) was determined under equivalent acylating conditions, by the picric acid method. The acylation reaction was faster in 20% DMSO/NMP than in polar, aprotic DMF. While the coupling yield reached 90% in one hour in the former solvent system, it was 69% in the latter.

Discussion

Solvation Properties of Resins. Correlation with Solvent Polarity

The combined swelling/EPR monitoring strategy, applied to the progressive growth of the TM-34 chain bound to a

Table 3. Effect of solvent upon EPR spectral parameters of peptide-resins swollen in DMF and 20% DMSO/NMP

Peptidyl resin	DMF		Solvent 20% DMSO/NMP	
	W_o (G)	h_{-1}/h_0	W_o (G)	h_{-1}/h_0
TM-8	2.41	0.36	2.21	0.29
TM-12	3.62	0.07	1.92	0.25
TM-16	2.53	0.43	2.10	0.32
TM-20	2.64	0.21	2.10	0.27
TM-28	3.02	0.15	2.18	0.22

solid support, reveals some details of the resin backbone in the swollen state. The swelling data for seven peptide-resins (Table 1 and Figure 1) indicate that the bead solvation range extends from 37% (TM-12 in DMF) to 82% (TM-16 in 20% DMSO/NMP). DMF shows the most pronounced variation in peptide-resin solvation as the sequence is built. The severe shrinking in this solvent for the 12-mer, and to a smaller extent for the 20-mer, emphasizes the complexity of solute-solvent interactions. The results show that DMF was incapable of disrupting peptide-peptide or peptide-matrix interactions in the 12-mer sequence. In contrast, DCM and, to a greater extent, 20% DMSO/NMP, had the capacity to prevent these interactions.

The special characteristics of DMF, differing from most of the other solvent systems, had already been observed^[15] during the synthesis of a well known strongly aggregating octapeptide sequence.^[21] These findings suggest caution in the use of this solvent, not only for SPPS, but also for other solid-supported methods. Interestingly, this conclusion seems to be in disagreement with the widely accepted concept that less polar solvents, such as DCM, are more capable of inducing strong peptide chain association.^[22] These contradictory results reinforce the notion that there are still open questions concerning the rules governing site-site interactions throughout the polymer network. In this context, the suggestion of the alternative use of other single or mixed polar aprotic solvents such as NMP or 20% DMSO/NMP seems pertinent.^[16b,23] When extended to more severe chain aggregation conditions, the use of very strong electron acceptor solvents (such as the polyfluorinated TFE or HFIP) as co-solvents, in association with weaker electron acceptor solvents (such as DCM or chloroform) to disrupt association forces has been suggested.^[24]

The purpose of a more complete swelling investigation of TM-12 and TM-24 peptide-resins (Figure 2) was not only to identify solvents more appropriate for their solvation (Table 2), but also to test the applicability of the previously proposed (AN+DN) polarity parameter as carried out with other polymeric materials.^[17] Both peptide-resins displayed improved solvation in less polar solvents, characterized by low ϵ or (AN+DN) values (Figure 2). As already stressed, DMF (solvent 5) induces strong shrinking of the TM-12 resin beads (see C in Figure 2), for which a low degree of swelling (37%) was measured. This effect was not observed for the 24-mer (see D in Figure 2). Nevertheless, the en-

hanced solvation attained by both resins in apolar solvent systems is clearly indicative of the dominant influence of the hydrophobic styrene-resin matrix in association with the presence of peptide segments.

Another objective of this study was to verify the relationship between the swelling properties of the peptide-resins and the polarity of the medium, measured either by the dielectric constant or by the (AN+DN) parameter. Analysis of Figure 2 shows better correlation with the (AN+DN) scale than with ϵ . Therefore, the amphoteric character of the sum of the solvent's Lewis acid and Lewis base properties (in a 1:1 proportion) seems to yield a more adequate parameter with which to monitor solute-solvent interactions. In contrast, the macroscopic parameter ϵ yields a worse fit of the solvation behavior, since only electrostatic interactions are taken into account, while the alignment of the solvent and the solute dipoles is not considered.^[25]

These findings thus endorse the appropriateness of the amphoteric (AN+DN) parameter for scaling polarity and are in accordance with the concept that the "two-parameter scale"^[26] is better than the "single-parameter" one, as demonstrated,^[17] for instance, when the Dimroth-Reichardt Et30 solvent term^[27] or Hildebrand's solubility parameter δ ^[28] were also compared. With this novel (AN+DN) acid-base polarity scale (or "amphoteric constant"), which ranges from zero to 129,^[17b] the slightly reduced swelling capacity of the mixed solvents 21 and 22 (Figure 2) can be interpreted in terms of the strength of association between their two components. The strong electron acceptor TFE tends to associate with a strong electron donor (DMF or DMSO) and not with the solute (peptide chains inside the bead), thus inducing less swelling. Such self-neutralizing effects of components are known, and characterize heterogeneous solvents.^[21] When the chain-chain interaction is strong, the reduction in the swelling capacity of solvents 21 and 22 is more pronounced, as already demonstrated for other peptide-resins containing aggregating sequences.^[17] In analogy to this effect, the reduced solvation capacity of DMF relative to 20% DMSO/NMP towards the aggregating resin-bound TM-12 sequence might therefore be credited to a much weaker chain-chain disruption capacity of the former polar aprotic solvent. Collectively, these findings reinforce the assumption that, under some circumstances, additional factors such as the characteristic inter- or intramolecular association forces of solute molecules must be taken into account when the appropriate concept of solvent polarity is to be applied. Efforts to clarify this issue are currently in progress.

Solvation and Degree of Chain Motion – The Spin Labeling EPR Approach

The pronounced difference in the swelling of TM-12-resin in DMF and in 20% DMSO/NMP can be analyzed in the light of the EPR data. A more strongly immobilized peptide chain population appears in DMF. Conversely, chain mobility is higher (narrower lines) and essentially constant throughout the TM-34 sequence elongation in 20% DMSO/NMP. The use of the W_o or the h_{-1}/h_0 parameters to assess the dynamics of labeled sites proved to be appropriate. For

the TOAC-labeled TM-12-resin, a more pronounced variation in peptide chain mobility was seen in DMF (Table 3). The W_0 term, which encompasses the contribution of the more and the less immobilized components, ranged from 2.4 to 3.6 G. In addition, the peak height ratio h_{-1}/h_0 varied from 0.07 to 0.43. This, and other spectral findings, provide evidence of the potential of spin labeling EPR for examination of the dynamic properties of neighboring labeled sites in the polymer network. Clearly, the potential of this approach extends to the optimization of, for instance, combinatorial chemistry methods, which depend on appropriate polymeric materials for chemical reactions.

Spin labeling EPR has been widely used, especially in the study of biological systems.^[29] Different spin labels have been employed,^[30] varying in the way in which they are introduced into the system under study: either by covalent binding, as in the case of proteins or nucleic acids, or by physical intercalation, as in the case of micelles and bilayers. With regard to the TOAC probe, a variety of applications have been described,^[31] including study of the structure-function relationships of partially^[14b,32] or fully^[33] biologically active peptides, of the conformation of model segments,^[34] and of membrane protein fragments,^[35] and also fluorescent peptides making use of the nitroxide's quenching effect.^[12d,32,36] More recently, a second type of paramagnetic amino acid probe containing an alternative pyrrolidine-type structure (POAC) was also reported in the literature.^[37]

Concerning SPPS, these results demonstrate the relevance of investigating the solvation behavior of peptide sequences by the combination of bead swelling studies with EPR spectral analysis. The study of the kinetics of coupling demonstrates the importance of peptide chain freedom for this polymer-supported technique. As expected, coupling was faster in 20% DMSO/NMP than in DMF. Moreover, emphasizing the direct relationship between these factors, the most prominent contaminant at the 16-residue stage was a peptide with deleted Leu¹³ and Ala¹⁶ residues (identified by mass spectrometry and amino acid analysis).

Although nearly four decades have elapsed since the inception of SPPS,^[1] the assembly of long, aggregating peptide sequences is still a challenge, with unknown factors precluding the complete control of this method. The inherent complexity of such heterogeneous products, typically represented by transmembrane segments, still leaves questions to be answered. The difficulties involved not only in the chemical synthesis itself, but also in the appropriate purification strategy have already been addressed.^[19,38] Various strategies to improve the critical coupling step have been proposed, involving different acylation components,^[39] solvent systems,^[16,23,24] addition of chaotropic agents,^[40] increase of the temperature,^[41] or the use of alternative protecting groups to avoid aggregation.^[42] For the relevant issue of purification of insoluble fragments, different sequence-dependent approaches have been already proposed.^[43]

In order to explain each degree of expansion of the network, it was recognized early in the polymer field that the swelling effect is subject to rules involving different thermodynamic properties.^[16a,44] The combined approach described

here, focusing on the stepwise monitoring of the growth of a long, hydrophobic transmembrane sequence, may represent a valid and useful strategy by which: i) to improve knowledge of the chemical processes occurring throughout the complex polymeric network, and ii) to facilitate the planning of alternative experimental conditions for the successful synthesis of difficult peptide sequences.

Conclusion

Solvent-dependent variation in the physicochemical characteristics of the polymer backbone was monitored during the chemical elongation of a resin-bound 34-mer transmembrane fragment (TM-34) of the B2 bradykinin receptor. The solvation behavior of peptide-resins containing minor TM-34 segments was gauged by measuring the swelling of beads and the degree of chain motion by means of EPR spectra obtained by labeling the peptides with the paramagnetic amino acid 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC). Pronounced chain aggregation in DMF at the 12-mer stage of the sequence was observed either by the prominent shrinking of beads or by the appearance of a significant amount of strongly immobilized chain population in this solvent. Accordingly, a slower coupling reaction and a lower yield of the synthesis was observed in DMF than in the mixed solvent 20% DMSO/NMP, which induced constant and optimized solvation throughout the TM-34 assembly in the resin. In addition, a more complete swelling investigation of two resin-bound segments of the TM-34 sequence (TM-12 and TM-24) allowed the potential of the simple sum of the solvent electron acceptor and electron donor properties, in 1:1 proportion, to be confirmed as an optional and sensitive polarity scale. In summary, the combined bead solvation-chain motion study developed here seemed to be sensitive and valuable not only for overcoming possible difficulties in some resin-supported reactions but also for enhancing knowledge of multiple factors that may govern the complex polymer network solvation phenomenon.

Experimental Section

General Remarks

Materials: Reagents and solvents for solid-phase peptide synthesis were of analytical grade and used from recently opened containers, without further purification. Boc-amino acids were purchased from Bachem (Torrance, CA), and the following side chain-protected amino acid were used: Asp (β -OcHex), Cys (MeBzl), Glu (γ -OcHex), Ser (Bzl), Thr (Bzl), Trp (For), and Tyr (2-Cl-Z).

Methods. Peptide Synthesis: TM-34 was synthesized manually by standard Boc-chemistry on a methylbenzhydrylamine-resin (0.34 mmol/g). The synthesis scale was 0.5 mmol/g, and the Boc protecting group was removed from the amine group with 30% trifluoroacetic acid in DCM (30 min), followed by washings with *i*PrOH containing 2% anisole and 10% DIEA in DCM for deprotonation of the peptide amine function. Coupling was performed with a 2.5-

fold excess of Boc-amino acid/TBTU/HOBt (1:1:1) in the presence of excess DIEA (5 equiv.) in DMF or 20% DMSO/NMP. Recouplings were performed under similar conditions when needed. All couplings were monitored by qualitative ninhydrin test and when positive, acetylation was performed with 50% acetic anhydride in DCM (15 min). Small aliquots of several shorter resin-bound TM-34 fragments were cleaved from the resin in HF/*o*-cresol/DMS/EDT (8.5:0.5:0.5:0.5, v/v). The reaction time was 90 min at 0 °C and the excess HF and scavengers were eliminated under vacuum. The peptide-resins were washed with ethyl acetate and dried, and the peptides were extracted with 5 to 50% acetic acid in water and lyophilized. Details of peptide synthesis yields, purification and analytical characterization by HPLC, amino acid analysis, and mass spectrometry were published previously.^[19] Crude peptides characterization: TM-8 ESI-MS: m/z = 905.92 [M + H⁺]. C₄₀H₆₃N₁₁O₁₃ (906.01): calcd. AAA: Asp 2.49 (3); Thr 1.08 (1); Ala 1.26 (1); Ile 2.20 (2); Phe 0.97 (1); TM-12 ESI-MS: m/z = 1407.16 [M + H⁺]. C₆₈H₉₄N₁₆O₁₇ (1407.59): calcd. AAA: Asp 3.25 (3); Thr 1.15 (1); Pro 0.96 (1); Ala 2.04 (2); Ile 1.85 (2); Phe 1.75 (2); Trp 0.92 (1); TM-16 ESI-MS: m/z = 1735.06 [M + H⁺]. C₈₂H₁₁₈N₂₀O₂₂ (1735.96): calcd. AAA: Asp 3.01 (3); Thr 0.97 (1); Ser 1.04 (1); Pro 0.93 (1); Gly 1.01 (1); Ala 3.03 (3); Ile 1.91 (2); Leu 1.09 (1); Phe 2.11 (2); Trp 1.03 (1); TM-20 ESI-MS: m/z = 2190.05 [M + H⁺]. C₁₀₄H₁₅₆N₂₄O₂₈ (2190.53): calcd. AAA: Asp 4.00 (4); Thr 1.04 (1); Ser 1.09 (1); Pro 0.97 (1); Gly 1.00 (1); Ala 3.11 (3); Ile 2.45 (3); Leu 3.18 (3); Phe 2.00 (2); Trp 0.92 (1); TM-24 ESI-MS: m/z = 2502.06 [M + H⁺]. C₁₁₈H₁₈₀N₂₈O₃₂ (2502.90): calcd. AAA: Asp 4.11 (4); Thr 1.08 (1); Ser 1.00 (1); Pro 0.96 (1); Gly 1.93 (2); Ala 4.77 (5); Ile 2.79 (3); Leu 4.00 (4); Phe 2.07 (2); Trp 0.98 (1); TM-28 ESI-MS: m/z = 2949.90 [M + H⁺]. C₁₃₉H₂₀₉N₃₃O₃₈ (2950.39): calcd. AAA: Asp 5.24 (5); Thr 1.13 (1); Ser 0.95 (1); Pro 0.99 (1); Gly 2.70 (3); Ala 4.80 (5); Ile 3.57 (3); Leu 4.59 (5); Tyr 0.95 (1); Phe 2.01 (2); Trp 0.99 (1); TM-34 ESI-MS: m/z = 3792.18 [M + H⁺]. C₁₇₃H₂₅₆N₄₀O₅₂S₂ (3792.32): calcd. AAA: Asp 5.30 (5); Thr 2.27 (2); Ser 1.36 (1); Glu 1.15 (1); Pro 0.95 (1); Gly 3.31 (3); Ala 6.04 (6); Val 0.88 (1); Ile 3.50 (4); Leu 4.72 (5); Tyr 0.75 (1); Phe 2.30 (2); Trp 0.84 (1).

Measurement of Peptide-Resin Swelling: Swelling studies of the narrowly sized bead populations were performed as published elsewhere^[16a,17,45] after the resins had been dried under vacuum using an Abderhalden-type apparatus. Briefly, 150 to 200 dry and swollen beads of each resin, allowed to solvate overnight, were spread over a microscope slide and measured directly with an Olympus model SZ11 microscope coupled with Image-Pro Plus 3.0.01.00 version software. The values of bead diameter distribution were estimated by geometric means and geometric standard deviations as published elsewhere.^[46]

EPR Studies: EPR measurements were carried out at 9.5 GHz in a Bruker ER 200 spectrometer at room temperature (22 ± 2 °C) in Wilmad flat quartz cells. Labeled peptidyl-resins were pre-swollen overnight in the solvent under study. The magnetic field was modulated with amplitudes less than one-fifth of the line widths, and the microwave power was 5 mW to avoid saturation effects. Details of the procedure for TOAC-labeling of resins have been reported.^[15]

Yield of the Coupling Reaction: The TM-12-resin (50–100 µmol) was elongated with the subsequent residue of the sequence (Boc-Leu-OH, 2.5 equiv.), in a reaction vessel thermostatted at 25 °C, by the BOP (2.5 equiv.)/DIEA (5 equiv.) coupling method in DMF or 20% DMSO/NMP (0.2 mm for Boc-Leu-OH and BOP, and 0.4 mm for DIEA) as solvent. The rate of rotation of the reaction flask was 20 rpm. The acylating reagents were dissolved in the solvent under investigation and added to the reaction vessel containing peptide-resin pre-swollen in the same solvent. The yield of coupling was

monitored by the picric acid method^[47] and each experiment was performed in duplicate.

Abbreviations: Abbreviations for amino acids and nomenclature of peptide structure follow the recommendations of the IUPAC-IUB (Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1971**, 247, 997). Other abbreviations are as follow: AAA: amino acid analysis; Boc = tert-butyloxycarbonyl; Bzl = benzyl; BOP = benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; 2-Cl-Z = chlorobenzyloxycarbonyl; CD = circular dichroism; DCM = dichloromethane; DIEA = diisopropylethylamine; DMF = *N,N'*-dimethylformamide; DMS = dimethyl sulfide; DMSO = dimethyl sulfoxide; EDT = ethanedithiol; EPR = electron paramagnetic resonance; EtOH = ethanol; HFIP = hexafluoro-2-propanol; HOBt = 1-hydroxybenzotriazole; Fmoc = 9-fluorenylmethyloxycarbonyl, HPLC = high-performance liquid chromatography; For = formyl; IR = infrared; MeBzl = methylbenzyl; MeOH = methanol; NMP = *N*-methylpiperidinone; NMR = nuclear magnetic resonance; β-OcHex = cyclohexyl; *i*PrOH = 2-propanol; SPPS solid-phase peptide synthesis; TBTU: 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEA = triethylamine; TFA = trifluoroacetic acid; TFE = trifluoroethanol; TOAC = 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid.

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Conformational Basis for the Biological Activity of TOAC-Labeled Angiotensin II and Bradykinin: Electron Paramagnetic Resonance, Circular Dichroism, and Fluorescence Studies

Abstract: *N*-Terminally and internally labeled analogues of the hormones angiotensin (AII, DRVYIHPF) and bradykinin (BK, RPPGFSPFR) were synthesized containing the paramagnetic amino acid 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC). TOAC replaced Asp¹ (TOAC¹-AII) and Val³ (TOAC³-AII) in AII and was inserted prior to Arg¹ (TOAC⁰-BK) and replacing Pro³ (TOAC³-BK) in BK. The peptide conformational properties were examined as a function of trifluoroethanol (TFE) content and pH. Electron paramagnetic resonance spectra were sensitive to both variables and showed that internally labeled analogues yielded rotational correlation times (τ_c) considerably larger than *N*-terminally labeled ones, evincing the greater freedom of motion of the *N*-terminus. In TFE, τ_c increased due to viscosity effects. Calculation of $\tau_{\text{C-peptide}}/\tau_{\text{TOAC}}$ ratios indicated that the peptides acquired more folded conformations. Circular dichroism spectra showed that, except for TOAC¹-AII in TFE, the *N*-terminally labeled analogues displayed a conformational behavior similar to that of the parent peptides. In contrast, under all conditions, the TOAC³ derivatives acquired more restricted conformations. Fluorescence spectra of AII and its derivatives were especially sensitive to the ionization of Tyr⁴. Fluorescence quenching by the

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nitroxide moiety was much more pronounced for TOAC³-AII. The conformational behavior of the TOAC derivatives bears excellent correlation with their biological activity, since, while the N-terminally labeled peptides were partially active, their internally labeled counterparts were inactive [Nakaie, C. R., et al., Peptides 2002, 23, 65–70]. The data demonstrate that insertion of TOAC in the middle of the peptide chain induces conformational restrictions that lead to loss of backbone flexibility, not allowing the peptides to acquire their receptor-bound conformation. © 2004 Wiley Periodicals, Inc. Biopolymers 74: 389–402, 2004

Keywords: TOAC; angiotensin II; bradykinin; EPR; circular dichroism

INTRODUCTION

Among the spectroscopic techniques available for the investigation of the conformation and dynamics of peptides, spin labeling electron paramagnetic resonance (EPR) has been used to a considerable extent. This approach enjoyed notable expansion following the introduction of the paramagnetic amino acid 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC),¹ which, for the first time, allowed the incorporation of the spin probe into the peptide *via* a peptide bond.^{2,3} At first, the use of the Boc methodology^{4,5} enabled labeling only at the N-terminus. Upon introduction of the Fmoc methodology,^{6,7} this restriction was abolished by the use of a Boc-Fmoc combined strategy, allowing for the insertion of TOAC at any desired position in the peptide chain.⁸

More recently, a new approach for the use of the Fmoc spin-labeled amino acid technology in peptide chemistry was introduced,⁹ namely the synthesis of the Fmoc derivative of the β -amino acid spin label 2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-amino-4-carboxylic acid.^{10,11} Stereochemical aspects of this pyrrolidine-based probe were also dealt with by Wright and co-workers.¹² The same group also described a chiral TOAC analogue where the amine and carboxyl functions were attached at positions 4 and 3 of the piperidine cyclic structure, respectively.¹³

The first analysis of the conformational properties of a TOAC-carrying peptide focused on the biologically vasoactive hormone angiotensin II (AII, DRVYIHPF). N-Terminally labeled analogues were found to be partly biologically active and their EPR spectra were sensitive to conformational transitions induced by changing physicochemical conditions.³ Following these early studies, TOAC was used in the investigation of many synthetic peptides.^{14–17} In particular, EPR spectra of doubly labeled peptides have been of great help in establishing the conformation of these compounds, allowing the distinction between α -helices and 3_{10} -helices, for instance.^{14–17} In these studies it was verified that TOAC itself favors the formation of helices and turns.¹⁸ TOAC has also been

employed to monitor the dynamics of the polymer network during peptide chain assembly.^{19–21}

With regard to biologically active peptides, work by the group of Toniolo has focused on trichogin GA IV, an antimicrobial lipopeptaibol that contains several aminoisobutyric acid (Aib) residues.^{22–25} Based on the consideration that both Aib and TOAC are disubstituted glycines, trichogin's Aib residues were replaced by TOAC and the conformational and functional properties of the analogues were investigated by X ray,²² EPR,^{23,24} and fluorescence.²⁵ It is noteworthy that the current hypotheses of the mechanism of action of several antimicrobial peptides propose that these molecules act at the membrane level. However, no protein receptor has been described for these compounds and they are believed to alter membrane structure and permeability by binding to the lipid bilayer.^{26–28} As a result, it is conceivable that the conformational requirements for these peptides are less restricted than those for receptor binding ligands.

Studies making use of TOAC-labeled peptides also focused on an N-terminally labeled fragment of a membrane-bound G-protein-coupled receptor (GPCR).^{29,30} Its EPR spectra were examined in solution and in the presence of model membranes. More recently, we investigated peptide hormones that bind to GPCR. In this context, N-terminally TOAC-labeled derivatives of the α -melanocyte stimulating hormone (α -MSH)³¹ and its potent long-acting analogue [Nle⁴, D-Phe⁷] α -MSH³² were synthesized and their biological and spectroscopic properties examined. These were found to be the first fully active TOAC-labeled peptide hormones. Recent work by Bettio et al. describes the synthesis, biological activity, and spectroscopic properties of several TOAC-containing derivatives of another GPCR binding peptide, neuropeptide Y.³³

TOAC derivatives of N-terminally and internally labeled AII and of another vasoactive hormone, bradykinin (BK, RPPGFSPFR), were synthesized. AII analogues carried TOAC before Asp¹ (TOAC⁰-AII), and replacing Asp¹ (TOAC¹-AII) or Val³ (TOAC³-AII), while BK analogues carried the spin-labeled amino acid before Arg¹ (TOAC⁰-BK) and replacing

Pro³ (TOAC³-BK).^{3,8,34–36} The biological properties of the native hormones were compared to those of the TOAC-labeled derivatives, including another analogue where TOAC replaced Pro⁷ in AII (TOAC⁷-AII). While the internally labeled analogues had no detectable biological activity, the N-terminally labeled hormones presented significant potency in different muscle preparations.³⁶ A preliminary account of the spectroscopic properties of these peptides was presented,³⁵ as well as studies of the interaction of BK and its TOAC derivatives with model membranes.³⁷

Here we report a detailed spectroscopic study of TOAC¹-AII, TOAC³-AII, TOAC⁰-BK, and TOAC³-BK. The EPR spectra of the peptides were examined in solution as a function of pH and of increasing content of the secondary structure-inducing agent, trifluoroethanol (TFE). Circular dichroism (CD) and static fluorescence spectra of the spin-labeled peptides were compared to those of the native hormones.

MATERIAL AND METHODS

Material

N^α-*tert*-butoxycarbonyl (Boc) or 9-fluorenylmethoxycarbonyl (Fmoc) amino acids were purchased from Bachem (Torrance, CA). Solvents and reagents were from Aldrich or Sigma Co. Dimethylformamide was distilled over P₂O₅ and ninhydrin under reduced pressure before use. All solvents were HPLC grade and all chemicals met ACS standards.

Methods

Peptide Synthesis. TOAC-containing peptides were synthesized manually according to the standard Boc/Bzl and Fmoc/*t*-butyl solid phase strategies, following the previously reported approach for TOAC incorporation.^{8,36} After the cleavage reaction with anhydrous HF, the crude spin-labeled peptides were submitted to alkaline treatment (pH 10, 1 h, 50°C) for complete reversion (monitored by analytical HPLC) of the N-O protonation that occurs during the HF reaction. The peptides were purified by preparative HPLC (C₁₈-column) using aqueous 0.02 M ammonium acetate (pH 5) and 60% acetonitrile solutions as solvents A and B, respectively (linear gradient of 30–70% B for 2 h, flow rate of 10 mL/min). The peptide homogeneity was checked by analytical HPLC, amino acid analysis, and LC/MS mass spectrometry (electrospray). The following peptides were synthesized: AII and AII analogues where TOAC replaced Asp¹ (TOAC¹-AII) or Val³ (TOAC³-AII); BK and BK analogues where TOAC was inserted before Arg¹ (TOAC⁰-BK) or replacing Pro³ (TOAC³-BK).

EPR Studies. EPR spectra were obtained at 9.5 GHz in a Bruker ER 200 spectrometer at room temperature (22

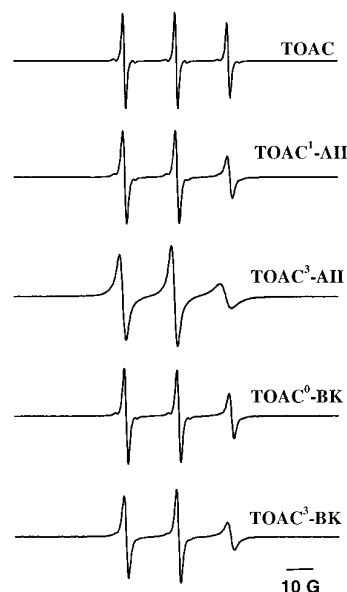


FIGURE 1 EPR spectra of (from top to bottom) 0.1 mM TOAC, TOAC¹-AII, TOAC³-AII, TOAC⁰-BK, and TOAC³-BK in 100% TFE.

± 2°C) using flat quartz cells from Wilmad Glass Co. (Buena, NJ). The magnetic field was modulated with amplitudes less than one-fifth of the line widths, and the microwave power was 5 mW to avoid saturation effects. Rotational correlation times, τ_B and τ_C , were calculated from spectral line heights and line widths making use of the equations given by Kivelson.³⁸

CD Studies. Circular dichroism spectra of samples containing 0.1 mM peptides were obtained in cuvettes of 0.2 mm path length at room temperature (22 ± 2 °C) using a Jobin Yvon CD6 spectropolarimeter. A thermostated water bath was used to control the cell temperature. The instrument was routinely calibrated with an aqueous solution of recrystallized *d*-10-camphorsulfonic acid. Data are expressed as mean residue ellipticity, $[\theta]$ (measured in deg cm² dmol⁻¹).

Fluorescence Studies. Static fluorescence spectra were obtained at room temperature (22 ± 2°C) in a Hitachi F4500 spectrofluorimeter, using cuvettes with excitation path length of 2 or 5 mm and emission path length of 10 mm. Excitation and emission slits were 5 nm. The peptide concentration varied from 1 to 2.5 × 10⁻⁵ M.

RESULTS

EPR Studies

EPR spectra of TOAC¹-AII, TOAC³-AII, TOAC⁰-BK, and TOAC³-BK were obtained in solution as a

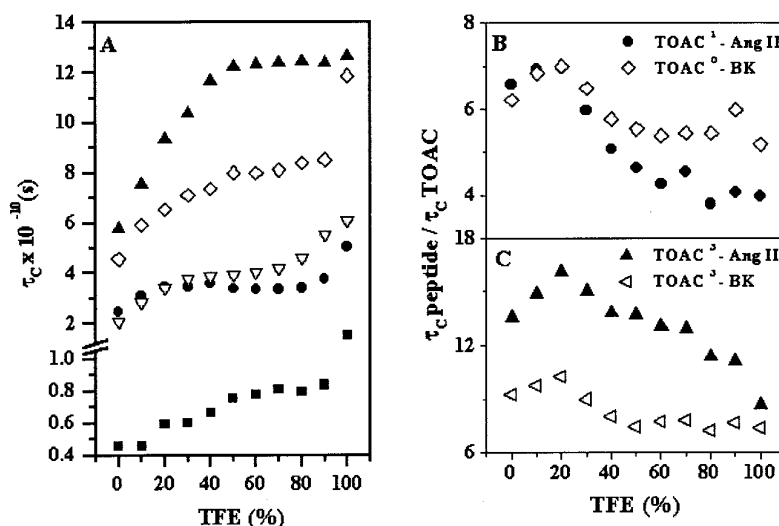
Table I Isotropic Hyperfine Splittings (a_N , Gauss) in the EPR Spectra of AII and BK TOAC-Labeled Analogues in Aqueous Solution, pH 7.0, and in 100% TFE

Analogue	a_N (Gauss)	
	In water	In TFE
TOAC ¹ -AII	16.51	16.17
TOAC ³ -AII	16.19	15.78
TOAC ⁰ -BK	16.59	15.80
TOAC ³ -BK	16.28	15.93

function of pH and TFE content. Figure 1 shows the spectra of TOAC and of the four peptides in pure TFE. It is seen that, while TOAC gives rise to a spectrum with narrow lines, typical of small molecules tumbling fast in solution, the spectra of the peptides display broader lines, evincing that the EPR time scale is able to distinguish between the amino acid (MW 215) and the peptides (MW ~1000). The spectra clearly show that internally labeled peptides yield spectra with broader lines than their N-terminally labeled counterparts, probably due to the greater freedom of motion of amino acids at the peptide N-termini. Table I shows that, as expected, the isotropic hyperfine splitting constant, a_N , decreases when the peptides go from water to the less polar solvent TFE.

The difference in the rate of motion can be quantitatively estimated by calculating rotational correlation times³⁸ for motionally narrowed spectra. According to theory, two equations can be used to calculate the rotational correlation time (τ) for fast tumbling, approximately spherical molecules, in isotropic medium. These equations yield two values, τ_B and τ_C , which should be very similar. It has been proposed that considerable differences between τ_B and τ_C could be taken as an indication of deviation from isotropic motion.³⁹ Figure 2A shows that τ_C increases with increasing TFE content and that τ_C values for TOAC are one to two orders of magnitude lower than for the peptides. In addition, while the rotational correlation times for the N-terminally labeled compounds vary from $ca. 2 \times 10^{-10}$ s to $ca. 6 \times 10^{-10}$ s, for internally labeled peptides these values range from $ca. 4.5 \times 10^{-10}$ s to $ca. 13 \times 10^{-10}$ s. Figure 2 also shows that, whereas the τ_C values are very similar for TOAC¹-AII and TOAC⁰-BK, they are considerably higher for TOAC³-AII than for TOAC³-BK, ranging from $ca. 6 \times 10^{-10}$ s to $ca. 13 \times 10^{-10}$ s for the former and from $ca. 4.5 \times 10^{-10}$ s to $ca. 8 \times 10^{-10}$ s for the latter in 90% TFE. For TOAC³-BK this value increases to 12×10^{-10} s in 100% TFE.

It is also worthwhile noticing that, while data in the literature show that for most peptides the TFE effect saturates between 30 and 50% TFE, in the present study this only happens for TOAC³-AII (Figure 2A). For all other compounds, although a tendency to sat-

**FIGURE 2** τ_C as a function of TFE content for TOAC (■), TOAC¹-AII (●), TOAC³-AII (▲), TOAC⁰-BK (◇), and TOAC³-BK (◊) (A). $\tau_{C \text{ peptide}} / \tau_{C \text{ TOAC}}$ as a function of TFE content for TOAC¹-AII and TOAC⁰-BK (B) and TOAC³-AII and TOAC³-BK (C). The symbols are the same as in A.

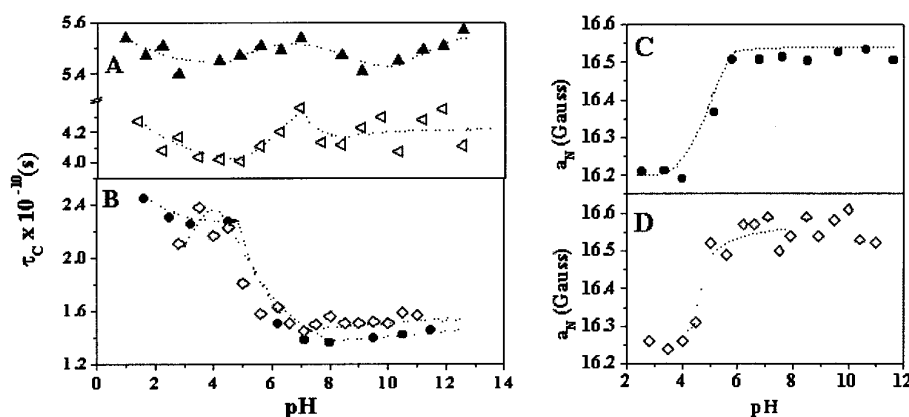


FIGURE 3 τ_C as a function of pH for TOAC³-AII ($\blacktriangle$) and TOAC³-BK ($\triangleleft$) (A) and for TOAC¹-AII ($\bullet$) and TOAC⁰-BK ($\diamond$) (B). a_N as a function of pH for TOAC¹-AII ($\bullet$) (C) and TOAC⁰-BK ($\diamond$) (D).

uration is seen between 20 and 30% TFE, a considerable increase in τ_C values occurs above 80 or 90% TFE. The fact that this behavior is also found for TOAC suggests that it could be due to an increase in medium viscosity. We have attempted to take viscosity effects into account by dividing the peptide τ_C values by those found for TOAC. Plots of $\tau_{C\text{peptide}}/\tau_{C\text{TOAC}}$ ratios as a function of TFE (Figures 2B and C) show that this ratio decreases for all peptides from 20% TFE on. Moreover, except for TOAC³-AII, this ratio reaches saturating values. For the latter peptide, a tendency to saturation seems to occur between 40 and 70% TFE. However, a steep decrease is observed for higher solvent content. The curves in Figures 2B and C are suggestive of an increase in the $\tau_{C\text{peptide}}/\tau_{C\text{TOAC}}$ ratio between 0 and 20% TFE. Figure 2 clearly shows that, in addition to being sensitive to the medium viscosity, the EPR spectra are capable of reporting on the conformational changes undergone by the spin-labeled peptides in the presence of the secondary structure-inducing solvent. The effective decrease in τ_C inferred from the data suggests that a more folded conformation is achieved by the peptides. This notion is corroborated by CD spectra (see below).

Calculated τ_B values for the spin-labeled hormones (not shown) revealed that, while τ_B and τ_C differed by no more than 4% for the N-terminally labeled derivatives, τ_C was $\sim 15\%$ higher for TOAC³-AII and 23–37% higher for TOAC³-BK in the whole range of TFE concentrations. These results are interpreted as indicating that when TOAC is at the N-terminus, due to its freedom of motion relative to the whole molecule, the difference between τ_B and τ_C does not reflect the molecule's anisotropy of motion. However, when

TOAC replaces residue 3, it reports more faithfully on the peptide's anisotropic motion.

EPR spectra of the TOAC-carrying peptides were obtained as a function of pH. The ionizable groups in each of the peptides are as follows: AII, terminal amino group, Asp¹ carboxyl group, Arg² guanidinium, Tyr⁴ phenol, His⁶ imidazole, and terminal carboxyl group; TOAC¹-AII, the terminal amino group is that of TOAC and the carboxyl group of Asp¹ is missing, the other groups are the same as in AII; TOAC³-AII, the ionizable groups are the same as in AII; BK, terminal amino group, Arg¹ and Arg⁹ guanidinium groups, and terminal carboxyl group; TOAC⁰-BK, the ionizable groups are the same as in BK, except that the terminal amino group is that of TOAC; TOAC³-BK, the ionizable groups are the same as in BK.

As previously observed,^{2,3,30} the spectra of TOAC at the N-termini of peptides are very sensitive to pH titration. Figure 3 presents the variation of τ_C and a_N as a function of pH. We have shown that curves such as those in Figure 3B reflect the titration of TOAC's amino group ($pK \sim 4.5$) and we have proposed that the different τ values below and above this pK correspond to different conformational states. Interestingly, when peptides carry TOAC at position 3, the changes in τ_C are small (Figure 3A). Since CD data do indicate pH effects on the peptides conformation, and since the peptides display TOAC-induced turns in all media used in this study (see below), we suggest that the pH-induced conformational changes do not significantly affect the peptides rotational correlation times.

Calculated τ_B values were on average 14 (TOAC¹-AII and TOAC³-BK) and 12% (TOAC³-AII) smaller than those of τ_C in the whole pH range, again evincing

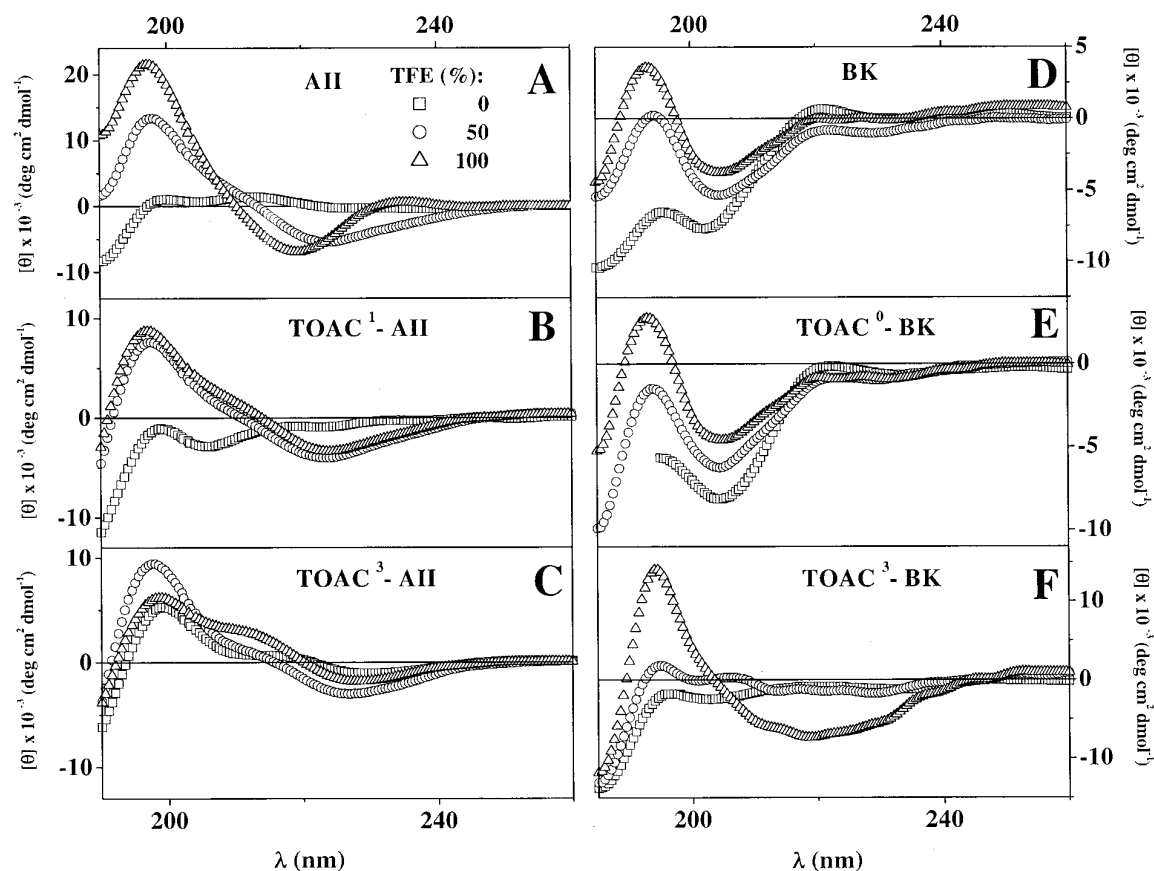


FIGURE 4 CD spectra of AII (A), TOAC¹-AII (B), TOAC³-AII (C), BK (D), TOAC⁰-BK (E), and TOAC³-BK (F) in the absence (□) and presence of 50 (○) and 100% (△) TFE. The aqueous solution was at pH 4.0.

the anisotropy of motion of the peptides. In the case of TOAC⁰-BK, there was essentially no difference between τ_B and τ_C .

The behavior of a_N as a function of pH (Figure 3C and D) resembles very closely that found for N-terminally TOAC-labeled peptides.^{2,3,30} The curves result from the slow proton exchange between the charged and uncharged forms of TOAC's amino group. The values of a_N (and g-factor) differ for both forms.^{2,3} Thus, at low pH the spectrum observed is that of the charged form, while the opposite is true at high pH. In the intermediate region, the observed spectra are weighted sums of the extreme spectra, according to the Henderson-Hasselbalch equation.^{2,3} Figures 3C and D show that the pK of TOAC's amino group is 4.5 in TOAC¹-AII and 4.9 in TOAC⁰-BK.

CD Studies

CD spectra of the spin-labeled peptides were obtained under the same conditions as those employed for EPR

and were compared to those of the native hormones. Figures 4 and 5 present the peptides spectra at variable TFE content and variable pH, respectively. The spectra of unlabeled AII (Figure 4A) and BK (Figure 4D) at pH 4.0 resemble those already reported in the literature (refs. [40–43] for AII and [44–49] for BK). Due to their flexible structure, the peptides exist as an equilibrium between different conformations. The spectra were shown to result from the contribution of the individual amino acids and to reflect transitions due to absorption by aromatic side chains.

TFE induced an increase in secondary structure of all peptides (Figure 4). The spectra of the unlabeled hormones in 100% TFE also resembled those previously reported in the literature (Figure 4A, refs. [40–42] for AII, and Figure 4D, refs. [44–49] for BK). In the presence of 50 and 100% TFE, the spectra of the native peptides and their labeled counterparts correspond to more ordered conformations, stabilized by solvent-induced intramolecular hydrogen bonding.

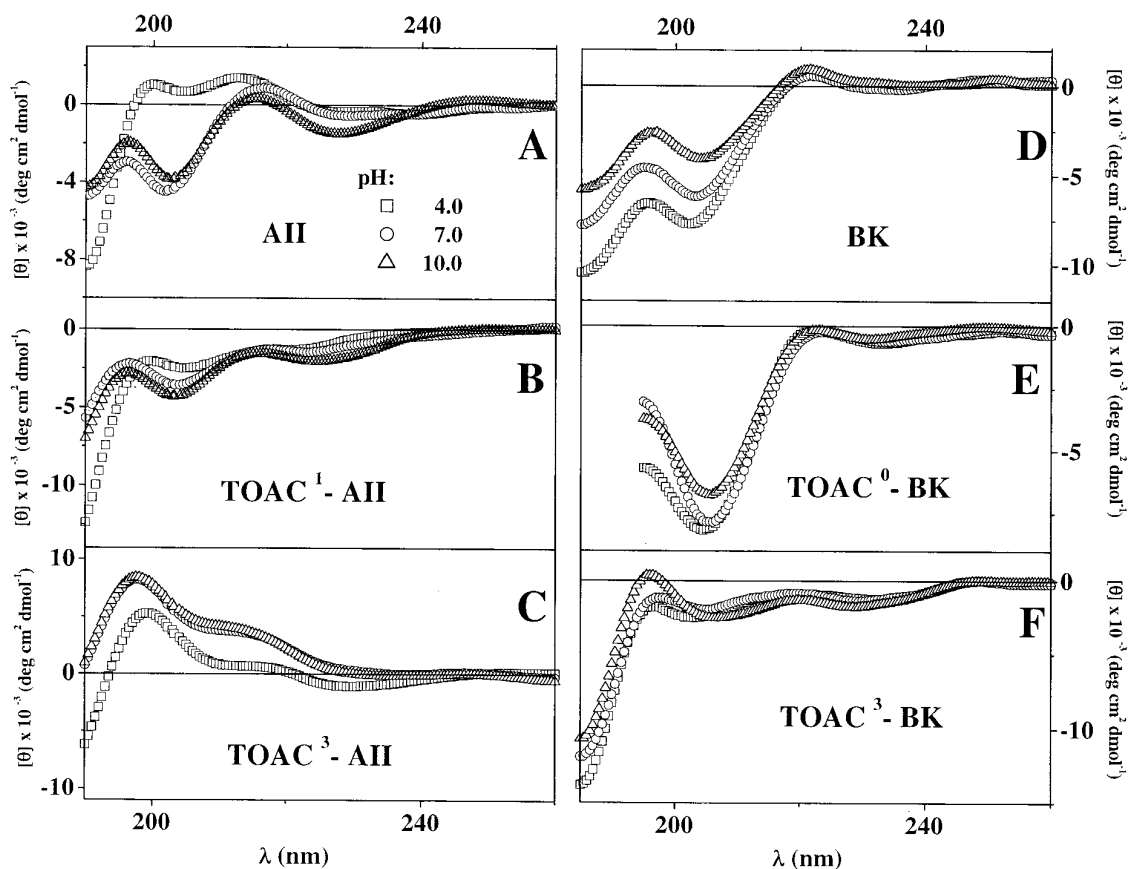


FIGURE 5 CD spectra of AII (A), TOAC¹-AII (B), TOAC³-AII (C), BK (D), TOAC⁰-BK (E), and TOAC³-BK (F) at pH 4.0 (□), 7.0 (○), and 10.0 (△).

The spectra of AII and its spin-labeled analogues (Figures 4A–C) are suggestive of turn formation, both in 50 and in 100% TFE. In contrast with all other conditions, the CD spectra of TOAC¹-AII (Figure 4B) did not resemble those of the parent compound (Figure 4A). Instead, the peptide yielded spectra similar to those of TOAC³-AII (Figure 4C).

TFE also induced an increase of secondary structure in BK and its spin-labeled analogues (Figures 4D–F). In this case, while N-terminally labeled BK (Figure 4E) yielded spectra very similar to those of the native hormone (Figure 4D), the internally labeled analogue gave rise to significantly different spectra (Figure 4F), indicating that the turns formed by the latter differ from those of BK and TOAC⁰-BK. Table II contains the wavelengths at which maximum and minimum ellipticities were measured, as well as the ellipticity values. It is seen that the internally labeled peptides were more ordered under all conditions. Interestingly, the folds induced by TOAC³ in both peptides are quite different.

Figures 6A and B show that the mean residue ellipticity at 197 nm (θ_{197}) increases with increasing TFE. For TOAC¹-AII and TOAC³-AII the ellipticity increases up to 30% TFE, reaching saturation above this concentration. In contrast, for native AII, θ_{197} displays a tendency to saturation between 30 and 80% TFE, increasing steeply thereafter (Figure 6A). With regard to BK, the θ_{197} values for the unlabeled hormone and its TOAC⁰ derivative were quite similar and, although there was no clear saturating effect, as usually observed for peptides, a tendency to saturation is seen (Figure 6B). The θ_{197} values were considerably higher for TOAC³-BK; moreover, θ_{197} increased slowly up to 60% TFE and then steeply up to 90% TFE (Figure 6B).

The effect of pH on the peptides CD spectra is shown in Figure 5. The spectra of the native hormones (Figure 5A, AII, and Figure 5D, BK) as well as those of the N-terminally labeled analogues (TOAC¹-AII, Figure 5B, and TOAC⁰-BK, Figure 5E) are suggestive of flexible conformations. Although the spectra of

Table II Wavelengths (nm) at which Maxima ($\lambda_{\max}$) and Minima ($\lambda_{\min}$) Appear in the CD Spectra of AII, BK, and Their TOAC-Labeled Analogues and Respective θ (10^3 , deg cm² dmol⁻¹) Values as a Function of TFE Content

%TFE	$\lambda_{\max}$	θ	$\lambda_{\min}$	θ	$\lambda_{\max}$	θ	$\lambda_{\min}$	θ	$\lambda_{\max}$	θ	$\lambda_{\min}$	θ
	AII				TOAC ¹ -AII				TOAC ³ -AII			
50	198	13.4	224	-5.49	197	7.59	223	-4.03	198	9.43	226	-3.07
100	197	21.5	219	-6.91	197	8.68	224	-3.22	199	6.18	228	-1.83
									211	2.99		
	BK				TOAC ⁰ -BK				TOAC ³ -BK			
50	195	0.15	205	-5.33	—	—	205	-6.26	195	1.73	214	-1.74
100	194	3.49	205	-3.76	193	2.66	205	-4.71	195	13.9	218	-7.53

N-terminally labeled peptides resembled more closely those of the unlabeled compounds, in no case they were exactly the same. The spectra of internally labeled peptides (TOAC³-AII, Figure 5C, and TOAC³-BK, Figure 5F) differ substantially from those of their unlabeled and N-terminally labeled counterparts. In the whole pH range both TOAC³ derivatives gave rise to spectra indicative of more ordered conformations. Table III presents the wavelengths at which maximum and minimum ellipticities were measured, as well as the ellipticity values.

The effect of pH upon θ_{197} is presented in Figure 6C for AII and its derivatives and in Figure 6D for BK and its derivatives. The curve for AII is similar to that obtained by Lintner et al.⁴² in plots of θ_{205} vs. pH. The different behavior of θ_{197} vs pH for TOAC¹-AII is probably due to the different pKs of the terminal amino groups (7.6 for AII⁵⁰ and 4.5 for TOAC¹-AII, ref. [3] and this work) and to the lack of Asp¹ in the latter peptide (see Discussion). Despite the fact that AII and TOAC³-AII carry the same ionizable groups, the curves differ substan-

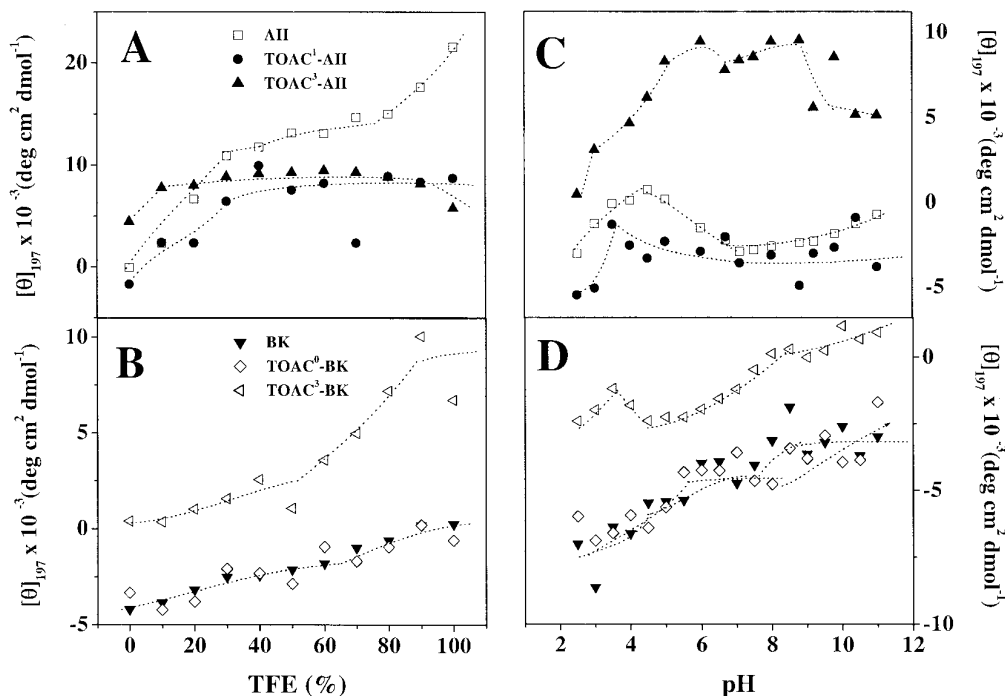
**FIGURE 6** Variation of θ_{197} as a function of TFE content (A, B) and pH (C, D) for AII ($\square$), TOAC¹-AII ($\bullet$), TOAC³-AII ($\blacktriangle$), BK ($\blacktriangledown$), TOAC⁰-BK ($\diamond$), and TOAC³-BK ($\triangleleft$).

Table III Wavelengths (nm) at which Maxima ($\lambda_{\max}$) and Minima ($\lambda_{\min}$) Appear in the CD Spectra of AII, BK, and Their TOAC-Labeled Analogues and Respective θ (10^3 , deg cm² dmol⁻¹) Values as a Function of pH

pH	$\lambda_{\max}$	θ	$\lambda_{\min}$	θ	$\lambda_{\max}$	θ	$\lambda_{\min}$	θ	$\lambda_{\max}$	θ	$\lambda_{\min}$	θ
	AII				TOAC ¹ -AII				TOAC ³ -AII			
4.0	200	0.99	228	-0.31	—	—	207	-2.34	199	5.23	206	-2.56
	213	1.39					222	-1.23				
7.0	—	—	203	-4.38	—	—	203	-3.08	198	8.27	—	—
			229	-0.53			223	-1.47				
10.0	—	—	203	-3.84	—	—	203	-3.51	198	8.47	—	—
			228	-1.45			225	-1.98				
	BK				TOAC ⁰ -BK				TOAC ³ -BK			
4.0	—	—	202	-7.65	—	—	205	-8.17	—	—	203	-2.65
7.0	—	—	203	-6.43	—	—	206	-7.85	—	—	205	-1.81
10.0	—	—	204	-5.15	—	—	206	-6.74	195	0.34	205	-2.53
											230	-1.75

tially (Figure 6C), in view of the conformational differences between both peptides.

The θ_{197} vs. pH plot for BK (Figure 6D) reflects the titration of the terminal amino and carboxyl groups and resembles that obtained by Lintner et al.⁴⁷ This plot is quite similar to that for TOAC⁰-BK (Figure 6D). The difference in the pH 8–9 range could be due to the different pKs of the terminal amino groups. As pointed out above, the pK of TOAC's amino group decreases to *ca.* 4.5–5.0 when this residue is at the N-terminus of peptides.^{2,3,30} Again, in the case of BK, although the native hormone and TOAC³-BK have the same ionizable groups, drastic differences occur in their pH behavior (Figure 6D), evincing that their conformational properties differ considerably.

Fluorescence Studies

Fluorescence spectra were obtained for AII and its TOAC derivatives as a function of TFE and pH. It is well known that the paramagnetic nitroxide moiety is a fluorescence quenching agent. Intramolecular quenching of Tyr⁴ fluorescence by TOAC was more efficient when it was next to the fluorophore than at the peptide's N-terminus. Although the fluorescence of the peptides was not very strongly influenced by TFE content, it was very sensitive to the presence of TOAC. While the fluorescence intensity (in arbitrary units) was of the order of 650 for AII, it dropped to *ca.* 150 for TOAC¹-AII and to *ca.* 50 for TOAC³-AII.

The peptide formal charge was calculated as a function of pH making use of the pKs of the ionizable groups determined by Juliano and Paiva.⁵⁰ For TOAC¹-AII the pK of the terminal amino group was

4.5. The effect of pH on formal charge and on the peptide fluorescence intensity is seen in Figures 7A (AII), B (TOAC¹-AII), and C (TOAC³-AII). Some features can be noticed: (i) there is a big difference between the curves corresponding to the formal charge and those corresponding to the fluorescence intensity in the pH region between 6 and 9; (ii) the spectra are more drastically affected by the ionization of the fluorophore, Tyr⁴; and (iii) the pK of this residue does not seem to differ much from that of the individual amino acid in all three peptides.

DISCUSSION

The solution structure of short biologically active peptides has been the subject of extensive investigation. The introduction of the unnatural amino acid TOAC in the sequence of the peptide hormones angiotensin II and bradykinin yielded molecules whose EPR, CD, and fluorescence spectra were sensitive to medium conditions.

Both N-terminally and internally TOAC-labeled AII and BK exhibited EPR spectra that changed as a function of TFE content and pH. Moreover, the spectra were also sensitive to the labeling position: N-terminally labeled AII and BK exhibited EPR spectra that differed considerably from those of their internally labeled counterparts (Figure 1). Calculated rotational correlation times yielded increasing τ_c values with increasing TFE (Figure 2A). τ_c was three- to fourfold greater for TOAC³-AII than for TOAC¹-AII and two- to threefold higher for TOAC³-BK than for TOAC⁰-BK, both in aqueous solution and in the pres-

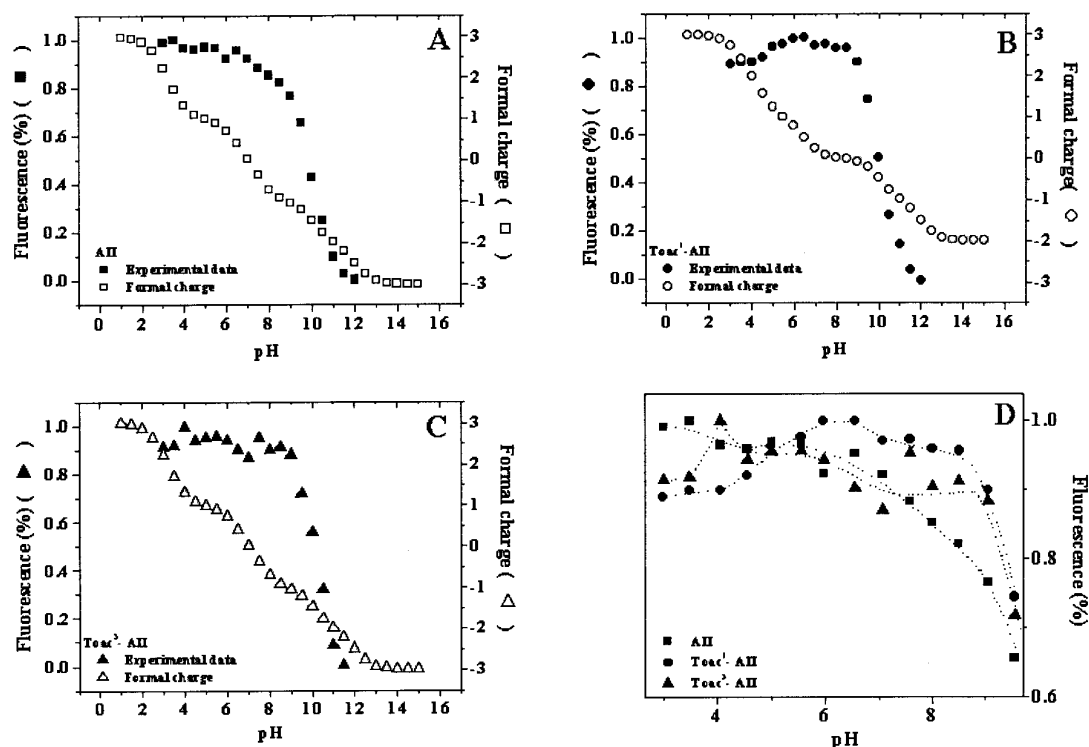


FIGURE 7 Normalized fluorescence intensity (full symbols) and formal charge (empty symbols) of AII (A), TOAC¹-AII (B), and TOAC³-AII (C) as a function of pH. In D the normalized fluorescence intensity is given between pH 3.0 and 9.5. The symbols are the same as in A–C.

ence of TFE. Thus, the peptide EPR spectra were capable of distinguishing between N-terminally and internally TOAC-labeled analogs, revealing the greater flexibility of the N-terminus (Figure 1). The decrease in the $\tau_{\text{Cpeptide}}/\tau_{\text{CTOAC}}$ ratio with increasing TFE (Figures 2B and C) suggested that the peptides acquired a more folded structure in the presence of the solvent. The spectral changes reported by EPR with increasing TFE correlated well with the acquisition of more ordered conformations in this solvent, as seen in the CD spectra (Figure 4).

Calculated rotational correlation times showed that the EPR spectra of the spin-labeled peptides were also sensitive to pH (Figure 3). As previously found,^{2,3,30} the spectra were strongly influenced by TOAC's ionization at the N-terminus (Figure 3B). The lesser pH sensitivity of the EPR spectra of the internally labeled peptides (Figure 3A) is ascribed to a smaller change in rotational correlation times with changing pH since these analogues are folded under all conditions examined (Figures 5C and F).

The EPR results correlated well with conformational information obtained by CD for the TOAC-labeled hormones and by fluorescence for the AII

analogues. In these latter studies the conformational properties of the analogues were compared to those of the parent hormones.

Angiotensin II and Analogues

A variety of spectroscopic techniques, as well as theoretical calculations, led to the proposal of different conformations for AII in aqueous solution.^{40–43, 50–63} As a result of these studies, it is widely accepted that AII has a flexible structure resulting from the equilibrium between several interconverting conformations. Recent NMR work at low temperature⁶³ indicated that AII in aqueous solution favors a hairpin conformation where the N- and C-termini are at a distance of 7.2 Å. The secondary structure-inducing solvent TFE has been found to increase the propensity of AII to acquire more stable conformations involving intramolecular hydrogen bond formation.^{40–42,54}

The present CD spectra showed that TFE promoted more folded conformations of AII and its spin-labeled analogues (Figure 4, Table II). As already suggested for the native hormone,⁵⁴ TFE seems to stabilize more than one folded conformation of the analogues. Inter-

estingly, the spectra in TFE closely resembled those obtained upon peptide binding to SDS micelles, showing that the two environments induce intramolecular hydrogen bonding, favoring the acquisition of similar folds. However, neither TOAC¹-AII nor TOAC³-AII gave rise to CD spectra similar to those of native AII. This was the only condition where the behavior of the N-terminally labeled analogue differed considerably from that of the parent compound. The reason for the difference may reside in the absence of Asp¹ in the labeled peptide, which would preclude conformations stabilized by proposed intramolecular interactions involving both the terminal amino group⁶² and the side chain carboxyl group of that residue.^{60,62,63} It is worth noting that the AII analogue in which Asp¹ was replaced by the disubstituted glycine dimethylglycine presented a CD spectrum in TFE distinct from that of the native hormone.⁴² However, the CD spectrum of TOAC¹-AII in TFE does not resemble that of dimethyl-Gly¹-AII either. Among the possible factors responsible for the different spectra one could invoke the greater constraint of the TOAC-bound amino group, as well as the large difference between the pK of the terminal amino group in the native (pK 7.6)⁵⁰ and labeled (pK 4.5) peptides, altering their ability to participate in hydrogen bonding and electrostatic interactions.

With regard to the fluorescence spectra, these were not strongly sensitive to TFE-induced conformational changes. As expected, TOAC was much more effective in quenching Tyr⁴ fluorescence when it replaced Val³ than Asp¹, due to proximity effects.

The replacement of Val³ by TOAC imparted considerable restriction on the peptide's flexibility. Under all conditions (variation of TFE content, Figure 4C, and of pH, Figure 5C), the CD spectra revealed the existence of several folded conformations in equilibrium. As will be discussed below, the presence of TOAC, a disubstituted glycine, imposes a bend, rendering the molecule less flexible. Previous studies indicated that BK analogues in which the disubstituted glycine Aib replaced for the native hormone's Pro residues favored a *cis* configuration of the peptide bond with the preceding residues.^{64–66} Interestingly, the CD spectra of Pro³-AII⁴² differ considerably from those obtained for TOAC³-AII.

In the pH titration, TOAC¹-AII's CD spectra were more similar to those of AII (Figures 5A and B). Although AII's Tyr⁴ has been implied in several intramolecular interactions, including the formation of a charge relay system with His⁶ and the Phe⁸ carboxyl group,⁶¹ the fluorescence pH titration studies did not provide any evidence for a significant pK change of the Tyr⁴ phenolic group. It is noteworthy that the

titration of ionizable groups other than Tyr⁴ had little effect on the peptide's fluorescence. Nevertheless, in the expanded pH 3.5 to 9.0 range (Figure 7D) AII, TOAC¹-AII, and TOAC³-AII responded differently to changes in pH. The fluorescence spectra were sensitive to the titration of TOAC's amino group in TOAC¹-AII and of His⁶ in TOAC³-AII. These differences are probably of conformational origin.

Bradykinin and Analogues

Similar to AII, the conformation of BK in aqueous solution is considered to consist of an equilibrium between interconverting states, resulting from the peptide flexibility.^{67–70} ¹³C and ¹H NMR studies indicated that the *trans* configuration about the X-Pro peptide bond is strongly favored.⁶⁷ A similar behavior was found for the peptide in dimethyl sulfoxide containing 1% water.⁷⁰ The effect of TFE on BK's CD spectra was previously reported.^{44–49} Previous CD and NMR studies of BK in 95% TFE led to the proposal that in this solvent the peptide exists in two main conformations, one extended and one folded,^{48,49} originating from solvent-induced intramolecular hydrogen bonding.

The CD spectra of BK and its TOAC-labeled derivatives in TFE showed that the introduction of TOAC before Arg¹ changed the peptide's conformation to a small extent (Figures 4D and E, Table II). The above-mentioned CD and NMR studies proposed that in 95% TFE a turn comprising the N-terminal portion is stabilized in BK's folded conformation with the Pro²-Pro³ peptide bond in the *cis* configuration.^{48,49} However, in studies of Aib-substituted BK analogs, Cann and co-workers pointed at the ability of Aib to mimic *cis*-Pro.^{64–66} An examination of the spectrum of TOAC³-BK in TFE (Figure 4F) reveals that it strikingly resembles that of Aib³-BK in that solvent.⁶⁵ This suggests that both Aib and TOAC mimic *cis*-Pro and that Aib³-BK and TOAC³-BK CD spectra reflect the bend imposed by this configuration. In contrast, the spectra show that the TFE-stabilized turns in BK and TOAC⁰-BK are different from that in TOAC³-BK (Figure 4, Table II). Moreover, the CD spectra of BK and its spin-labeled derivatives in TFE closely resemble those obtained upon binding the peptides to sodium dodecyl sulfate (SDS) micelles.³⁷ NMR studies of SDS-bound native BK indicate that a β -turn is formed in the peptide C-terminus, but that the N-terminal portion retains a certain degree of flexibility.^{71,72} Thus, we suggest that the spectra of BK and of TOAC⁰-BK in TFE do not correspond to folds containing a major contribution of conformers with Pro³ in the *cis* configuration.

As in the TFE study, the conformational behavior of TOAC⁰-BK (Figure 5E) as a function of pH closely resembled that of the parent peptide (Figure 5D). In contrast, that of TOAC³-BK differed from the former and the CD spectra (Figure 5F, Table III) seem to correspond to an equilibrium of folded conformations. It is worth noting that the spectra of TOAC³-BK in aqueous solution also bear a striking resemblance to those of Aib³-BK in this solvent.⁶⁵

Structure-Function Correlation

A vast literature exists aiming at understanding the agonistic and antagonistic and lack of activity of biologically active peptides and their analogs on the basis of their conformational and dynamic properties. In this regard, a large amount of work has been produced regarding both angiotensin II and bradykinin.

The flexibility of short peptide hormones has been considered an important feature that allows them to acquire their receptor-bound conformation. In view of the difficulties in studying the conformational aspects of receptor-ligand complexes, in particular in the case of G-protein-coupled receptors, the conformational properties of ligands were examined in organic solvents and in the presence of model membranes. Under these conditions, the peptides were found to acquire preferred conformations stabilized by intramolecular interactions favored by the nonaqueous media. It has often been speculated that one of these conformations could correspond to that of the receptor-bound state. It should also be considered, however, that receptor-ligand-specific interactions could play a role in the final receptor-bound conformation.

The flexibility requirement in order to achieve the receptor-bound conformation is clearly demonstrated by the numerous studies showing that analogues designed to display restricted conformations are frequently inactive. The present study shows that the incorporation of TOAC, a disubstituted glycine, like Aib, at position 3 of AII and BK imparts conformational restrictions that are absent in the parent compounds, possibly by giving rise to a *cis* peptide bond between residues 2 and 3. These more restricted conformations were found both in aqueous media and in mixtures containing variable proportions of TFE. In the presence of the secondary structure-inducing solvent all peptides acquired more ordered conformations. However, in no case were the folded structures acquired by the internally labeled analogues similar to those of the native hormones.

In agreement with the conformational studies, a recent report showed that while the AII and BK ana-

logs labeled at the N-terminus were still able to bind to their respective receptors and trigger a biological response, TOAC³-AII and TOAC³-BK were inactive in biological assays.³⁶ Interestingly, the Aib³ analogues of both AII^{73,74} and BK⁶⁴ were essentially inactive. In addition, another AII derivative containing a cyclic amino acid at position 3, 1-aminocyclopentane carboxylic acid, also displayed essentially no activity.⁷⁵ In contrast, an analog of AII containing Pro at position 3 still maintained some biological activity.⁷⁶ This could imply that in the receptor-bound state the proline residue of this analog is in the *trans* configuration. A similar reasoning could apply to native BK.

In conclusion, the results presented in this work corroborate the notion that the flexibility of the unbound hormones is important to allow them to achieve their proper conformation in the bound state and that substitutions that prevent this process lead to inactive analogs. The data also show that care should be taken when planning the design of TOAC-substituted analogs in conformational studies of biologically active peptides.

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Anexo 16

Conformational Flexibility of Three Cytoplasmic Segments of the Angiotensin II AT_{1A} Receptor: A Circular Dichroism and Fluorescence Spectroscopy Study

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Abstract: The conformation of three synthetic peptides encompassing the proximal and distal half of the third intracellular loop (Ni3 and Ci3) and a portion of the cytoplasmic tail (*f*CT) of the angiotensin II AT_{1A} receptor has been studied using circular dichroism and fluorescence spectroscopies. The results show that the conformation of the peptides is modulated in various ways by the environmental conditions (pH, ionic strength and dielectric constant). Indeed, Ni3 and *f*CT fold into helical structures that possess distinct stability and polarity due to the diverse forces involved: mainly polar interactions in the first case and a combination of polar and hydrophobic interactions in the second. The presence of these various features also produce distinct intermolecular interactions. Ci3, instead, exists as an ensemble of partially folded states in equilibrium. Since the corresponding regions of the angiotensin II AT_{1A} receptor are known to play an important role in the receptor function, due to their ability to undergo conformational changes, these data provide some new clues about their different conformational plasticity. Copyright © 2002 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: angiotensin II AT_{1A} receptor; circular dichroism; fluorescence; G-protein

Abbreviations: AT_{1A}, type 1A angiotensin II receptor; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; CD, circular dichroism; DCM, dichloromethane; DIEA, N,N-diisopropylethylamine; DMF, N,N-dimethylformamide; *f*CT, segment of the AT_{1A} C-terminal cytoplasmic tail (residues 300–320); AcOH, acetic acid; GPCR, G-protein coupled receptor; HATU, N-[(dimethylamino)-1*H*-1,2,3-triazolo [4,5-*b*] pyridin-1-ylmethylene]-N-methylmethanaminium N-oxide; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; i3, third intracellular loop of AT_{1A}; MBHAR, 4-methylbenzhydrylamine resin; Ni3 and Ci3, N-terminal (residues 213–231) and C-terminal (residues 227–242) segments of i3, respectively; NMR, nuclear magnetic resonance; TBTU, 2-[(1*H*-benzotriazol-1-yl)1,1,3,3-tetramethyluronium; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; Z, benzyloxycarbonyl.

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INTRODUCTION

G-protein coupled receptors (GPCRs) form one of the largest and most important families of membrane-bound receptors [1]. It is widely accepted that, to untangle their structure–function relationship, an effective approach is to investigate segments of these large proteins [2–6].

The third intracellular loop (i3) and a portion of the C-terminal cytoplasmic tail of the

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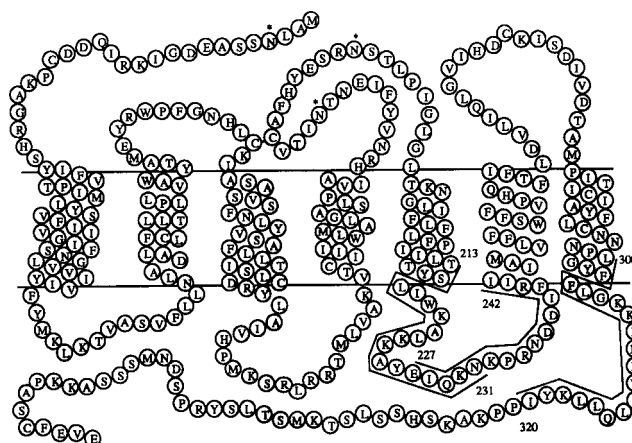


Figure 1 Amino acid sequence and schematic representation of the rat AT_{1A} receptor. The synthetic peptides studied in this work, named Ni3 (residues 213–231), Ci3 (residues 227–242) and *f*CT (residues 300–320), are indicated by solid lines.

angiotensin II AT_{1A} receptor (AT_{1A}) are known to be involved in receptor activation and G-protein selection and coupling [7–11]. Based on this evidence, we concentrated on three segments of those cytoplasmic domains (Figure 1) that site-specific mutagenesis and chimera expression [10–13] indicate are essential for the receptor's function: (i) the proximal part of the i3 loop (residues 213–231, TSYTLWKALKKKAYEIQKN-NH₂, Ni3), (ii) a segment of the C-terminal tail (residues 300–320, LFYGFGLGKKFKKYFLQLKYI-NH₂, *f*CT) and (iii) the distal part of the i3 loop (residues 227–242, EIQKNKPRNDDIFRII-NH₂, Ci3). Segments (i) and (ii), as free peptides, are able to compete with the receptor for G-protein activation [7, 8] while segment (iii), although inactive when tested as a free peptide, turns out to be an essential part of the i3 loop for AT_{1A} activation and coupling [7].

Previous studies carried out by ¹H-NMR [14, 15] showed that the synthetic receptor's segments, in water at acidic pH, exist in a random coil conformation and that, in 30% TFE, while *f*CT and Ni3 are able to fold into an α -helical structure, Ci3 exhibits an ill-defined conformational behaviour.

Recognizing that the expression of some biological function implies the existence of stable or inducible structural determinants, we have been prompted to investigate, by CD and fluorescence spectroscopies, whether physiological parameters such as pH, ionic strength and dielectric constant, can elicit *in vitro* secondary and tertiary structures representative of the molecular events that *in vivo* are expected to be associated with the function of the receptor complex machinery.

MATERIALS AND METHODS

Peptide Synthesis

The peptides TSYTLWKALKKKAYEIQKN (Ni3), EIQKNKPRNDDIFRII (Ci3) and LFYGFGLGKKFKKYFLQLKYI (*f*CT) (Figure 1) were synthesized manually accordingly to the standard Boc/Bzl strategy [16, 17] *p*-Methylbenzhydrylamine-resin (0.79 mmol/g) in a 0.4 mmol scale was used as the solid support and the side-chain protecting groups employed were: Bzl (S and T), 2-Br-Z (Y), cyclohexyl (D and E), 2-Cl-Z (K), *p*-toluenesulphonyl (R) and formyl (W). Briefly, the α -amino group deprotection and neutralization steps were performed in 30% (v/v) TFA/DCM for 30 min and in 10% (v/v) DIEA/DCM for 10 min. The coupling step was carried out using a three-fold excess of the acylating reagents TBTU/DIEA [18] and HATU/HOBt [19] when recoupling was necessary. On the basis of a previous report [20] correlating the solvation of resin beads with several factors such as the peptide content, the peptide sequence and the polarity of the medium, during the coupling in the resins containing up to about 30% peptide (weight/weight) or higher, we used as solvent systems, 50% v/v DCM/DMF and 30% DMSO/NMP, respectively. Cleavage of the peptide from the resin was carried out in HF: *o*-cresol: dimethylsulphide (9:0.5:0.5) at 0 °C for 90 min. In the case of the Trp-containing Ni3 sequence, ethanedithiol was added to the HF mixture in 5% (v/v) final solution. The resins were rinsed with ethyl acetate and peptides were extracted with 5% AcOH and lyophilized. Purification of the peptides was carried out on a Waters 510 HPLC instrument using a Vydac C₁₈ semi-preparative column

(25 × 250 mm, 300 Å pore size, 15–20 µm particle size). The peptides were dissolved in 5% AcOH, sonicated and centrifuged at 10 000 × *g*. After filtration, the solution was loaded onto the column and eluted with solvent A, H₂O containing 0.1% TFA and solvent B, H₂O containing 60% acetonitrile and 0.08% TFA. The flow rate was 10 ml/min and absorption was detected at 210 nm. Linear gradients ranging from 45% to 85% of B, for Ni3 and *f*CT, and 25%–65% of B, for Ci3, were applied over a period of 2 h.

Analytical HPLC

A Vydac C₁₈ column (4.6 × 150 mm, 300 Å pore size, 5 µm particle size) was used with solvent A, H₂O containing 0.1% TFA and solvent B, H₂O containing 60% acetonitrile and 0.08% TFA. Flow rate was 1.5 ml/min and the absorption was detected at 220 nm. A linear gradient ranging from 5% to 95% of B was applied over a 30 min interval. In these conditions, the peptides Ni3, Ci3 and *f*CT eluted in 19, 17 and 25 min, respectively.

Amino Acid Analysis

The peptides Ci3 and *f*CT were hydrolysed with 6 N HCl and the Trp-containing Ni3 with 3 N *p*-toluenesulphonic acid (both procedures at 110 °C for 72 h) followed by neutralization with 2 N NaOH, in a Pyrex tube with plastic Teflon-coated screw cap. The hydrolysed products were analysed in a Beckman 6300 Amino Acid Analyzer. The analyses showed the expected amino acid composition for each of the peptides.

Mass Spectrometry

Peptides were analysed by Matrix Assisted Laser Desorption Ionization (MALDI) on a Micromass Spectrometer, model ToFSpec SE, using α -cyano-4-hydroxycinnamic acid as matrix. The molecular weights found for peptides Ni3, Ci3 and *f*CT were, *m/z*: 2297.9 Da (*M*⁺ + 1) (theoretical value: 2297.7), 1998.4 Da (*M*⁺ + 1) (theoretical value: 1998.3) and 2657.8 Da (*M*⁺ + 1) (theoretical value: 2657.3), respectively.

Circular Dichroism Spectroscopy

Far-UV CD spectra were recorded on a Jasco J-715 equipped with a Peltier system PTC-348 WVI for cell temperature control and on a Jobin Yvon CD6 spectropolarimeter. Ellipticity is reported as the mean

residue molar ellipticity, $[\theta]$ (deg cm² dmol⁻¹). Peptide concentration was determined either based on the peptide content derived from the amino acid analyses or spectrophotometrically using the extinction coefficients of the Tyr and/or Trp residues at 276 nm, $\epsilon_{\text{Tyr}} = 1390 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{\text{Trp}} = 5455 \text{ M}^{-1} \text{ cm}^{-1}$, respectively [21]. The percentage of α -helix was estimated using the mean residue molar ellipticity at 222 nm ($[\theta]_{222}$) [22].

Fluorescence Spectroscopy

Fluorescence spectra were recorded using a Hitachi 1050 spectrofluorimeter at 25 °C. In the case of *f*CT the excitation wavelength was set at 274 nm and, to avoid the interference of the water Raman peak at 302 nm, the intensity of the Tyr fluorescence was measured at 310 nm. In the case of tyrosinate, the fluorescence intensity was measured at 370 nm to eliminate any artefact due to residual Tyr fluorescence. For Ni3, Trp was excited at 295 nm. Emission and excitation slits were set at 5 nm band-pass; cells were 1 cm pathlength. Corrections were made for inner filter effects [23]. Fluorescence quenching data were analysed using the Stern-Volmer approach for pure collisional or pure static quenching. In the case of mixed type quenching the data were fitted according to Eftink and Ghiron [24].

Analysis of the pH Titration

The data of the pH titration for both the CD and fluorescence experiments were fitted by a non-least square regression analysis using the Henderson–Hasselbach equation with two *pK_a* values.

RESULTS AND DISCUSSION

The C-Terminal Tail (The *f*CT Segment)

Figure 2A shows that at pH 4.0 *f*CT exists in an extended form and folds into an α -helical conformation upon increasing the pH. The conformational transition begins at about pH 6.5 and the negative ellipticity increases steadily up to pH 7.7 (Figure 2A, inset). At pH 8.0 a sudden increase of the spectral intensity occurs. Subsequently, at pH 8.8, there is an abrupt decrease of the ellipticity associated with a significant change of the CD profile (Figure 2B) suggesting the onset of self-association. Overall, the data confirm the expected pH dependence of the peptide conformation due to the

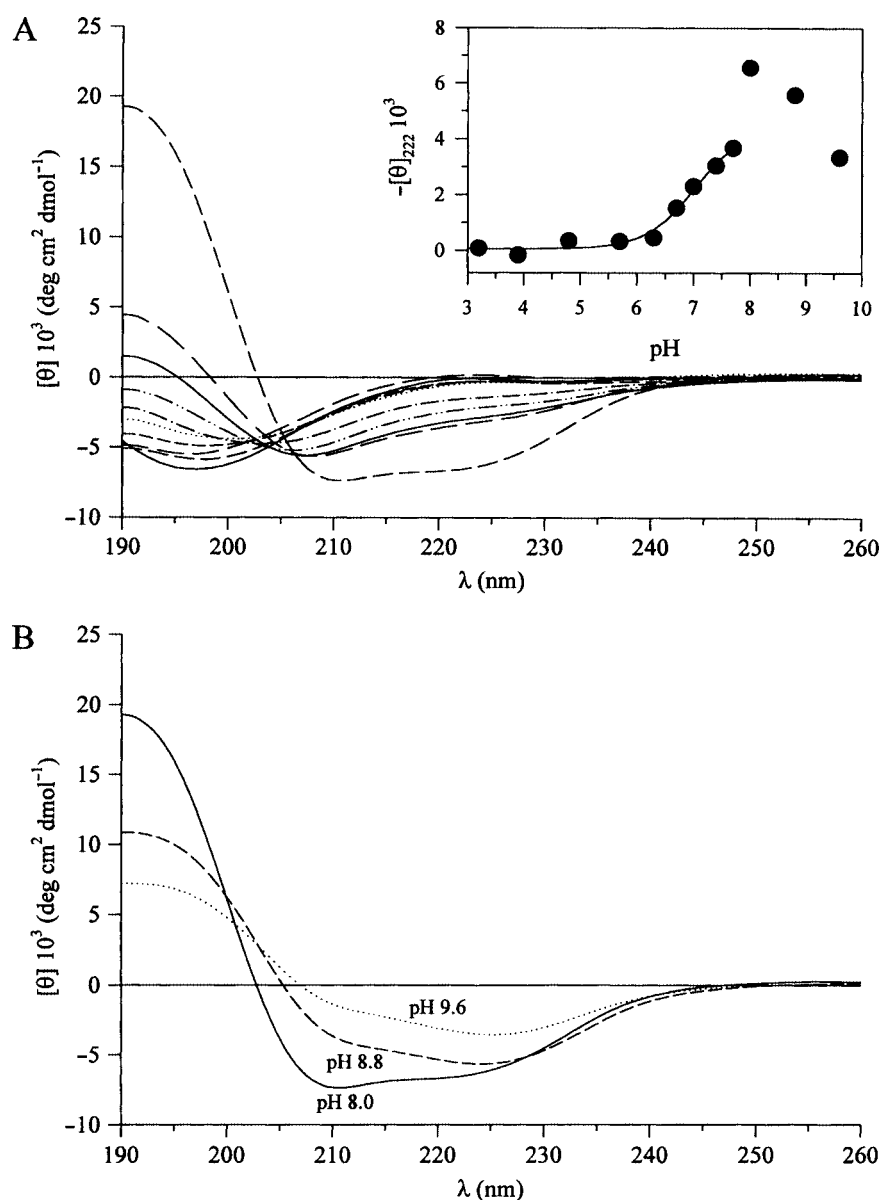


Figure 2 CD spectra of 0.12 mM fCT in water as a function of pH at 20 °C. A: pH range 3.0–8.0 (acid to basic pH from top to bottom at 222 nm). Inset: $-[\theta]_{222}$ as a function of pH; B: pH values of 8.0 (—), 8.8 (---) and 9.6 (·····).

presence of several ionizable side chains. They also indicate that hydrophobic interactions between the Tyr and Leu residues at positions $(i, i + 3)$ or $(i, i + 4)$, (Tyr3-Leu6, Tyr13-Leu17, Ley17-Tyr20), combined with electrostatic interactions, most likely between the Lys and Tyr residues, might be involved in helix stability and may also favour coiled-coil formation [25–29]. In addition, the intra- and intermolecular salt bridges, besides being important for helix stabilization [30], contribute to neutralize the peptide surface, further justifying its enhanced aggregation capacity.

To corroborate this hypothesis we examined the pH dependence of the peptide fluorescence (Figure 3). The phenol side chain of the Tyr residues is in equilibrium with its dissociated form, with a $pK_a \approx 10$ which drops to $pK_a \approx 4.5$ in the excited state [31]. Since the fluorescence emission maximum of the former lies between 303–305 nm while the tyrosinate form emits in the range 340–350 nm [32], we have been able to monitor the pH behaviour of the two species. As indicated in the Materials and Methods section, to avoid spectral interference, the fluorescence intensity was

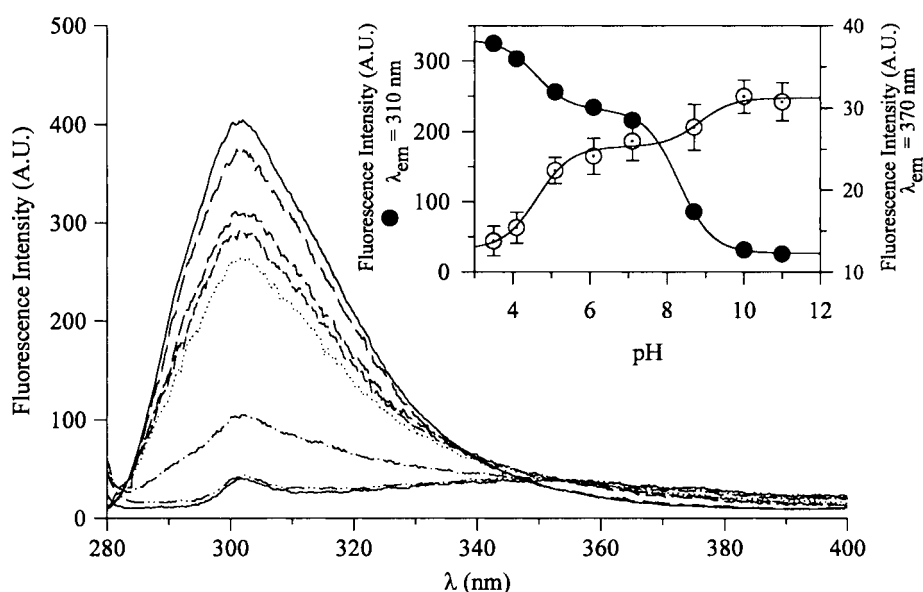


Figure 3 Fluorescence spectra of *f*CT (1.18×10^{-5} M) in water as a function of pH, (acid to basic pH from top to bottom); excitation: 274 nm. Inset: Plot of the fluorescence intensity as a function of pH at the emission wavelengths 310 nm (●) and 370 nm (○).

measured at 310 nm and at 370 nm for Tyr and tyrosinate, respectively. The first decrease in the fluorescence intensity, measured at 310 nm, at about $\text{pH } 4.6 \pm 0.1$, coupled with a fluorescence increase measured at 370 nm (Figure 3, inset), is indicative of the ionization of the Tyr hydroxyl group in the excited state. As for the second decrease of the fluorescence intensity at 310 nm at about $\text{pH } 8.5 \pm 0.3$, and the concomitant increase detected at 370 nm, it can be associated with ionization in the ground state. The pK_a decrease, observed in the ground state, is suggestive of the onset of inter- and/or intramolecular interactions. These results are consistent with the CD data and support the hypothesis that hydrophobic and electrostatic side chain interactions are responsible for the peptide tendency to form intermolecular aggregates.

The Stern-Volmer plots of the Tyr fluorescence quenching, carried out at pH 4.0, 7.4 and 8.5, using acrylamide, indicate that the quenching mechanism is purely collisional (data not shown). The quenching constants (Table 1), show that acrylamide efficiency decreases from pH 4.0 to pH 8.5, and that, already at neutral pH, the quencher accessibility to the Tyr residues is reduced indicating that partial aggregation is already present.

To further ascertain the role of electrostatic and hydrophobic interactions in the regulation of the peptide folding pathway, the dependence of the secondary structure on ionic strength was

Table 1 Stern-Volmer Quenching Constants, K_{sv} , for the Tyr Residues in *f*CT and the Trp Residue in Ni3 Obtained using Acrylamide as Quencher

pH	$K_{sv}(\text{M}^{-1})$	
	<i>f</i> CT	Ni3
4.0	14.4	15.3
7.4	10.6	8.1
		3.0 ^a
8.5	9.3	—
9.3	—	7.5

^a At pH 7.4 the Stern-Volmer plot for Ni3 is not linear. Therefore the data have been analysed according to [24].

studied at pH 4.0 and 7.4 (Figure 4). At pH 4.0, the data evidence the existence of a two-step transition (Figure 4, inset). Up to 1.5 mM NaCl there is a smooth transition from the extended to a stable intermediate state. This behaviour, combined with the absence of an isodichroic point, suggests the presence of various conformations in equilibrium. A second, steeper transition takes place between 2.5 and 4.0 mM NaCl, indicating a further enhancement of the peptide ordered secondary structure.

Interestingly, when the titration is carried out at pH 7.4, only a smooth transition is observed

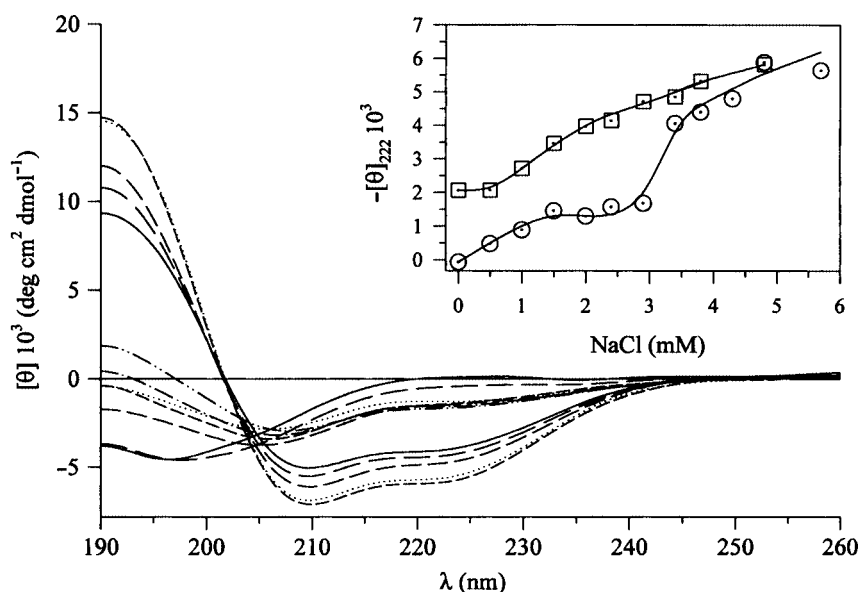


Figure 4 CD spectra of 0.12 mM *fCT* in water at pH 4.0 and 20°C as a function of NaCl concentration (NaCl concentration increasing from top to bottom at 222 nm). Inset: $-[\theta]_{222}$ as a function of NaCl concentration at pH 4.0 (O) and pH 7.4 (□).

starting at 1 mM NaCl, which is followed by a steady increase of the ellipticity up to 4.5 mM NaCl, where it reaches a value similar to the one obtained when carrying out the salt titration at pH 4.0. The loss of biphasic behaviour (Figure 4, inset) suggests that the intermediate state observed at pH 4.0 depends on electrostatic interactions.

Overall, the results show that at physiological pH *fCT* exists as an ensemble of conformations characterized by partial helical folding and capable of acquiring supramolecular organization.

The i3 Loop

The Ni3 segment. In water, at pH 3.8, Ni3 displays a CD spectrum typical of a peptide in a predominantly random conformation (Figure 5A). Different from *fCT*, the variation of the peptide concentration in the range 0.15–1.5 mM does not cause any aggregation (data not shown).

Nonetheless, because of its charged residues and similarity to *fCT*, Ni3 is expected to be sensitive to pH and ionic strength. In fact, on increasing the pH, we observed a fold of Ni3 into a helical conformation up to a maximum helical content of ca. 66% (Figure 5A). An initial minor conformational transition at $\text{pH } 6.7 \pm 0.1$ can be related to the dissociation of Glu15. The increase of its apparent pK_a may reflect the formation of salt bridges with the protonated Lys residues in positions $(i+3)$ and $(i-3)$. The second transition, at $\text{pH } 9.5 \pm 0.1$,

suggests that the dissociation of the four Lys residues and the consequent reduction of the helix destabilizing electrostatic repulsions [25] plays a crucial role in the folding process. Interestingly, the pH at which this conformational transition takes place is significantly higher than for *fCT*.

The behaviour of the Trp7 fluorescence as a function of pH is reported in Figure 5B. The first weak decrease of the fluorescence intensity at $\text{pH } 6.0 \pm 0.4$ is likely to reflect an initial interaction with Tyr3 as a result of the conformational change induced by the ionization of Glu15. The second main decrease, centred at $\text{pH } 9.4 \pm 0.1$, might be due to a stronger interaction, favoured by the peptide folding, with the nearby Tyr3 and Lys8. However, considering that the absence of a blue shift in the fluorescence emission spectrum indicates that, while the peptide folds, the Trp residue remains in a polar environment, another factor justifying the fluorescence decrease could be the movement of the Trp side chain closer to the peptide bonds.

The Stern-Volmer constants for the acrylamide quenching experiments, carried out at various pHs, are reported in Table 1. The quenching mechanism at pH 4.0 and 9.3 is of the collisional type. At pH 7.4, the non-linearity of the plot (data not shown) indicates the presence of an additional static component. The analysis of the data [24] yielded a K_{SV} of 8.1 M^{-1} and 3.0 M^{-1} for the collisional and static components, respectively. This result is

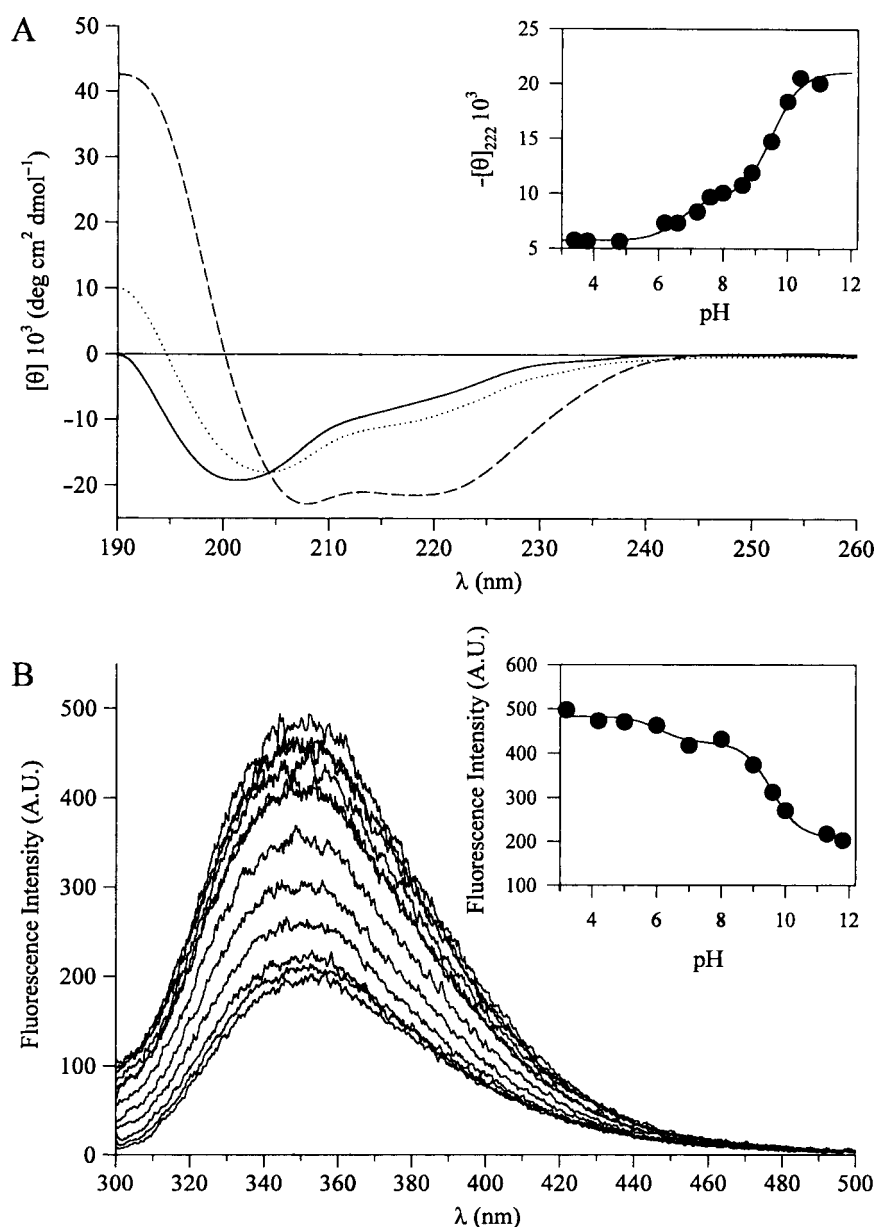


Figure 5 A. CD spectra of 0.15 mM Ni3 in water as a function of pH, at 20°C; pH values of 3.8 (—), 7.2 (·····) and 10.4 (---). Inset: $-[\theta]_{222}$ as a function of pH; B. Fluorescence spectra of Ni3 (2.2×10^{-5} M) in water (acid to basic pH from top to bottom), λ_{exc} 295 nm. Inset: fluorescence intensity at 350 nm as a function of pH.

therefore suggestive of a heterogeneous population of Trp residues. Since each Ni3 peptide molecule carries only one Trp residue, these data also indicate the coexistence of various conformational states in agreement with the absence of an isodichroic point in the CD spectra. In addition, because the CD data do not indicate any aggregation process, the decreased accessibility at basic pH can be considered a confirmation of the proposed onset of intramolecular interactions.

As for the dependence of the peptide conformation on ionic strength, although Ni3 is positively charged, the alterations of the CD spectra, both at pH 4.0 and 7.4, are not significant (data not shown). It is possible that conformational changes might occur at a higher salt concentration. However, for the purpose of the present work, it is noteworthy that, in the same ionic strength range, Ni3 and fCT, though they both contain a patch of Lys residues (Figure 6), behave in a distinct manner.

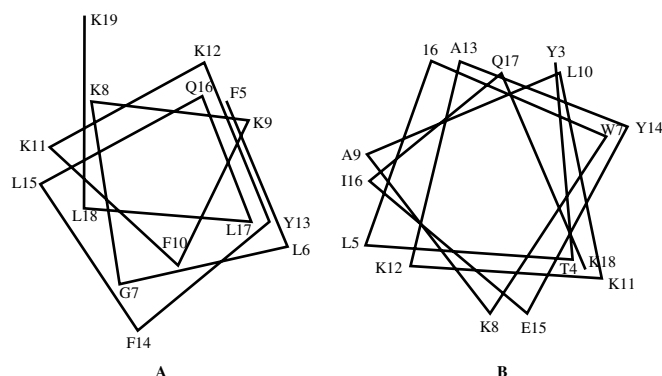


Figure 6 Helical wheels based on the structure determined by NMR in 70% H₂O: 30% TFE. A. *f*CT at 5 °C (14); B. Ni3 at 25 °C (15).

Indeed, the NMR-based helical wheel, obtained in 30% TFE [15] (Figure 6B), evidences that in the patch of Lys residues there are also Glu and Thr residues that, because of their significative electron density, at the low salt concentrations tested could account for the reduced ion–peptide interaction.

To better define the intrinsic propensity of the peptide to fold into a helical structure, we tested the effect of TFE as a co-solvent [33, 34]. The TFE/H₂O titration at pH 4.0 (Figure 7A) shows that the coil to helix transition has already started at 10% TFE. The extent of the helical conformation increases up to 30% TFE, reaching a value of about 59%. In the TFE range 20%–100% the CD spectra show an isodichroic point at 201.2 nm, confirming the existence of a two-state transition. Moreover, the sigmoidal shape of the plot of $[\theta]_{222}$ vs % TFE (Figure 7A, inset) is suggestive of a strong cooperativity of the folding. The CD profile of the peptide was investigated at various temperatures in 30% TFE to evaluate the helix stability. This experimental condition was chosen because, while at this TFE concentration the peptide appears to have already reached a stable folded conformation, yet enough water molecules are present to guarantee an efficient interaction with the peptide backbone. Figure 7B shows a linear increase of the ellipticity with decreasing temperature. However, the relatively small variation of the helical content (from 35% to 59%), suggests a very stable helical structure. Moreover, since the decrease in ellipticity is associated with an insignificant shift of the two π - π^* transitions (192 nm and 208 nm), we can hypothesize that, upon increasing the temperature, rather than an unfolding process, what is occurring is only a

loosening of the helical structure that causes a weakening of the helical macrodipole.

In summary, Ni3 turns out to be a peptide characterized by a certain degree of conformational flexibility in water. However, if its conformational space is restricted, as happens in TFE, it acquires a quite stable helical structure able only to fluctuate between a loose and a tight state.

Comparison of *f*CT and Ni3 Helicity

Although *f*CT and Ni3 share some common features such as the presence of Lys patches and the ability to form amphipathic helices, they show a distinct behaviour with respect to their response to ionic strength and their ability to elicit intermolecular interactions.

The helical content for Ni3 and *f*CT in H₂O at pH 8.0 is 38% and 28%, respectively. However, in 30% TFE at 5 °C the two peptides have the same helicity (ca. 60% helical content).

This apparent inconsistency can be explained if the CD data are analysed using, as a measure of the α -helix content, the ratio R_2 between the ellipticity at 222 nm and that of the negative maximum in the range 195–210 nm [35]. Knowing that R_2 tends to 1 for 100% helix and decreases for a lesser helical content we find, in contrast to the previous estimate based on $[\theta]_{222}$ that, at pH 8.0, *f*CT presents a higher content of helical structure than Ni3, $R_2 = 0.88$ and 0.52, respectively. Moreover, the measurable limiting values of R_2 (Figure 8) suggest that, in water, the two peptides possess comparable helicity.

These results can be rationalized considering that when the *f*CT molecules begin to fold, due

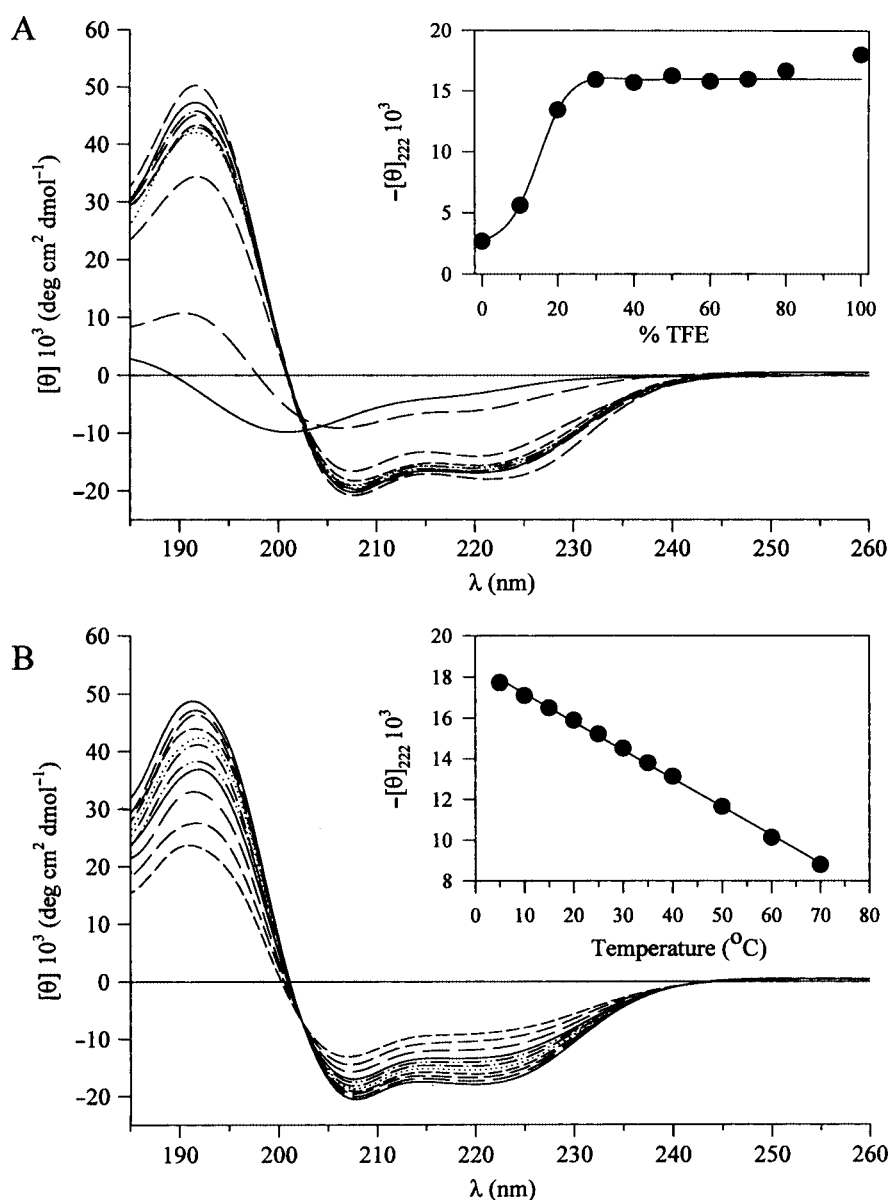


Figure 7 A. CD spectra of 0.15 mM Ni3 in water at pH 4.0 and upon addition of TFE in the concentration range 0–100% (v/v) at 20°C (TFE concentration increasing from top to bottom at 222 nm). Inset: $-\theta]_{222}$ as a function of TFE percentage; B. CD spectra of 0.15 mM Ni3 in the 70%:30% H₂O - TFE solvent mixture as a function of temperature (temperature decreasing from top to bottom at 222 nm). Inset: $-\theta]_{222}$ as a function of temperature.

to the amphipathic nature of the helix and to the presence of the Tyr Leu pairs, they initially form loose helix associations that can lead to a dampening of the optical activity. As the pH increases, the supramolecular clusters become tighter and, between pH 7.7 and 9.6, an α -helical coiled-coil may form. These molecular events are supported by the changes in the CD spectra consisting of a red shift of the cross-over point and in a drastic reduction of the π - π^* transition

that leads to $[\theta]_{222}/[\theta]_{\min} > 1$. The lack of a clear red shift of the π - π^* transition [36] is probably due to the fact that it is not a simple two-state equilibrium.

In conclusion, in the case of *f*CT, the driving force responsible for its folding is a combination of various factors such as intramolecular hydrophobic interactions between Leu and Tyr chains [26] as well as the reduction of the repulsive forces between the charged Lys residues [25] and the formation of

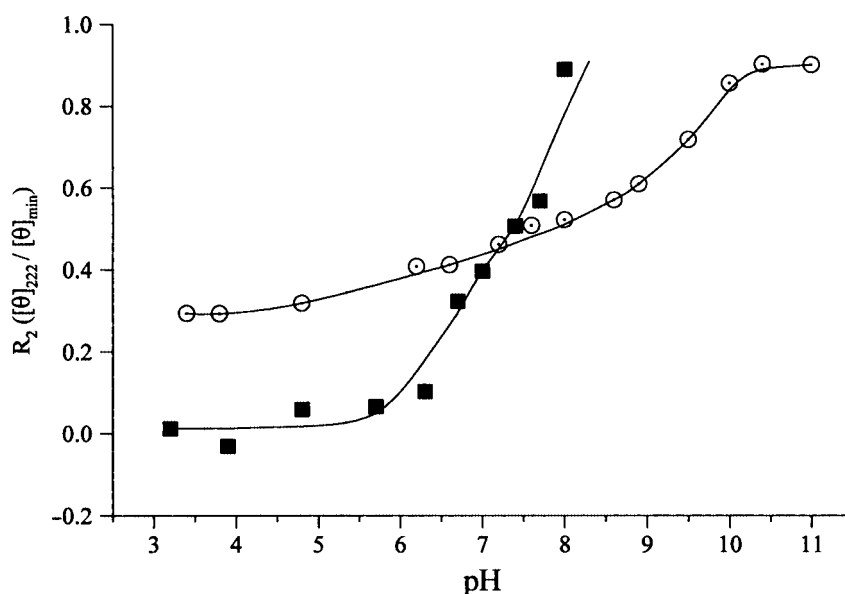


Figure 8 R_2 vs pH for fCT (■) and Ni3 (○). R_2 is the $[\theta]_{222}/[\theta]_{\text{neg,max}}$ ratio, according to [35].

salt bridges between Tyr and Lys residues [27, 28]. These last factors contribute to neutralize the helix surface and to enhance the intermolecular hydrophobic interactions, thus determining the strong aggregation tendency of the peptide that leads to the formation of supramolecular clusters and/or helical coiled-coils [29, 37].

The folding of Ni3, on the other hand, is mostly driven by intramolecular electrostatic interactions. This is evidenced by the shape of the pH titration, where the two conformational transitions centred at $\text{pH } 6.5 \pm 0.1$ and 9.4 ± 0.1 (Figure 5A, inset) reflect the dissociation of ionizable residues. In addition, the peptide insensitivity to ionic strength suggests a helical structure with a surface possessing a net high electron density that gives rise to repulsive intermolecular interactions and prevents aggregation.

In TFE, however, where the aggregation processes are strongly hampered, the two peptides behave in a very similar manner. Thus, we can conclude that, although both peptides possess similar helicity, the global folding is driven by diverse chemical interactions that lead to distinct molecular behaviour in aqueous environment.

The Ci3 segment. In water at pH 4.0, Ci3 exists in an extended conformation [15] and, similarly to Ni3, its conformation is insensitive to changes in peptide concentration (data not shown). However, in this case variations of pH and ionic strength do not

induce any regular folding of the peptide (data not shown).

The titration with TFE (Figure 9A) shows that, in agreement with NMR data [15], the solvent can favour the formation of some helical determinants, as suggested by the enhancement of the negative band at 220–222 nm. However, the overall folding process occurs in a quite different manner and to a different extent when compared with Ni3 and fCT. As a matter of fact, the CD profile does not permit a unique interpretation. In fact, it is possible that the negative band at about 205 nm exists as the result of the coexistence of the α -helix π - $\pi_{\parallel}^*$ transition with the random coil π - π^* transition, therefore being compatible with a peptide containing α -helix and random coil portions [38]. Such an interpretation is supported by the fact that the first negative band, that in the case of a pure α -helix is at 222 nm, is shifted here to about 220 nm and by the observation that the positive π - $\pi_{\perp}^*$ transition at 195 nm is strongly dampened. Alternatively, the two major negative bands in the 202–204 nm and 218–222 nm regions could reflect the presence of structural determinants reminiscent either of a 3_{10} -helix [39] or of turns belonging to a helical backbone and giving rise to a β -spiral-like motif [40, 41].

To further verify the peptide difficulty in acquiring a well-defined secondary structure, we examined the temperature dependence of its structure in 100% TFE. Figure 9B shows that throughout the

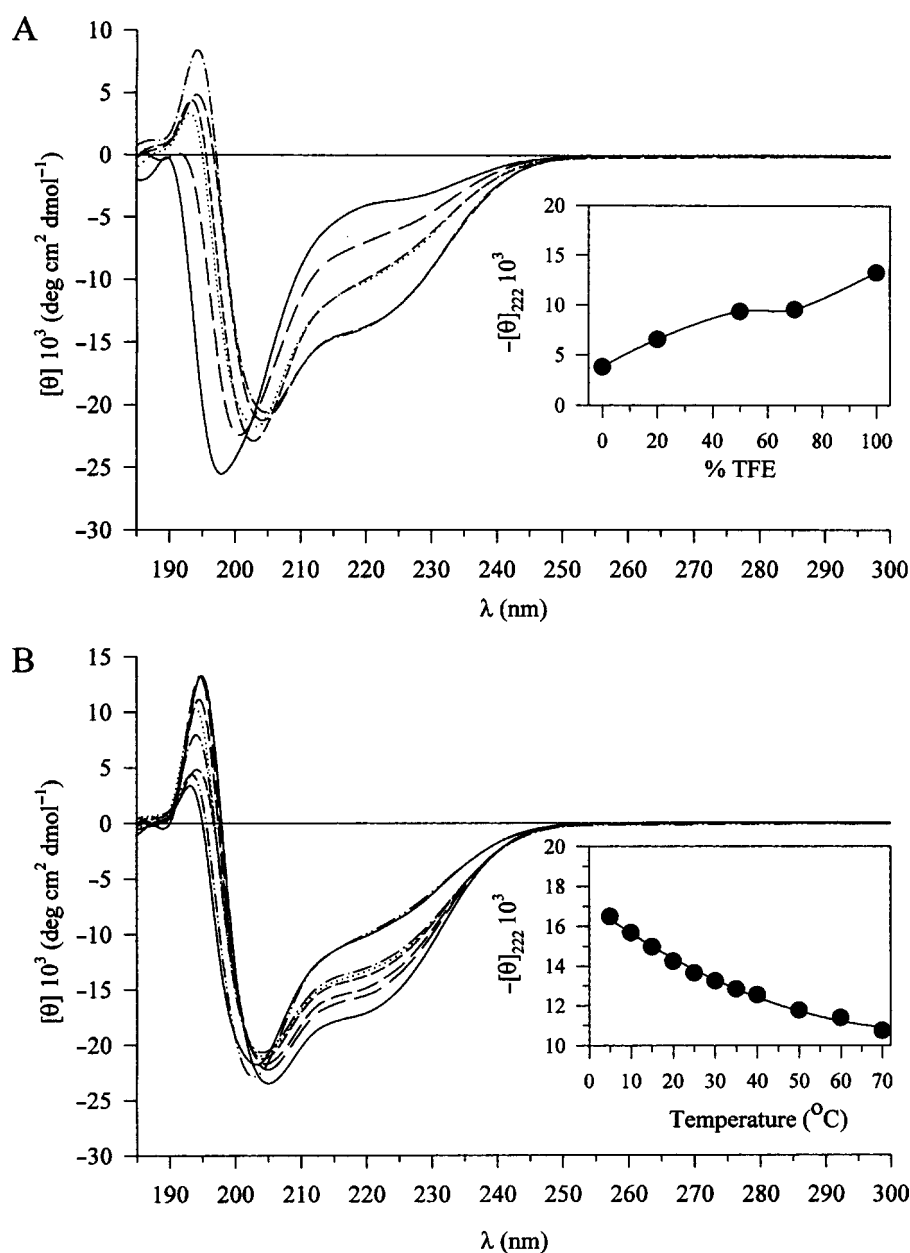


Figure 9 A. CD spectra of 0.15 mM Ci3 in water at pH 4.0 and upon addition of TFE in the concentration range 0–100% (v/v) at 20 °C (TFE concentration increasing from top to bottom at 222 nm). Inset: $-[\theta]_{222}$ as a function of TFE percentage. B. CD spectra of 0.15 mM Ci3 in 100% TFE as a function of temperature (temperature decreasing from top to bottom at 222 nm). Inset: $-[\theta]_{222}$ as a function of temperature.

entire temperature range (5°–70 °C) the CD profile is not indicative of any preferential conformation. In addition, the non-linear variation of the molar ellipticity at 222 nm (Figure 9B, inset) suggests that changes in temperature induce conformational modifications involving the extension and combination of the various secondary structure elements present in the peptide.

CONCLUSION

The present view of the molecular mechanism of signal transduction consists of a sequence of events in which ligand binding induces a concerted reorganization of the spatial distribution of the trans-membrane helices [42]. Such a structural change would propagate to the cytosolic loops leading

to a rearrangement of their conformation, the receptor activation. The resulting alteration of the receptor-G protein contacts constitutes the receptor G-protein coupling, responsible for G-protein signalling [42, 43].

Overall conformational flexibility is a key feature for the function of the GPCR machinery. Here we have studied three peptides encompassing functional regions of the AT_{1A} receptor showing that, using *in vitro* conditions that mimic their *in vivo* microenvironment, they can undergo distinct conformational changes that are expected to reflect their specific role in the various steps of AT_{1A} operation.

We believe, therefore, these studies may be useful for better understanding the complex structure-function relationship of large proteins such as GPCRs and, in particular, they may provide insights that will allow the description in a more precise manner of those molecular processes that are often indicated as generic conformational changes.

Acknowledgements

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Anexo 17

Synthesis and Immunological Activity of a Branched Peptide Carrying the T-cell Epitope of gp43, the Major Exocellular Antigen of *Paracoccidioides brasiliensis*

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Abstract

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The 43 kDa glycoprotein (gp43) of *Paracoccidioides brasiliensis* is the major diagnostic antigen of paracoccidioidomycosis (PCM), a prevalent fungal infection in South America. A 15-mer sequence from gp43, denominated P10, induced T-CD4⁺ T helper 1 cellular immune responses in mice of three different haplotypes and protected against intratracheal challenge by a virulent isolate of *P. brasiliensis*. In an attempt to improve delivery of P10, a promiscuous antigen also presented by human leucocyte antigen-DR alleles, aiming at immunotherapy, we synthesized a multiple antigen peptide with the protective T-cell epitope expressed in a tetravalent 13-mer analog of P10 (M10). M10 induced specific lymph node cell proliferation in mice preimmunized with peptides in complete Freund's adjuvant (CFA). In addition, M10 immunization without CFA significantly protected intratracheally infected mice. We conclude that M10 is a candidate for an anti-PCM vaccine. In this report we describe: (1) the synthesis of M10; (2) the induction of M10-elicited T-cell response and (3) *in vivo* protection of mice immunized with M10 and challenged by a virulent strain of *P. brasiliensis*.

Introduction

Paracoccidioidomycosis (PCM) is a systemic granulomatous disease caused by *Paracoccidioides brasiliensis*, a thermal dimorphic fungus. It is widespread in South and Central America, mainly affecting rural workers but can also reach urban centres upon migration of infected individuals [1]. According to McEwen *et al.* [2], approximately 10 million people may be infected with this fungus and up to 2% of them may develop the disease. The incidence may increase due to forest destruction and rise in iatrogenic immunosuppression procedures [3]. The disease has multiple manifestations, and two progressive forms (acute and chronic) are recognized [4]. In most cases, the disease involves primarily the lungs and then disseminates to other organs and systems [4].

The glycoprotein gp43 from *P. brasiliensis*, which is secreted exocellularly by the infective yeast phase [5, 6], is the main PCM diagnostic antigen [7], being recognized by virtually all sera from infected patients as determined by using different serological methods [8, 9]. Gp43 also

contains epitopes that elicit positive delayed-type hypersensitivity (DTH) in both infected guinea pigs [10] and human patients [11]. Depletion of gp43 by immunoaffinity chromatography from a complex antigenic preparation generally used for skin tests, resulted in negative DTH reaction [10].

Gp43 reactive T lymphocytes are stimulated by a 15-mer peptide designated as P10 (QTLIAHTLAIRYAN). The HTLAIR inner core of P10 is the essential domain of the epitope. Immunization with both gp43 and P10 led to protection against intratracheal infection by a virulent isolate of *P. brasiliensis* [12]. The protective effect of P10 is mainly attributed to interferon- γ (IFN- γ)-mediated immune response involving monocytic cell recruitment and granuloma formation. Unlike gp43, which induces an antibody response compatible with both T helper 1 (Th1) and Th2 lymphocyte activation in infected BALB/*c* mice, immunization with P10 does not induce a detectable humoral response [12]. P10 is presented by I-A molecules from three mouse haplotypes and also by a significant number of human

leucocyte antigen-DR alleles (unpublished results). A DNA-based vaccination using the gp43 gene that also induces a long-lasting humoral and cellular immune response [13] is also protective.

Over the past years, considerable progress has been made toward the structural design and development of complex immunogenic polypeptides. Several studies used the multiple antigen peptide (MAP) construction as a powerful alternative to amplify the immunogenicity of synthetic peptides carrying B-cell and T-cell epitopes.

MAPs are constructions based on a small immunologically inert core molecule of radial-branching lysine residues onto which growing peptides are synthesized, starting with the C-terminal amino acid. MAPs may have high molar ratios of peptide antigen to the core molecule and do not require a carrier protein which has been associated with toxicity and immunogenicity [14].

Vaccines using MAPs have been used successfully against bacteria [15], virus [16] and parasite [17] infections. To elicit humoral and cellular immune responses, MAPs have been injected with adjuvants such as incomplete and complete Freund's adjuvant (CFA) [15], AlPO_4 and muramyl dipeptide, without macromolecular carriers. In some cases, the MAPs induced high neutralizing titres of antibodies and cellular reactivity superior to the recombinant antigen, as for instance the HIV-1 envelope protein [18]. As the immunization with P10 in CFA generated a protective immune response in mice subsequently infected by a virulent isolate of *P. brasiliensis* [12], we investigated the possibility of using MAPs incorporating the P10 antigen or a truncated analog containing the T-cell epitope, for increased immunoprotection in the absence of the CFA.

Materials and methods

Abbreviations. Abbreviations for amino acids and nomenclature of peptide structures follow the recommendations of the IUPAC-IUB (Commission on Biochemical Nomenclature) [19]. Other abbreviations are as follows: Boc, tert-butyloxycarbonyl; Bzl, benzyl; 2-BrZ, 2-bromobenzyloxycarbonyl; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MeOH, methanol; NMP, *N*-methylpiperidinone; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEA, triethylamine; TFA, trifluoroacetic acid.

Strains. *P. brasiliensis* isolate 1914 was obtained primarily from a patient with the chronic form of the disease by M. B. Albornoz, Instituto de Biomedicina, Caracas, Venezuela. This isolate has been maintained *in vitro* by subculturing in Sabouraud medium.

Mice. Male or female BALB/c (H-2K^d) mice, 6–8 weeks of age, were obtained from CEDEME animal facility at Federal University of São Paulo (UNIFESP),

SP, Brazil. They were immunized, infected and killed using procedures approved by the Ethics Committee of UNIFESP.

Synthesis of P10. Peptides were synthesized by the 9-fluorenylmethoxy-carbonyl technique [20] based on the method described by Atherton and Sheppard [21] with an automated benchtop simultaneous multiple solid-phase peptide synthesizer (PSSM 8 System; Shimadzu, Tokyo, Japan). Peptides were purified by HPLC with a C-R7A Shimadzu UV-vis Detector and a Shimadzu RF-535 Fluorescence Detector coupled to a Vydac C₁₈ analytical column. Amino acid analysis was carried out in a Beckman 6300 amino acid analyzer following hydrolysis in 6N HCl with 5% phenol at 110 °C for 48 h. Matrix-assisted laser desorption/ionization mass spectrometry was performed on a ToFSpec E instrument from Micromass, with an α -cyano-4-hydroxycinnamic acid matrix.

MAPs (M10 and MC) synthesis. The peptides were synthesized manually according to the standard Boc/Bzl strategy [22]. Briefly, the α -amino group deprotection and neutralization steps were performed in 30% TFA/DCM (30 min) and in 10% DIEA/DCM (10 min), respectively. The scale of synthesis was 0.4 mmol using a 0.2 mmol/g methylbenzhydrylamine resin and all Boc amino acids being coupled with TBTU [23], in the presence of HOBt and DIEA (with 3.3-fold and fourfold excess over the amino component in the resin, respectively). The following side chain-protecting group was used: *p*-toluenesulfonyl (tosyl) for His and Arg, 2-Br-carbobenzoxyl for Tyr and Boc for α - and ϵ -lysyl amine groups. After 2 h coupling time, the qualitative ninhydrin test was performed to estimate the completeness of the reaction. Cleavage reactions were carried out with the low-high HF procedure. The resin was rinsed with ethyl acetate and the peptide extracted in 10% aqueous acetic acid solution and lyophilized. M10 peptide was purified in preparative HPLC (C₁₈-column) using as solvent A, aqueous 0.1% TFA and B, 90% acetonitrile in water containing 0.08% TFA. A linear gradient of 35–65% B in 60 min, flow rate of 10 ml/h and detection at 220 nm were employed. The homogeneity of purified M10 was confirmed by analytical HPLC, matrix-assisted laser desorption/ionization mass spectrometry and amino acid analysis.

The 13-mer amino acid sequence from P10, LIAHTLAIRYAN, was used for the synthesis of M10, and as a control, a truncated peptide containing the last five amino acids, IRYAN, was synthesized and designated as MC.

Swelling of beads. Before the microscopic measurement of dry and swollen bead sizes, the M10 resin and its minor fragments were treated with TEA for deprotonation of the N-terminal amine group. Resin beads were dried in vacuum using an Abderhalden-type apparatus and refluxed in MeOH. Swelling studies were performed as published earlier [24, 25]. Briefly, 150–200 dry and swollen beads of

each resin were allowed to solvate overnight, spread over a microscope slide and measured directly at low magnification. As the sizes in a sample of beads are not normally, but log-normally, distributed, the central value and the distribution of the particle diameters were estimated by the more accurate geometric mean values and geometric SD.

Lymphoblast proliferation. Male or female BALB/c mice were subcutaneously immunized with 20 µg of P10 or 1, 10 or 20 µg of M10 in CFA. The emulsion (50 µl) was inoculated in one rear footpad. Control mice were injected with CFA alone.

Inguinal and popliteal lymph nodes from control (CFA immunized) and P10- or M10-immunized mice were removed after 7 days, and the cells were manually dispersed and centrifuged at 500 *g* (Sorvall RT 6000 D Centrifuge, Asheville, NC, USA) for 5 min; this process was repeated twice. The cell pellet was suspended in 1 ml of RPMI 1640 supplemented with 20 mM NaHCO₃, 10 mM HEPES, 100 U of penicillin/ml, 100 µg of streptomycin/ml, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 5 mM sodium pyruvate, 100 mM nonessential amino acids and 1% normal human serum. The cells were counted in 1:1 dilutions in 0.1% Trypan Blue. Viable cells were cultured at a density of 4×10^5 /well (Costar, Corning, NY, USA). Different concentrations of P10 or M10 from 0.15 to 1.5 µM were added, and the reaction was incubated at 37 °C and 5% CO₂ for 144 h. Controls were run with complete culture medium or with 20 µg/ml of Concanavalin A. Twelve to 16 h before the cells were harvested, 1 µCi/well of [³H]-thymidine (Amersham Pharmacia, Piscataway, NJ, USA) was added. Proliferation was determined by incorporation of the radioactivity by the cells, and the results (expressed in counts per minute) are the means of triplicate determinations. All antigenic solutions were tested for endotoxin with the E-toxate kit (Sigma, St. Louis, MO, USA). The experiments were performed at least three times, and each value is the average of results from five wells.

Cytokine detection in culture supernatants. Lymph node cells from immunized mice and unimmunized controls were obtained as described above. A cell suspension of 4×10^6 cells/ml was distributed in 24-well plates, and M10 or MC at 1.5 µM was added. After different times of incubation, the supernatants were collected and assayed for interleukin-2 (IL-2) and IFN-γ using enzyme-linked immunosorbent assay kits (BD Pharmingen, San Diego, CA, USA). The experiment was repeated at least three times.

Immunization with M10 or MC. Groups of 10 male or female BALB/c mice were immunized subcutaneously once or four times with 1 µg of M10 or MC in 50 µl of phosphate-buffered saline (PBS). As a control, animals were immunized with the same volume of PBS. Immunized animals were infected intratracheally, 15 days after the last immunization. Sera from immunized, but

uninfected, mice were collected and tested for antibody response against M10 and MC both by chemiluminescent (CL) ELISA and dot blotting. The solid phase was sensitized with 200 ng/well of M10, MC or gp43, and CL-ELISA was used as described previously [26, 27]. The experiments were repeated three times.

Intratracheal infection of BALB/c mice. BALB/c mice were anaesthetized with a solution of Ketamine (Parke-Davis, São Paulo, SP, Brazil) and Rompum (Bayer, São Paulo, SP, Brazil), their necks were hyperextended, the trachea exposed at the level of the thyroid and each animal injected with 3×10^5 yeast cells in PBS using a 26-gauge needle. The incisions were sutured with 5–0 silk.

Fungal burden in organs of infected animals. Colony-forming units (CFUs) were counted after 1 and 3 months of infection. The entire lung, liver and spleen were removed, weighed and then homogenized using a tissue grinder in 10 ml of sterile PBS. Serial dilutions of the homogenate were prepared in brain heart infusion agar supplemented with 4% fetal calf serum and 5% spent culture medium of *P. brasiliensis* as growth factor. Garamycin (Gentamicin Sulfate, Schering-Plough, Rio de Janeiro, Brazil) and Cycloheximide (Sigma) were added at 40 and 500 mg/l, respectively. The mixture was plated and incubated at 36 °C. Isolated colonies were counted after 20 days.

Histopathology. Groups of five to 10 immunized or control BALB/c mice were infected intratracheally and killed 1 or 3 months after infection. The lungs, spleens and livers were excised, fixed in 10% buffered formalin and embedded in paraffin for sectioning. The sections were stained with hematoxylin and eosin and examined microscopically (Optiphot-2; Nikon, Tokyo, Japan).

Statistical analysis. All data were analysed by Student's *t*-test and Kruskal–wallis test (Primer of Statistics-The Program, McGraw Hill Co., New York, NY, USA). *P* values below 0.05 were considered significant.

Results

P10 and M10

In order to increase the efficiency of immunotherapy by P10 without using CFA as a necessary adjuvant and examine additional protective protocols including the combined use of cytokines (e.g. IL-12) and additional specific fungal antigens, we are investigating different delivery systems for immunoprotective P10. In the present work, the strategy of complex molecules incorporating antigenic peptides or the MAP constructs introduced earlier [28, 29] was used with oligomeric sequences derived from P10 and composed of four equal peptide chains (LIAHTLAIRYAN) attached to a three-branched lysine core containing glycine at the C-terminal position (Fig. 1). Owing to the forced proximity amongst these chains which induces serious

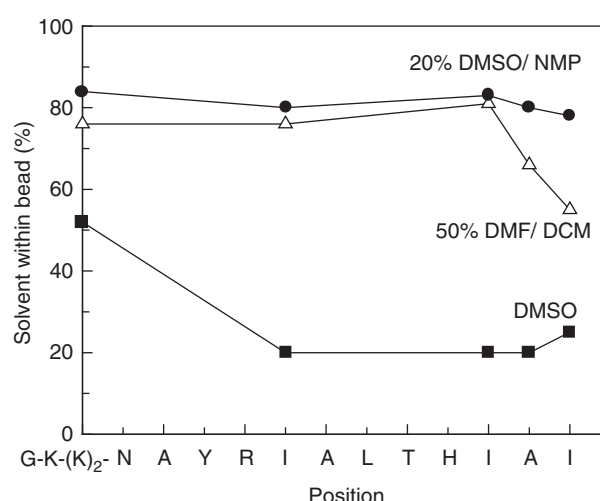


Figure 2 Swelling of M10 resin at different synthetic chain position. DCM, dichloromethane; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; NMP, *N*-methylpiperidinone.

similar synthesis yield for (1–5) and (1–10) fragments was obtained, after peptide cleavage from the resin. Figure 3A,B depicts the HPLC profiles of cleaved M10 minor segments synthesized in 20% DMSO/NMP. The purity yields of about 70 and 50%, respectively, for these two fragments were calculated, irrespective of the solvent used. In contrast to the unsuccessful M10 synthesis in DCM/DMF, the enhanced solvation in 20% DMSO/NMP allowed, although in lower yield (4%), the synthesis of the desired branched M10 peptide. Figure 3C,D shows the HPLC profiles of the crude and purified M10 product synthesized by the optimized condition described.

Lymphoblast proliferation and cytokine production induced by M10 and P10

To show the ability of M10 to elicit a specific cellular immune response, BALB/c mice were immunized subcutaneously with 20 μg per animal of P10 in CFA, as standardized previously [12], or with three different doses of M10: 1, 10 or 20 μg per animal also in CFA. After 7 days, inguinal and popliteal lymph node cells were harvested and cultivated *in vitro* in presence of different doses of the respective immunogen. Figure 4 shows the lymph node cell proliferation obtained with the immunizing dose of 1 μg of M10 comparatively with 20 μg of P10. Immunization with 1 μg of M10 induced a strong proliferative response, even more intense than the response induced by 20 μg of P10. The response was specific, as no proliferation was observed with lymph node cells from mice immunized with CFA alone, either incubated with M10 or P10 (data not shown). Mice immunized with 10 or 20 μg of M10 did not increase their proliferative responses beyond that with 1 μg of M10 (data not shown).

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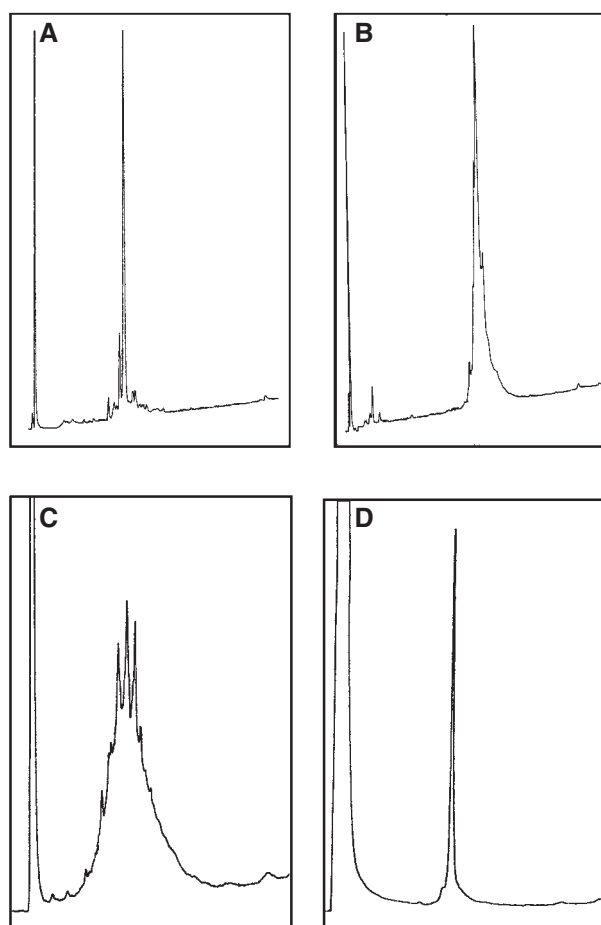


Figure 3 High-performance liquid chromatography (HPLC) profiles of crude truncated: (A) [IRYAN]4-(K)2-K-G-amide (B) [IHTLAIRYAN]4-(K)2-K-G-amide fragments synthesized in 20% *N*-methylpiperidinone (NMP)/dimethylsulfoxide (DMSO). HPLC profiles of crude (C) and pure (D) M10-(K)2-K-G-amide peptide synthesized in 20% NMP/DMSO.

Lymphocytes restimulated with M10, but not MC, produced IFN- γ and IL-2 (data not shown) similar to the response previously described for P10 [12].

CFUs in organs from infected and immunized BALB/c mice

As survival analysis in experimental PCM requires at least 1 year observation, we explored the induction of protective mechanisms by measuring the fungal burden in mice vaccinated with a single dose of M10, MC (both at 1 μ g per animal in PBS) or PBS after 1 and 3 months of infection. Analysis of CFUs in whole organs after 1 month of i.t. infection revealed dissemination to spleen and liver in all groups of mice. Despite some dissemination, the group of mice immunized with M10 showed a significant reduction in the number of isolated colonies from the lungs, spleen and liver when compared with the other groups (Fig. 5). After 3 months of infection, the fungal burden increased in MC or PBS-treated mice but not in M10-treated mice (Fig. 5). Furthermore, mice that had been immunized four times using MC or M10, with 1-week intervals between injections, exhibited similar CFUs compared with those immunized with a single dose (data not shown).

Histopathology of immunized, infected BALB/c mice

Lungs of control animals, immunized with MC or PBS, showed intense cellular infiltration mainly of macrophages, lymphocytes and epithelioid cells. Around the foci of epithelioid granulomas, giant cells were also observed. Multiplying fungal cells were seen; some of them were internalized in polymorphonuclear cells and present in a great number. In contrast, M10-treated mice exhibited very few granulomas with virtually no fungal cells seen and large areas of preserved lung tissue (Fig. 6). These results were similar to the previously observed reduction of fungal burden and immunoprotection mediated by P10 [12].

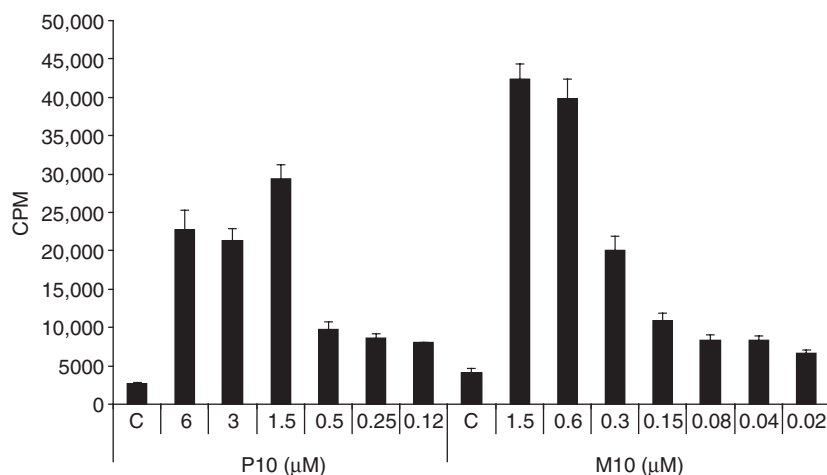
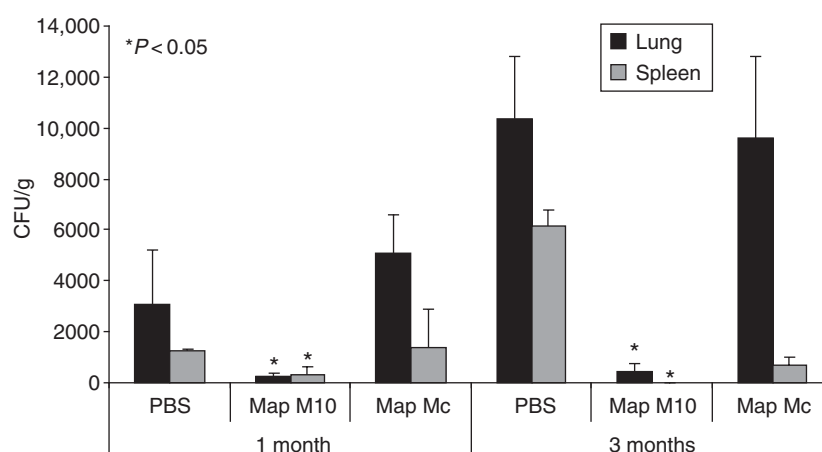


Figure 4 Inguinal and popliteal lymph node cell proliferation from P10- and M10-immunized mice after 7 days. Control 'C' mice received complete Freund's adjuvant alone. Each bar represents the average of five determinations and error bars indicate SDs.

Figure 5 Organ colony-forming units from mice immunized with M10 or MC and infected with 3×10^5 yeast cells, after 1 month and 3 months. Control mice received only phosphate-buffered saline (PBS) and were infected with the same number of yeast cells. Experiments were carried out in triplicate. Each bar represents the average counts of organs from 10 mice and error bars indicate SDs.



Antibody response to M10

A serum pool from immunized mice was assayed for anti-M10 and anti-gp43 antibody reactivity, using chemiluminescence ELISA for increased sensitivity [26]. No antibody reactivity was detected when gp43 was immobilized on a solid phase; however, a discrete antibody response to M10 and MC was detected, probably as a result of the branched lysine nonspecific stimulation (data not shown).

Discussion

MAP constructs have been used to induce B-cell and T-cell responses in animal models and human patients with parasitic diseases and cancer [15–17, 31–34]. They have not so far being used in the immunoprotection against systemic mycoses.

Presently, we describe the synthesis of the M10 complex composed of a total of 56 amino acid residues, with a molecular weight of more than 6 kDa.

Assembling of MAPs is not an easy chemical synthesis, because accumulated errors can take place in the process, making difficult their purification and characterization [35]. The synthesis of M10 was not different, requiring improved resin solvation, as also needed in several other instances such as those of peptide synthesis in heavily peptide-loaded conditions [36], of hydrophobic transmembrane receptor segments [37] or of very strong self-aggregating sequences [38, 39], with the aid of a chain motion paramagnetic amino acid monitor [40].

There are cases when linear peptides were as efficient as different MAP structures in the generation of specific T-cell responses [41, 14]. To increase the efficiency of these molecules, a new lipophilic MAP system for immunogens that contains a built-in lipophilic adjuvant and is able to elicit cytotoxic T-lymphocytes (CTLs) has been introduced [42]. Interestingly, the new design of MAPs containing lipidated built-in adjuvant can be delivered by oral administration to elicit systemic and mucosal immunoglobulins as well as CTLs [43].

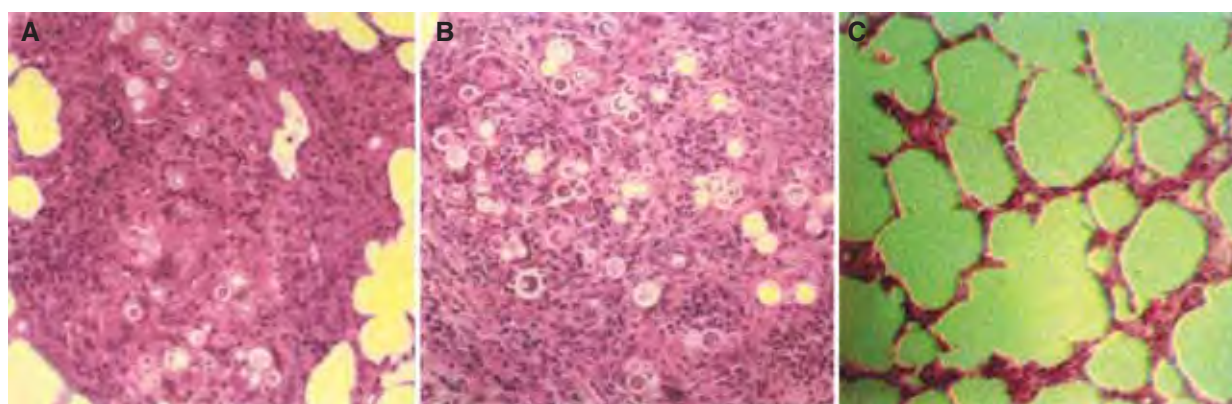


Figure 6 Protective effect of M10 immunization in intratracheally infected mice. (A) Lung section from a control MC-immunized mouse. (B) Lung section from a nonimmunized mouse. (C) Apparently resolved granuloma, containing few macrophages and no fungal cells, in a lung section of M10-immunized mouse. HE 10 $\times$.

In the *P. brasiliensis* system, we compared the immunization with P10 and M10, both injected with CFA, and there was no apparent advantage of using the MAP versus the linear peptide with respect to the observed intensity of the lymphoproliferation. On a molar basis, several experiments showed that lymphoproliferation *in vitro* with 1 µmol of MAP matched with 4–5 µmol of P10. However, M10 could be preferable to P10, if also active in the absence of CFA.

We found indeed that M10 induced a significant protective immune response in mice challenged i.t. with virulent *P. brasiliensis* yeast forms, when injected only with PBS. Reduction of CFUs in the lung and spleen were significant after 1 month of infection, and these results were similar after 3 months, in contrast with the control group. It is remarkable that a single dose of M10 was able to reduce both the CFUs in the lung and spleen.

Apart from the difficulties in its synthesis, M10 is a candidate to be used in human immunotherapy, because with P10, it does not induce a humoral response and the T-cell epitope in the 13-mer peptides included in the construct is promiscuously presented by several mouse and human major histocompatibility complex class II haplotypes [12]. MAP delivery systems already used in mice, but equally acceptable for human immunization using alum and granulocyte-macrophage colony-stimulating factor [31], could be a possibility amongst others to be investigated.

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Anexo 18

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Model Peptides Mimic the Structure and Function of the N-terminus of the Pore-Forming Toxin Sticholysin II

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Abstract: To investigate the role of the N-terminal region in the lytic mechanism of the pore-forming toxin sticholysin II (St II), we studied the conformational and functional properties of peptides encompassing the first 30 residues of the protein. Peptides containing residues 1–30 (P1–30) and 11–30 (P11–30) were synthesized and their conformational properties were examined in aqueous solution as a function of peptide concentration, pH, ionic strength, and addition of the secondary structure-inducing solvent trifluoroethanol (TFE). CD spectra showed that increasing concentration, pH, and ionic strength led to aggregation of P1–30; as a consequence, the peptide acquired β -sheet conformation. In contrast, P11–30 exhibited practically no conformational changes under the same conditions, remaining essentially structureless. Moreover, this peptide did not undergo aggregation. These differences clearly point to the modulating effect of the first 10 hydrophobic residues on the peptides aggregation and conformational properties. In TFE both the first ten hydrophobic peptides acquired α -helical conformation, albeit to a different extent, P11–30 displayed lower α -helical content. P1–30 presented a larger fraction of residues in α -helical conformation in

TFE than that found in St II's crystal structure for that portion of the protein. Since TFE mimics the membrane environment, such increase in helical content could also occur upon toxin binding to membranes and represent a step in the mechanism of pore formation. The peptides conformational properties correlated well with their functional behavior. Thus, P1–30 exhibited much higher hemolytic activity than P11–30. In addition, P11–30 was able to block the toxin's hemolytic activity. The size of pores formed in red blood cells by P1–30 was estimated by measuring the permeability to PEGs of different molecular mass. The pore radius (0.95 ± 0.01 nm) was very similar to that of the pore formed by the toxin. The results demonstrate that the synthetic peptide P1–30 is a good model of St II conformation and function and emphasize the contribution of the toxin's N-terminal region, and, in particular, the hydrophobic residues 1–10 to pore formation. © 2005 Wiley Periodicals, Inc. *Biopolymers* 84: 169–180, 2006

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Keywords: Sticholysin II; actinoporin; pore-forming toxin; hemolytic peptide; circular dichroism

INTRODUCTION

Sticholysin II (St II) is the most hemolytic isoform of the pore-forming proteins (PFP) produced by the sea anemone *Stichodactyla helianthus*.¹ St II forms cation-selective hydrophilic pores² both in natural and model lipid membranes of around 1 nm hydrodynamic radius.³ Despite the extensive work attempting to clarify how this water-soluble protein inserts, oligomerizes, and eventually disrupts target membranes, little is known about the actual amino acid sequence(s) involved in each event.^{2–7} St II belongs to the actinoporin family, a unique class of eukaryotic PFP exclusively found in sea anemones. They are cysteineless proteins, with M_r around 20 kDa, and a preference for sphingomyelin (SM)-containing membranes.^{2,5,8}

The structures of the soluble state of equinatoxin II (EqT II), a highly homologous actinoporin (66% identity and higher than 80% similarity) purified from the sea anemone *Actinia equina*,⁹ and St II¹⁰ were recently solved by X-ray crystallography. The solution structure of EqT II was also solved by NMR.¹¹ Both toxins contain a hydrophobic β -sandwich core, flanked on the opposite sides by two α -helices. In St II, the two α -helices comprise residues 14–23 and 128–135.¹⁰ The first thirty N-terminal residues, which include one of the helices, are thought to be the best candidates for pore formation. This region contains an amphipathic stretch, well conserved in all actinoporins, clearly similar to some membrane-interacting peptides such as melittin and fusogenic viral peptides,¹² and is the only portion of the molecule that can change conformation without perturbing the general protein fold.^{9,10} Furthermore, it has been demonstrated that a flexible N-terminal region and a stable β -sandwich are prerequisites for proper pore formation by EqT II.¹³

Recently, a cysteine-scanning mutagenesis of the region encompassing residues 10–28 of EqT II suggested that this portion adopts α -helical conformation in the membrane and that the α -helix is longer than in the soluble form. In the membrane-bound state this portion lays on the water–lipid interface and it inserts into the membrane to line the pore interior forming an angle of around 20° with the bilayer normal.¹⁴ A small increase in helicity of sticholysins occurs upon binding to lipid membranes, as found by FTIR spectroscopy.¹⁵ However, a more definitive picture of this mechanism would require the determination of the structure of the oligomerized toxins in membranes. A model for St II pore formation was proposed based on low resolution electron microscopy data of the lipid-bound toxin.¹⁰

The study of actinoporins is important not only to understand their envenoming properties, but also, in a more general sense, to investigate basic mechanisms of lipid–protein interaction, as well as the sequences responsible for their biological activity. Furthermore, these toxins—or their functionally relevant amino acid sequences—could also be potential tools for the construction of immunoconjugates addressed to cancer or other undesirable cells. In fact, they have already been used for the construction of antitumoral and antiparasite chimeric molecules.^{16–21} The understanding of the basic molecular mechanisms involved in toxin–cell interaction is essential for the rational design of these immunotoxins.

To clarify the role of St II's N-terminal sequence to protein function we have synthesized and conducted conformational and functional studies of two peptides containing residues 1–30 (P1–30) and 11–30 (P11–30) of the toxin. The peptides conformational properties were examined in aqueous solution by circu-

lar dichroism (CD) as a function of peptide concentration, pH, ionic strength, and addition of the secondary structure-inducing solvent trifluoroethanol (TFE). The peptides' functional properties were assessed by means of their hemolytic activity (HA). The results demonstrate that the hydrophobic N-terminal sequence (residues 1–10) plays an important role in the hemolytic activity and that the synthetic peptide P1–30 is a good model of both St II structure and function.

MATERIALS AND METHODS

Materials

All natural 9-fluorenylmethyloxycarbonyl (Fmoc) amino acids and Rink-amide MBHAR resin were purchased from Advanced Chemtech (Louisville, KY, USA) and Novabiochem (San Diego, CA, USA). Solvents and reagents were from Sigma–Aldrich Co (St. Louis, MO, USA) and Fluka (Switzerland). Polyethylene glycol was obtained from Fluka (Buchs, Switzerland). Sticholysin II was purified from the sea anemone *S. helianthus* according to Lanio et al.¹

Methods

Peptide Synthesis. The peptides (with amidated C-terminus) were synthesized manually according to the standard N^α-Fmoc protecting-group strategy.²² The following side chain protecting groups were used: Boc (*t*-butoxycarbonyl) for K; *t*But (*t*-butyl) for D, S, T, and E; and Pmc (2,2,5,7,8-pentamethyl-chromane-6-sulfonyl) for R. After the coupling of the C-terminal amino acid to 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-(4-methylbenzhydramine) resin (Rink-amide-MBHAR), the successive α -amino group deprotection and neutralization steps were performed in 20% piperidine/dimethylformamide (DMF) for 20 min. The amino acids were coupled at threefold excess using diisopropylcarbodiimide/*N*-hydroxybenzotriazole (HOBt) in DMF and, if necessary, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexafluoro-phosphate/HOBt/diisopropylethylamine in 20% dimethyl sulfoxide (DMSO)/*N*-methylpiperidinone. After a 2-h coupling time, the ninhydrin test was performed to estimate the completeness of the reaction. Cleavage from the resin and removal of the side chain protecting groups were simultaneously performed with Reagent K (82.5% trifluoroacetic acid (TFA), 5% thioanisole, 2.5% ethanedithiol, and 5% water) over 2 h. In this procedure, the crude peptides were precipitated with anhydrous ethyl ether, separated from soluble nonpeptide material by centrifugation, extracted into 5% acetic acid in water, and lyophilized. The peptides, after dissolution in 0.045% TFA/H₂O (solvent A), were purified by semipreparative HPLC on a Shimadzu system (Tokyo, Japan) using a reverse phase C₁₈ column with a linear gradient 20–70% solvent B (0.036% TFA/acetonitrile) over 150 min. The flow rate was 5 mL/min. UV detection was

carried out at 220 nm. The peptides' homogeneity was checked by analytical HPLC (Varian, Walnut Creek, CA, USA), using solvents A and B with a linear gradient of 5–95% solvent B for 30 min, at a flow rate of 1.5 mL/min and UV detection at 220 nm. The identity of the peptides was confirmed by electrospray mass spectrometry on a ZMD model apparatus (Micromass, Manchester, UK) and amino acid analysis (Shimadzu model LC-10A/C-47A, Tokyo, Japan).

CD Studies. Circular dichroism spectra were obtained in 0.5 mm path length cuvettes, at room temperature (22 ± 2 °C) using a Jobin Yvon CD6 spectropolarimeter (Longjumeau, France). The instrument was routinely calibrated with an aqueous solution of recrystallized D-10-camphorsulfonic acid. Data are expressed as mean residue molar ellipticity, $[\theta]$ (in deg · cm² · dmol⁻¹). pH titration was performed using 5 mM phosphate–borate–citrate (PBC) buffer. The pH was adjusted by adding small amounts of NaOH. Variable ionic strength experiments were performed at pH 4.0; the ionic strength was adjusted by adding increasing amounts of NaCl. As for variable TFE concentration, samples were prepared by mixing appropriate amounts of two peptide stock solutions, one in 100% TFE and the other in aqueous solution, pH 4.0.

Hemolytic Activity. HA was evaluated turbidimetrically at 600 nm at room temperature (22 ± 2 °C) in a Labsystems microplate reader (Helsinki, Finland) as previously described.⁴ Erythrocyte suspensions were prepared using pooled fresh human red blood cells (RBC), washed, and resuspended in physiological Tris-buffered saline (TBS, 145 mM NaCl, 10 mM Tris–HCl, pH 7.4). The RBC suspensions were diluted to an absorbance of 0.1 at 600 nm. Peptide samples were twofold serially diluted in saline buffer in a flat-bottom 96-well microplate, rendering peptide concentrations ranging from 0.075 to 110 μ M. The reaction was started by adding the same volume of erythrocyte suspension to each well (200 μ L final volume) and the decrease in absorbance was recorded as a function of time with intermittent shaking. The loss of turbidity was quantitatively related to the toxin HA.⁴ In some cases, the HA was quantitatively expressed by HC₅₀, the toxin concentration needed to elicit 50% disruption of the RBC ensemble.²³ Peptides were dissolved in DMSO (0.5%, vol:vol) to improve their solubility, in particular that of P1–30.

Inhibition of St II HA by P11–30. Inhibition of St II HA by sublytic P11–30 concentrations was assayed by preincubating the cell suspension with a fixed peptide concentration for 30 min at 25 °C. Hemolysis was started by adding the cell suspension to wells with two different St II concentrations to attain 1.1 and 2.6×10^5 peptide/St II molar ratios.

Pore Size Determination. Pore size was determined in RBC prepared as described above. Briefly, in each well, a fixed concentration (20 or 160 μ M) of P1–30 was present, in a final volume of 100 μ L of TBS, with or without 60 mM of a polyethylene glycol series: PEG200, PEG400, PEG600, PEG900, PEG1000, and PEG1500.³ The reaction was

started by adding 100 μ L of RBC, at a titer corresponding to an initial absorbance at 600 nm of around 0.1. The microplate was stirred and read every 10 s during 45 min. Addition of large osmoticants increased the half-time of P1–30-induced hemolysis ($t_{1/2}$), in a size-dependent manner. The difference $t_{1/2} - t_{1/2}^{\circ}$, the half-times in the presence and absence of osmotic protectants, respectively, was used as an estimate of the introduced delay; this parameter measures the time necessary for the osmolite to diffuse inside the cell through the toxin-induced lesions. Accordingly, $1/(t_{1/2} - t_{1/2}^{\circ})$ is an estimate of the permeability of the PEGs through the pore, which can be used to build a Renkin plot^{24,25} reporting the relative permeability of the molecule versus its size. This measurement allows the estimation of the size of the lesion originated by P1–30 in erythrocytes.³

RESULTS AND DISCUSSION

Conformational Properties of St II's N-terminal Peptides: CD Studies

St II is the most potent cytolysin produced by the sea anemone *S. helianthus* exerting hemolytic activity via pore formation in the membrane.³ It is likely that the N-terminal sequence plays an important role in this event by means of the interaction with the target cell membrane. This is suggested by the presence of hydrophobic (residues 1–10) and highly amphipathic (residues 14–35) sequences in the St II N-terminus^{26,27} and is in agreement with evidence collected for homologous EqT II.¹⁴ The toxin N-terminal region would be responsible for insertion, destabilization of the target membrane, and organization of the pore. To investigate the role of the N-terminal region of St II in the mechanism of pore formation, two peptides from the N-terminal sequence of St II were synthesized in an attempt to model the corresponding regions in the whole protein. An analogous experimental approach has been used to characterize the contribution of different regions of another hemolysin, the lectin Cel-III.²⁸ We also used this approach to study another family of membrane-bound proteins, G protein-coupled receptors (GPCR), as well as the α subunit of the G protein. We found that fragments of GPCR corresponding to extramembranous loops tend to display conformational propensities similar to those found or calculated for the same sequences in the whole protein.^{29–34} Similar results were obtained for the C-terminus of the G protein α subunit.³⁵

The 1–30 sequence of St II is ALAGTHIAGASLTFQVLVDKVLLEELGKVSRLK.²⁶ The peptides used in this study contained either residues 1–30 (P1–30) or residues 11–30 (P11–30). While the first ten residues correspond to an essentially hydrophobic stretch,²⁷ residues 14–23 correspond to the toxin's N-

terminal α -helix, as determined by X-ray crystallography.¹⁰ This helix is of amphipathic nature, as calculated using the algorithm of Eisenberg et al.^{36,37} (results not shown).

Making use of the algorithm of Rost and Sander,³⁸ theoretical predictions (Figure 1A and B) indicate that both peptides display a high propensity to adopt an α -helical conformation between residues 14 and 24 (of the protein), in excellent agreement with the X-ray data, which show that the amphipathic α -helix occurs in the F¹⁴–E²³ stretch.¹⁰ Moreover, in P1–30 (Figure 1A), amino acids 11–13 and 24 also display a considerable propensity to acquire α -helical conformation. Helical propensity of a similar magnitude (> 60%) is also predicted for residues 25–30 (Figure 1A). Due to end effects, the absence of the first ten residues in P11–30 leads to a decrease of the average propensity of residues 11–30 to acquire α -helical conformation. Thus, while the average α -helical content of these residues in P1–30 is 92.5%, this value decreases to 68.3% in P11–30. This analysis shows that the first ten residues influence the ability of the subsequent residues to form an α -helix. The helical wheel projection³⁹ in Figure 1C corroborates the α -helix amphipathic nature: while D¹⁸, K¹⁹, E²², E²³, and K²⁶ form the polar face, F¹⁴, V¹⁶, L¹⁷, V²⁰, L²¹, and L²⁴ give rise to the helix apolar face.

At pH 7.0, the CD spectrum of P1–30 in aqueous solution (Figure 2A) presents a positive band centered at 195 nm, a negative band at 220 nm, a shoulder at ca. 208 nm, and a crossover point at 205 nm, indicating that P1–30 adopts a β -sheet conformation, very likely resulting from peptide aggregation. This process is probably favored by the highly hydrophobic 1–10 sequence. β -Sheet peptides usually give rise to CD spectra displaying a positive band between 195 and 200 nm, characteristic of the amide $\pi \rightarrow \pi^*$ transition, and a negative band around 216 nm due to the amide $n \rightarrow \pi^*$ transition.⁴⁰ In addition, a crossover point between 204 and 207 nm is usually observed.⁴¹ On the other hand, the CD spectra of P11–30 present two negative bands, one more intense centered at 202 nm and another at 225 nm, showing that this fragment preferentially adopts a flexible conformation under these experimental conditions. The negative band around 225 nm suggests that the peptide is partially folded.⁴⁰

The X-ray crystallographic structure of St II's soluble monomer¹⁰ revealed a small β -strand in the N-terminal region between residues I⁶ and A⁸, followed by an α -helix extending from F¹⁴ to E²³. The absence of this region in P11–30 is probably responsible for its enhanced aqueous solubility. For this reason, we investigated the effect of concentration on

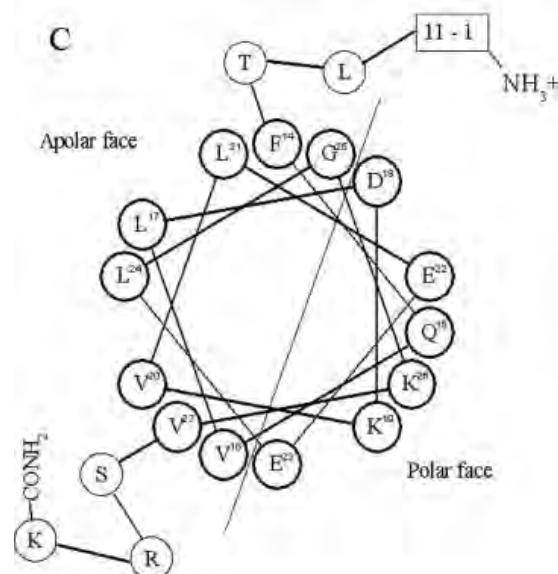
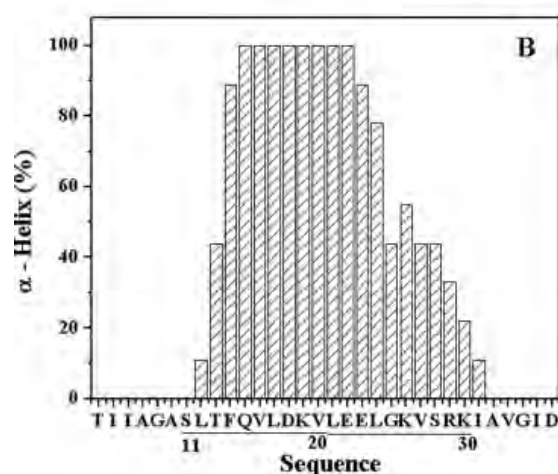
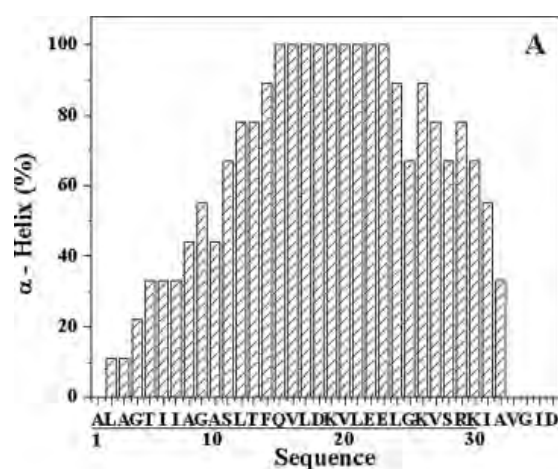


FIGURE 1

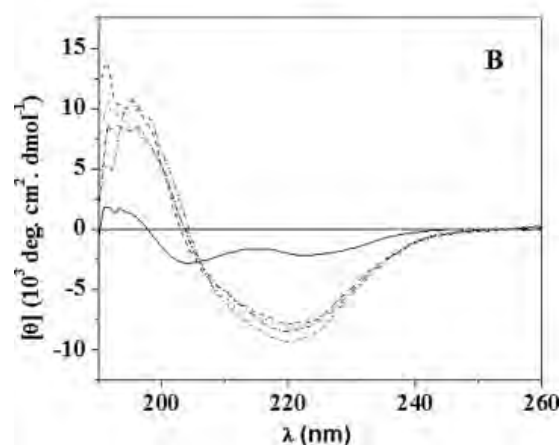
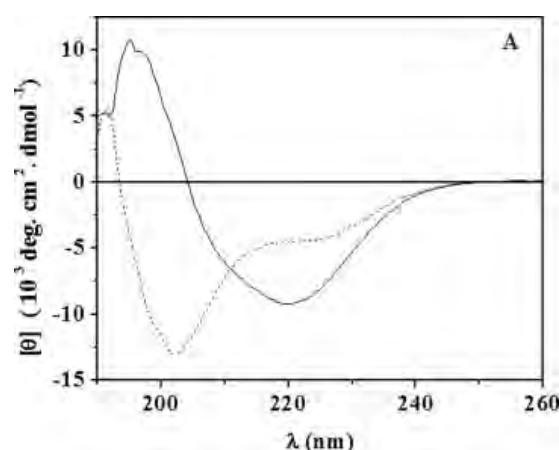


FIGURE 2 (A) Far-UV CD spectra of P1-30 (—) and P11-30 (---) in aqueous solution, pH 7.0. Peptide concentration: 80 μ M. (B) CD spectra of P1-30 as a function of peptide concentration (μ M): 5 (—); 20 (---); 40 (·····); 60 (-----); 80 (·····).

the peptides conformation. The CD spectra of P11-30 remained practically unaltered in the whole concentration range studied (5–80 μ M, data not shown), corroborating the notion that this peptide does not have a tendency to aggregate. On the other hand, P1-30 displays a β -sheet conformation between 20 and 80 μ M, as a consequence of peptide aggregation (Figure 2B). For 5 μ M peptide, the lowest concentration assayed, a spectrum suggestive of α -helical conformation was obtained (Figure 2B); however, the noise prevented a more detailed spectral analysis. It is con-

FIGURE 1 Secondary structure prediction according to Rost and Sander³⁸ for P1-30 (A) and P11-30 (B), and helical wheel diagram for residues 1–30 (C) according to Schiffer and Edmundson.³⁹

ceivable that under physiological conditions aggregation might play an important role in the mechanism of actinoporins pore formation. It has been proposed that the N-terminal segment of actinoporins plays a relevant role in pore formation, an event that would be preceded by protein oligomerization.^{14,42}

To gain insight into the nature of the forces involved in peptide folding and aggregation, we also studied the effect of pH on peptide conformation. Both peptides share the same ionizable groups: the terminal amino group, D¹⁸, K¹⁹, E²², E²³, K²⁶, R²⁹, and K³⁰ (the C-terminus is amidated); however, only P1–30 exhibited pH-dependent conformational changes (Figure 3A). At pH 4.0 and below, the CD spectra of P1–30 display two negative bands, one at 202 nm and another at 225 nm, suggesting the contribution of two populations, one extended and the other partially folded.⁴⁰ As the pH is increased to 6.0, despite the propensity of P1–30 to acquire α -helical conformation (Figure 1A), the CD spectrum changes to that of a β -sheet, displaying a positive band centered at 198 nm, a negative band at 221 nm, and a shoulder at ca. 208 nm. No further conformational changes were observed with increasing pH; above pH 8.0, flattening of the positive band occurred, which might be related to light scattering due to peptide aggregation. In fact, at alkaline pH, particles could be visually detected in suspension, evincing aggregation. The observed conformational change is probably induced by changes in the extent of dissociation of ionizable groups, modifying the charge balance (see below). However, despite the fact that P1–30 and P11–30 have the same ionizable groups, the latter peptide displayed a disordered conformation throughout the pH range studied (data not shown). The different spectral behavior of both peptides is most likely due to the N-terminal hydrophobic segment. Thus, changes in the ionization degree and the presence of the 1–10 hydrophobic stretch, which acts as a nucleation site, lead to P1–30 aggregation, giving rise to the β -sheet features in the peptide's CD spectra at pH 6.0 and above.

Figure 3B shows the effect of pH on the mean molar residue ellipticity at 222 nm ($[\theta]_{222}$) and on the peptides formal charge calculated based on the pK_a s of the individual ionizable groups. This calculation does not take into account possible changes in pK_a due to charge effects of neighboring residues.³⁴ For P1–30, the greatest changes in $[\theta]_{222}$ occur in the pH range between 3.0 and 6.0. The titration curve shows a midpoint at ca. pH 4.5. A smaller change is seen between pH 8 and 11, with a midpoint at ca. pH 9.3. The increase in $[\theta]_{222}$ in this region is probably due to flattening of the spectrum resulting from peptide aggrega-

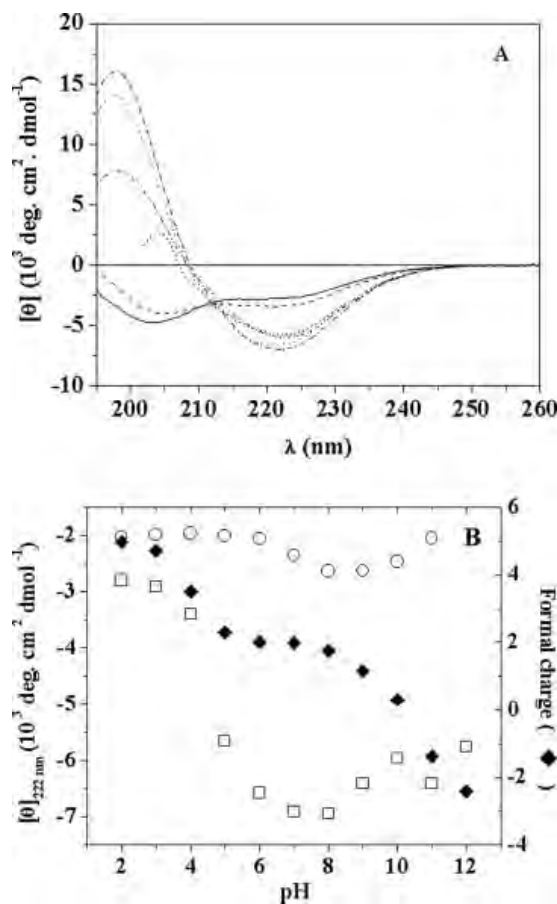


FIGURE 3 (A) CD spectra of 80 μ M P1–30 in aqueous solution as a function of pH. pH: 2 (—); 4 (---); 6 (·····); 8 (— · — ·); 10 (— · — · — ·); 12 (----). (B) Formal charge (◆) and mean molar residue ellipticity at 222 nm ($[\theta]_{222}$) in the CD spectra of P1–30 (□) and P11–30 (○) as a function of pH.

tion, which causes light scattering. It is noteworthy to analyze the occurrence of the midpoints in connection with the formal charge versus pH curve. At pH ca. 4.5 and 9.3, the peptide's formal charge would be approximately +2.5 and +1, respectively. On the other hand, the formal charge curve indicates the peptide would bear a net charge of zero at a pH close to 10. The fact that extensive aggregation is detected at pH 8 suggests that pK shifts occurred due to the mutual influence of the charged residues on each other's ionization and that, very likely, the zero net charge was reached at a pH lower than that predicted using the pK_a s of the individual amino acids. In contrast with the above results, in the case of P11–30, $[\theta]_{222}$ remained practically unaltered between pH 2.0 and 6.0; a small decrease of molar ellipticity was observed between pH

6 and 8, followed by a slight increase between pH 8 and 11. The differences between P1–30 and P11–30 again point to the role of residues 1–10 in the conformational behavior of both peptides.

Studies at varying ionic strength corroborated the effects found with variable pH. The CD spectrum of P1–30 at pH 4.0 continually changed with increasing ionic strength from one of a random conformation in the absence of salt to that of a predominantly β -sheet structure at 100 mM NaCl. Concomitantly, $\lambda_{\min}$ at 201 nm shifted to higher wavelengths (Figure 4A). A second minimum around 224 nm is observed at 10 mM NaCl; as the salt concentration increases, this minimum is blue shifted to 220 nm. At pH 4.0 the peptides are positively charged and, therefore, charge repulsion prevents P1–30 from acquiring a higher content of secondary structure; as the ionic strength increases, charge screening occurs, enabling the peptide to aggregate and fold into a β -sheet conformation. Interestingly, the spectra show that the aggregation process takes place stepwise and that increasing the ionic strength has a less pronounced effect than increasing the pH. On the other hand, P11–30 displayed minimal changes of $[\theta]_{222}$, evincing the retention of an extended conformation (Figure 4B). The differences in the CD spectra of P1–30 and P11–30 when obtained at the same pH or same ionic strength clearly point to the modulating effect of the first ten hydrophobic amino acids on the peptide conformational properties.

We also examined the effect of TFE, a well-known secondary structure inducer,⁴³ on the conformation of St II peptides. Figure 5 shows the CD spectra of P1–30 as a function of TFE. In aqueous solution at pH 4.0, the peptide adopts an extended conformation, as seen by the negative band around 200 nm. The peptide acquires increasing α -helical conformation with increasing TFE, as indicated by the gradual decrease of $[\theta]_{222}$ up to 40% TFE.⁴⁰ The inset in Figure 5 shows the α -helical content of both P1–30 and P11–30 as a function of TFE concentration, calculated according to Rohl and Baldwin.⁴⁴ It is seen that the secondary structure content of both peptides increased up to around 40% TFE, remaining constant thereafter. Moreover, P1–30 presents a higher helical content, corroborating theoretical predictions (Figure 1). According to the inset in Figure 5, at 100% TFE P1–30 has around 60% α -helical content, which corresponds to about 18 residues.

We have previously shown that binding of St II to model membranes results in small changes in the toxin's secondary structure.⁷ Moreover, FTIR data indicated a 5% increase in St II α -helical content upon lipid binding.¹⁵ Such an increase corresponds to

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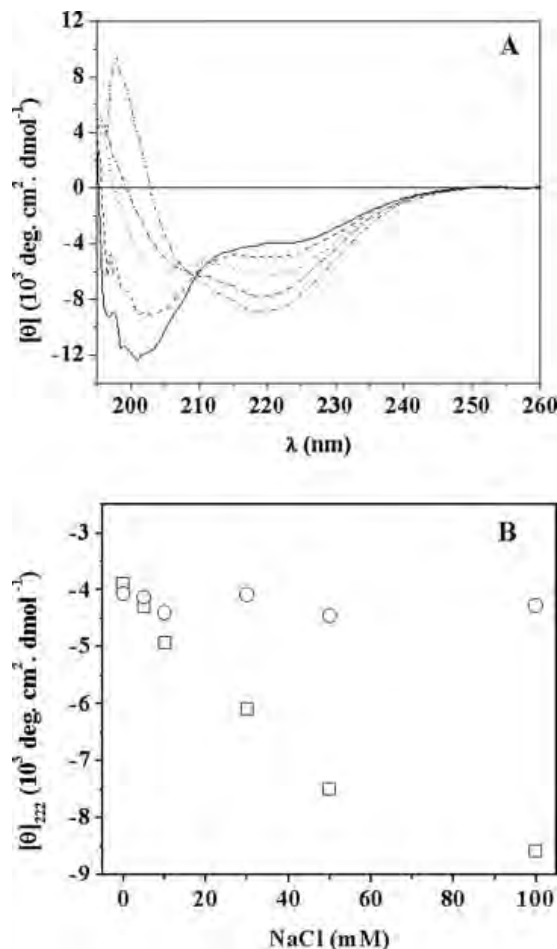


FIGURE 4 (A) Effect of ionic strength on CD spectra of P1–30 in aqueous solution, pH 4.0. [NaCl] (mM): 0 (—); 10 (---); 30 (·····); 50 (— · —); 100 (— · — · —). (B) Mean molar residue ellipticity at 222 nm ($[\theta]_{222}$) as a function of ionic strength for P1–30 ($\square$) and P11–30 ($\circ$). Peptide concentration: 80 μ M.

approximately 9 residues (the protein has 175 amino acids). We suggest that the ca. 18 residues of P1–30 found in α -helical conformation in TFE consist of the F¹⁴–E²³ stretch found in the toxin's X-ray structure plus 8 additional residues. The Rost and Sander plot³⁸ of Figure 1A shows that residues 11–13 and 24 also display a considerable propensity to acquire α -helical conformation. Moreover, it has been shown that amino acids such as G, I, and V that do not display a propensity to α -helical conformation in aqueous solution, do so in a bilayer or membrane mimetic environment such as TFE.⁴⁵ Therefore, the hydrophobic sequence in the N-terminal region enhances the propensity of P1–30 to adopt α -helical conformation. On

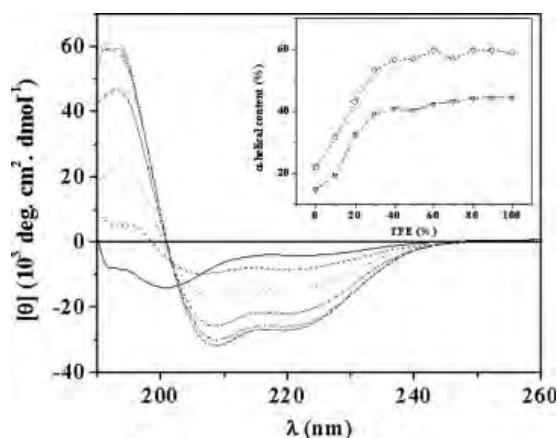


FIGURE 5 Effect of TFE on P1–30 CD spectra. TFE (%; v/v): 0 (—); 10 (---); 20 (.....); 30 (-----); 40 (.....); 80 (-----); 100 (.....). (Inset) α -Helical content (%) as a function of TFE, calculated according to Rohl and Baldwin.⁴⁴ Peptide concentration: 80 μ M. P1–30 (○) and P11–30 (▽).

the other hand, P11–30 acquires approximately 45% α -helical content in TFE, indicating that around 9 residues are involved in the α -helical structure. The lack of the first ten residues in P11–30 could account for its significantly lower α -helical content, evincing the importance of the N-terminus for helix stabilization.

The increased length of the helix in the membrane mimetic environment compared to that in the crystal structure of the monomeric toxin (18 versus 10 residues, respectively) could represent a step in the mechanism of pore formation. It has been proposed that, upon binding to the membrane, the actinoporins N-terminus detaches from the protein surface and the amphipathic α -helix lays flat on the membrane surface.¹⁴ A subsequent event would be the insertion of the 1–10 hydrophobic stretch in the bilayer accompanied by a membrane-induced increase of α -helical content of this stretch. Oligomerization of this structure would finally give rise to the functional pore. It should be noticed that helix formation by residues downstream to L²⁴ would keep the amphipathic nature of the helix (Figure 1C). At this point we cannot define whether residues from the C-terminal portion also participate in the lengthening of the helix. It is also worthwhile noticing that residues 4–8 contain the GXXXA motif, which is similar to the GXXXG motif present in the transmembrane helix of glycophorin A and other proteins, considered to be important for protein dimerization.^{46,47} Although it has been found that the G to A mutation exerts an unfavorable effect in the energetics of glycophorin A dimer-

ization, the mutated sequence is also considered to be a dimerization motif.⁴⁸ It is conceivable that this motif also plays a role in P1–30 aggregation and in peptide and toxin oligomerization during pore formation.

Hemolytic Activity of St II N-terminal Peptides

Native St II exhibits hemolytic activity in the picomolar concentration range.⁴ Hence, we decided to test the ability of the peptides to promote human red

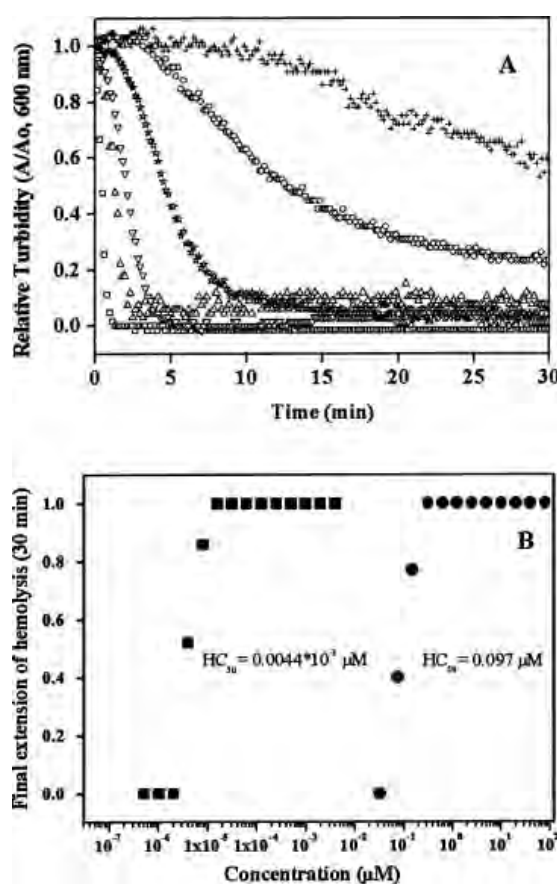


FIGURE 6 (A) Time course of P1–30–induced hemolysis of human RBC. The time course of hemolysis was followed by the decrease in turbidity of a cell suspension initially adjusted to an apparent absorbance of 0.1 at 600 nm. The peptide dissolved in 0.5% DMSO was added at concentrations (μ M): 0.075 (+), 0.15 (○), 0.3 (☆), 0.6 (▽), 1.2 (△), 5.0 (□). Hemolysis was started by adding the RBC suspension. (B) Comparison between the hemolytic activity of St II (■) and P1–30 (●). HA was estimated from the extent of hemolysis after 30 min and plotted as a function of St II or P1–30 concentration.

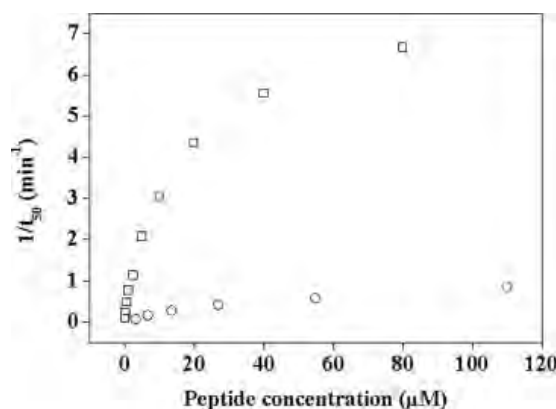


FIGURE 7 Rates of P1-30 and P11-30-induced hemolysis. Hemolysis rates were estimated from plots of the inverse of t_{50} (min^{-1}), the time necessary to lyse 50% of the RBC ensemble in the assay) versus peptide concentration. P1-30 (□); P11-30 (○).

blood cell lysis. As previously shown, since P1-30 has a strong tendency to aggregate in aqueous solution (Figure 2B), we attempted to overcome this problem by dissolving the peptides in a solution containing DMSO so that the solvent final concentration in the HA assay was 0.25%. This DMSO concentration neither affected the apparent red blood cell stability nor the activity of St II (data not shown).

Figure 6A shows the time course of P1-30-elicited hemolysis. The rate of cell integrity loss and the extent of lysis were dose dependent. The peptide effect occurred in the concentration range between 0.075 and 2 μM , while that of the toxin is in the nanomolar range (0.001–0.04 nM); therefore St II is ca. 10^5 -fold more active than P1-30 (Figure 6B).

The fact that P1-30 exhibits HA, albeit in a higher concentration range, reinforces the notion that this peptide can mimic not only the conformational, but also the functional behavior of St II. Differences in the order of the HA very likely arise from the absence in P1-30 of the exposed aromatic cluster in actinoporins^{9,10} that mediates the initial attachment to membranes, considered to be the first step of the pore-forming mechanism.^{14,42} In fact, steric shielding of the aromatic cluster or mutation of W¹¹² and W¹¹⁶ to F significantly reduced the EqT II–lipid interaction.⁴²

The rate of P1-30 and P11-30-elicited hemolysis was expressed as $1/t_{50}$ versus peptide concentration, where t_{50} is the time necessary to attain 50% cell rupture. This parameter was obtained from hemolysis time courses such as those in Figure 6A. Both peptides displayed concentration-dependent HA but to dif-

ferent degrees. The hemolysis rate of P1-30 was around sevenfold that of P11-30 (Figure 7).

It has been proposed that the second step of EqT II binding to membranes involves the N-terminal helix, which translocates into the lipid phase, thus creating the lining of the transmembrane pore.¹⁴ In a study of the role of EqT II N-terminal sequence by N-truncation mutagenesis it was found that deletion of 10 ($\Delta 10$) and 33 ($\Delta 33$) residues decreased HA with increasing truncation.⁴⁹ The deletion of the first 33 residues produced a mutant completely devoid of HA whereas the activity of EqT II $\Delta 10$ was reduced to 31% that of the wild-type protein. Interestingly, P1-30, which corresponds to the analogous truncated sequence in EqT II $\Delta 33$, showed much higher HA compared to P11-30 (Figure 7), stressing the importance of the hydrophobic leader sequence for St II-promoted hemolysis.

To obtain insight into the molecular mechanism underlying the differential HA of St II peptides, blocking experiments were performed by preincubating RBC with P11-30 at sublytic concentrations. St II was added to attain two different peptide:toxin molar ratios. P11-30 was able to partially block St II HA in a dose-dependent manner, with maximal inhibition being found at a 2.6×10^5 peptide:St II molar ratio (Figure 8). When the inhibition was calculated comparing t_{15} (the time necessary to produce a 15% decrease of the RBC suspension absorbance) in the presence and absence of the peptide, P11-30 was

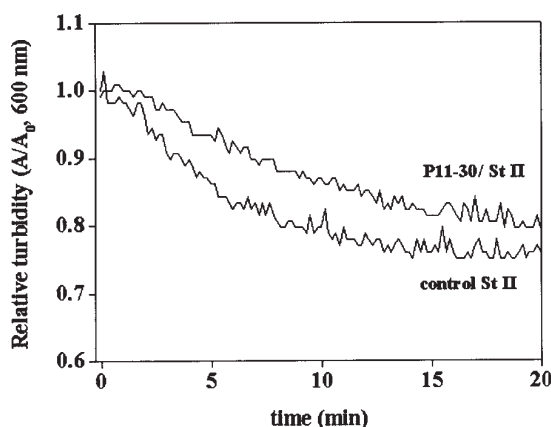


FIGURE 8 Blocking of St II HA by P11-30. The partial blocking activity of the peptide was assessed by preincubating RBC with P11-30 (30 min, 25°C); the reaction was started by adding the cell suspension and the peptide to wells containing St II. The final volume in each well was 200 μL and the apparent initial absorbance was 0.1 at 600 nm. P11-30 : St II molar ratio: 2.6×10^5 .

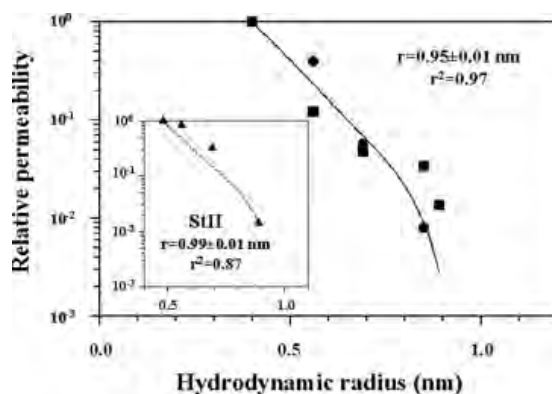


FIGURE 9 Radius of the pore formed by P1–30 in human RBC estimated by the Renkin plot. The rate of colloid osmotic lysis promoted by two P1–30 concentrations (20 and 160 μ M) was measured in the presence and absence of 30 mM final concentration of PEGs of different size. The relative permeability of each PEG was derived and the pore radius estimated with the Renkin equation.^{24,25} The solid line is the best fit of the Renkin equation for all experimental values obtained (squares and circles correspond to 20 and 160 μ M P1–30, respectively). r , hydrodynamic radius (nm); r^2 , determination coefficient.

found to inhibit the toxin HA by a factor of two. P1–30 showed HA in a lower micromolar concentration range than P11–30 (Figure 7), hence it was not possible to assay its blocking activity in the same concentration conditions. Furthermore, no synergistic effect was observed when both P1–30 and St II were present in the hemolytic assay (data not shown).

We hypothesize that the blocking activity of P11–30 could result from either the interaction of the peptide with the RBC membrane, resulting in partial saturation of the toxin binding sites, or the formation of peptide–St II heterooligomers capable of binding to the membrane but unable to form competent pores. In this case, nonfunctional P11–30/St II oligomers would form, most probably via an interaction between the peptide and St II's N-terminus. Interhelical contacts would stabilize the complex and, at the same time, shield part of the toxin residues required for pore formation. Such complexation could prevent the detachment of the St II N-terminus from the whole protein fold, not allowing the binding of this region to the membrane surface and its further insertion.

In view of the ability of P1–30 to mimic St II and to lyse RBC in a dose-dependent manner, we decided to estimate the size of the lesion in the membrane, taking advantage of the colloid–osmotic characteristics of St II-induced hemolysis. The method was essentially the same previously used to estimate the

radii of the pores formed by St I and St II,³ as well as by EqT II.⁵⁰ Briefly, we determined the rate of P1–30-promoted hemolysis in the presence and absence of 30 mM PEGs of different sizes. An estimate of each PEG relative permeability was derived from the inverse of the delay in hemolysis compared to control (absence of PEG). Permeability was found to be inversely related to PEG size. The following PEGs were used, with their hydrated radii in parentheses.⁵¹ PEG200 (0.40 nm), PEG400 (0.56 nm), PEG600 (0.69 nm), PEG900 (0.85 nm), PEG1000 (0.89 nm), and PEG1500 (1.1 nm).

Dividing the data by the permeability of a reference polymer, in this case PEG200, the values were fitted to a Renkin plot,^{24,25} which provided an estimate of the pore radius of 0.98 ± 0.02 and 0.94 ± 0.01 nm for the experimental values obtained for 20 and 160 μ M P1–30, respectively. The similarity of the values obtained for both concentrations could mean, as was demonstrated for St I and St II,³ that the lesion radius is independent of toxin concentration, and, consequently, has a fixed peptide predominant structure. Figure 9 shows the experimental values obtained in both series of experiments and the best fit of the Renkin equation for the data. This fit yielded a radius of 0.95 ± 0.01 nm, a value similar to that found for St II under the same experimental conditions (0.99 ± 0.01 nm; Figure 9, inset) and to that previously obtained for St I (0.96).³

Taken in conjunction with previously mentioned data that showed a progressive decrease of EqT II activity with increasing truncation of the N-terminal region,⁴⁹ the present results provide a clear demonstration of the role of the N-terminal hydrophobic segment and the amphipathic α -helix of actinoporins in pore formation. In particular, the results stress the contribution of residues 1–10 to St II's hemolytic activity, which is probably related to the increase in α -helical content in the membrane-bound state of its N-terminus. This molecular process is probably common to the mechanism of pore formation of actinoporins in general. This contribution is the first report of a model 3 kDa peptide that can reproduce the pore-forming activity of an actinoporin (20 kDa) and opens a new approach for the study these proteins, namely, the use of smaller molecules that mimic their function. Furthermore, the synthetic peptide P1–30 is not only a good model of St II structure and function but, due to its reduced molecular size, could also be useful as a biotechnological tool. Studies of the interaction of this peptide and properly engineered analogues with biological and model membranes should provide insight into the molecular details of the mechanism of peptide–membrane interaction and

should allow the optimization of their potential applications.

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Anexo 19

Sticholysins I and II interaction with cationic micelles promotes toxins' conformational changes and enhanced hemolytic activity

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Abstract

The effect of three cationic surfactants bearing the same polar head group and different chain length (cetyltrimethyl ammonium bromide (CTAB); tetradecyltrimethylammonium bromide (TTAB); dodecyltrimethylammonium bromide (DTAB)) on the conformation and function of the sea anemone pore-forming toxins sticholysins I and II (St I and St II) was studied by fluorescence and circular dichroism spectroscopy and evaluation of hemolytic activity (HA). Preincubation of the toxins with the longer chain surfactants CTAB and TTAB at concentrations slightly above their critical micelle concentration (CMC) leads to an enhancement of their HA. Significant increases in the fluorescence intensity with a slightly red shift in λ_{max} were observed at concentrations close to the surfactants' CMC, suggesting changes in the environment of the tryptophan residues. The changes in the fluorescence intensity are more noticeable and take place at lower surfactant concentrations for St I, irrespective of the surfactant alkyl chain length, although the differences between St I and St II increase as the surfactant alkyl chain length increases. This is evinced not only by the higher fluorescence intensity values and the lower surfactant concentrations required to reach them, but also by the higher acrylamide-quenching constant values (K_{sv}) for St I. However, the surfactant's effects on the toxins' HA were not found to be directly related to the observed changes in fluorescence intensity, as well as near- and far-UV-CD spectra. In particular, the latter spectra indicate that changes in HA and in fluorescence behavior take place without noticeable modifications in St I and St II secondary and tertiary structures. The results suggest that the interaction with the surfactants induces only subtle conformational changes in the toxins that favor the formation of lytic competent structures.

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Keywords: Sticholysins; Cationic surfactants; Hemolytic activity; Circular dichroism; Fluorescence

1. Introduction

Sticholysins I and II (St I and St II) are two closely related isotoxins purified from the Caribbean Sea anemone *Stichodactyla helianthus*. The

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high hemolytic activity (HA) of these macromolecules is strongly dependent on the characteristics of the red blood cell (RBC) membrane and the toxins' conformation (Alvarez-Valcarcel et al., 2001; Martinez et al., 2001). In previous works, we reported the effect of anionic and zwitterionic surfactants on the toxins' conformation and HA (Lanio et al., 2002, 2003). Both isoforms were found to be similarly affected by the surfactants, but the effects depended on the surfactant head group charge and concentration. In fact, negatively charged sodium dodecyl sulfate (SDS) strongly binds to the toxins, blocking their HA in the concentration range below the CMC. However, at SDS concentrations higher than the CMC, partial recovery of HA was observed for both toxins, as well as an increase in α -helical content and loss of tertiary structure (Lanio et al., 2003). On the other hand, the zwitterionic surfactant *N*-hexadecyl-*N,N*-dimethyl-3-ammonio-1-propane sulfonate did not significantly affect the toxins' HA, even at concentrations above the CMC, in spite of considerable changes in the toxins' intrinsic fluorescence and tertiary structure (Lanio et al., 2002).

Here, we report a study of the effect of cationic surfactants on St I and St II structure and function. The surfactants carry the same polar head groups and alkyl chains of different lengths: cetyltrimethylammonium bromide (CTAB), tetradecyltrimethylammonium bromide (TTAB), and dodecyltrimethylammonium bromide (DTAB). The results show that incubation of the toxins with TTAB or CTAB at their CMC, or slightly above them, enhances the toxins' HA, while essentially no effect is observed for DTAB. In addition, the toxin-surfactant interaction produces changes in the protein's intrinsic fluorescence spectra, without noticeable effect on their far- and near-UV-CD spectra.

2. Materials and methods

St I (Swiss Protein Data Bank P81662) and St II (P07845) were purified from *S. helianthus*, characterized according to Lanio et al. (2001), and stored at -20°C . Protein concentration was evaluated using absorption coefficients of 2.13 and $1.87\text{ mL mg}^{-1}\text{ cm}^{-1}$ at 280 nm for St I and St II, respectively (Lanio et al., 2001). CTAB, TTAB, DTAB, cetyltrimethylammonium chloride (CTAC), and dodecyltrimethylammonium chloride (Aldrich Chemical Co. Deisenhofen, Germany, 99% pure) were used as received. The CMCs of CTAB, TTAB,

and DTAB in the absence of salt are 0.92, 3.5, and 15 mM, respectively (Fendler and Fendler, 1975).

2.1. Hemolytic assay

HA was evaluated turbidimetrically, as described previously (Harshman and Sugg, 1985; Martinez et al., 2001). Erythrocyte suspensions were prepared using fresh human RBCs, washed and resuspended in physiological buffer (Tris-buffered saline (TBS): 145 mM NaCl, 10 mM Tris-HCl, pH 7.4). The RBC suspension was diluted to an absorbance of 0.1 at 600 nm in a 96-well microplate reader Multiskan "EX" (Labsystems, Helsinki, Finland) at 25°C . The surfactants' effect was evaluated in two types of experiments:

- (a) The proteins ($3.5\text{ }\mu\text{M}$ in TBS) were preincubated in the presence of surfactant. The concentrations employed (2 mM CTAB, 4 mM TTAB, 15 mM DTAB) were at or slightly above the surfactants' CMC. The solutions were rapidly diluted (between 20 and 100 times) and an aliquot dispensed into the flat-bottom microplate wells containing $200\text{ }\mu\text{L}$ of the RBC suspension. The decrease in absorbance was recorded as a function of time (Martinez et al., 2001; Pazos et al., 1998). Experiments without previous incubation with surfactants were carried out as controls. In order to verify the effect of the surfactants on RBC stability, assays were carried out in the absence of toxins using samples at the same surfactant concentrations as those samples containing the toxins. The final surfactant concentration was below $40\text{ }\mu\text{M}$ in all the assays.
- (b) RBCs were preincubated for 5 min with the surfactants at the concentrations employed in the hemolytic assays (below $40\text{ }\mu\text{M}$). The corresponding aliquots of the protein in buffer were added to the RBC suspension in the microplate wells.

2.2. Fluorescence spectroscopy

Fluorescence spectra were obtained in a 1681 Spex-Fluorolog (Jobin YVON-SPEX, Edison, NJ, USA) fluorescence spectrometer equipped with a thermostated cell holder. Intrinsic fluorescence emission spectra were recorded under a variety of conditions. Excitation was carried out at 295 nm to selectively irradiate tryptophan (Trp). Fluorescence

intensity was recorded as a function of surfactant concentration at 334 nm. Fluorescence quenching by acrylamide was evaluated from the decrease in fluorescence intensity as a function of acrylamide concentration in the absence or presence of surfactants at concentrations slightly above the CMC. The quenching constant, K_{SV} , was obtained from slopes of Stern–Volmer plots (Lakowicz, 1986).

2.3. Circular dichroism spectroscopy

CD spectra were recorded on a Jobin Yvon CD6 spectropolarimeter (Longjumeau, France) coupled to a multiscan computer (D&D technology) and baseline corrected by using control samples of similarly prepared solutions devoid of protein. Spectra were obtained in the far-UV range (190–250 nm) in 1 mm path length quartz cuvettes, and in the near-UV range (250–350 nm) in 5 mm path length quartz cuvettes.

3. Results

3.1. Hemolytic activity

Three types of experiments were performed to investigate the effect of surfactants on the HA of St I and St II:

- (i) Evaluation of the toxins' HA in the absence of surfactant.
- (ii) Evaluation of HA employing the toxins preincubated with surfactants at concentrations above or equal to the CMC. These solutions were diluted at least 400 times at the moment of the HA evaluation, leading to final surfactant concentrations lower than 40 μ M.
- (iii) Determination of the effect of the surfactants on RBC integrity. In this case, RBCs were preincubated with a concentration of surfactant equal to that of the HA determination in type ii experiments, and the toxins in buffer were added after the incubation period.

The results obtained in type i and type ii experiments are shown in Fig. 1 and Table 1. From the time course of hemolysis induced by the toxins and the extent of hemolysis at 300 s, the following conclusions can be drawn:

- (a) preincubation of the toxins with CTAB and TTAB significantly increased the HA of both toxins;

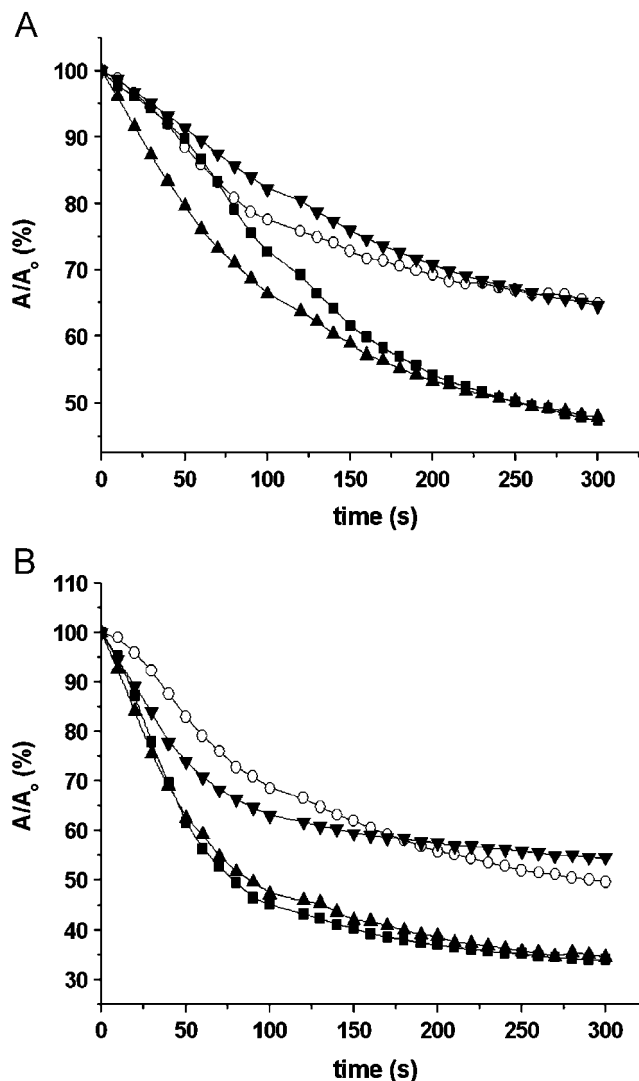


Fig. 1. Effect of St I (A) and St II (B) preincubation with surfactants on hemolytic activity. 3.5 μ M St I or St II was preincubated for 5 min with the surfactants at the following concentrations: CTAB, 2 mM, TTAB, 4 mM, DTAB, 15 mM. After appropriate dilution, 10 μ L of the preincubated toxin was added to 200 μ L of a RBC suspension in a microplate well. A/A_0 is the percentage of the initial absorbance at 600 nm remaining at time t . ($\circ$) Control; ($\blacksquare$) CTAB; ($\blacktriangle$) TTAB; ($\blacktriangledown$) DTAB. Each curve represents the mean value of three independent experiments.

- (b) preincubation with DTAB did not affect the toxins' HA;
- (c) the effect of preincubation with the surfactants was similar for both toxins.

Increased hemolysis observed in the presence of TTAB and CTAB is not due to a direct effect of the surfactants on erythrocyte membrane since incubation of RBC with surfactants did not produce any detectable hemolysis (data not shown). Furthermore, in other set of experiments (type iii),

Table 1

Effect of preincubation with surfactants on the percentage of intact RBC remaining after exposure to St I and St II for 300 s

Treatment	St I	St II
None	64.4 ± 2.6	49.0 ± 2.5
Toxin + CTAB (2 mM) ^a	46.9 ± 4.0	33.7 ± 0.8
Toxin + TTAB (4 mM) ^a	47.0 ± 3.1	33.4 ± 2.5
Toxin + DTAB (15 mM) ^a	63.9 ± 16.3	54.7 ± 4.0
RBC + CTAB ^b	74.6 ± 4.5	51.7 ± 8.5
RBC + TTAB ^b	78.7 ± 1.0	45.3 ± 2.1
RBC + DTAB ^b	65.7 ± 4.9	54.6 ± 4.9

^aPrior to HA assay, the toxin was incubated with surfactant at the indicated concentration. The solution was diluted and an aliquot (10 µL) taken to perform the HA assay for 300 s. Dilutions were adjusted to obtain comparable activities for both toxins (St I and St II final concentrations were 8.7 and 1.7 nM, respectively).

^bRBCs were preincubated for 300 s with surfactant at the same concentration as that in the HA assay: 5 and 1 µM CTAB, 10 and 2 µM TTAB, 37.5 and 7.5 µM DTAB for St I and St II, respectively. The toxins were added after the incubation.

preincubation of RBC with surfactants prior to addition of the toxins did not show any effect on St II HA; moreover, a significant protection against St I-induced hemolysis was observed for CTAB and TTAB, the longest alkyl chain surfactants (Table 1). These results imply that preincubation of RBC with relatively low surfactant concentrations (5 µM CTAB or 10 µM TTAB) renders the cell membrane less susceptible to the damage elicited by St I. In order to assess whether this effect is due to an irreversible association of the toxin with the long-chain surfactants in solution and/or modification of the cell membrane by these surfactants, the cell suspension was washed once after incubation with TTAB. Subsequently, RBCs were resuspended in TBS and exposed to St I. The results (Fig. 2) indicate that the toxin HA is intermediate between that found in untreated RBC and in unwashed RBC previously exposed to the surfactant.

It is worthwhile noting that preincubation of the RBC with surfactant does not preclude a nearly quantitative St I association with the membrane. In fact, the supernatant remaining after the hemolytic assay with the washed cells did not show any residual HA when added to a fresh RBC pellet (data not shown). The differences between the hemolytic response of RBC preincubated with low detergent concentrations and control RBC suggest that at these concentrations the surfactants could modify lipid membrane organization, precluding the ability of the toxin to form active pores. These facts suggest

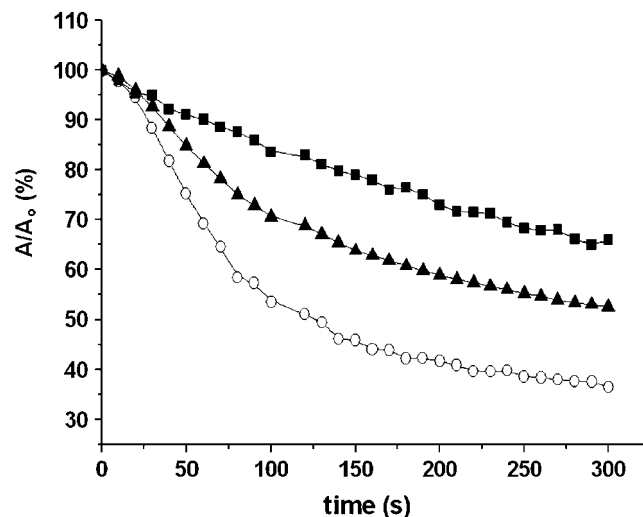


Fig. 2. Hemolytic activity of 0.3 nM St I in an RBC suspension preincubated with 10 µM TTAB. The toxin was added to the pretreated suspension after sample centrifugation and resuspension in buffer. (○) Control: toxin added to RBC (no surfactant); (▲) RBC preincubated with TTAB and washed. (■) Unwashed RBC previously exposed to TTAB. The data correspond to the average of three determinations.

that at least part of the observed protection is due to an irreversible modification of the RBC. In this regard, it has been previously reported that cationic amphiphiles protect erythrocytes against hypotonic hemolysis at a concentration of about 15% of that inducing 50% hemolysis, i.e., at very low concentrations (Isomaa et al., 1986). The relationship between amphiphile concentrations required for protection and alkyl chain length led to the proposal that membrane–aqueous phase partitioning is the mechanism whereby amphiphile monomers intercalate into the bilayer (Isomaa et al., 1986).

The absence of protection observed in St II experiments could be a consequence of the lower surfactant concentrations employed in the St II experiments (Table 1).

3.2. Fluorescence studies

Surfactant-induced toxin conformational changes were assessed by measuring the maximum emission wavelength (λ_{max}) and the intensity of the intrinsic fluorescence following excitation of tryptophan residues.

Addition of surfactant produced an initial increase in fluorescence intensity that reached a nearly constant value at high surfactant concentrations. Typical results are shown in Fig. 3. The data show that significant changes in the environment of Trp

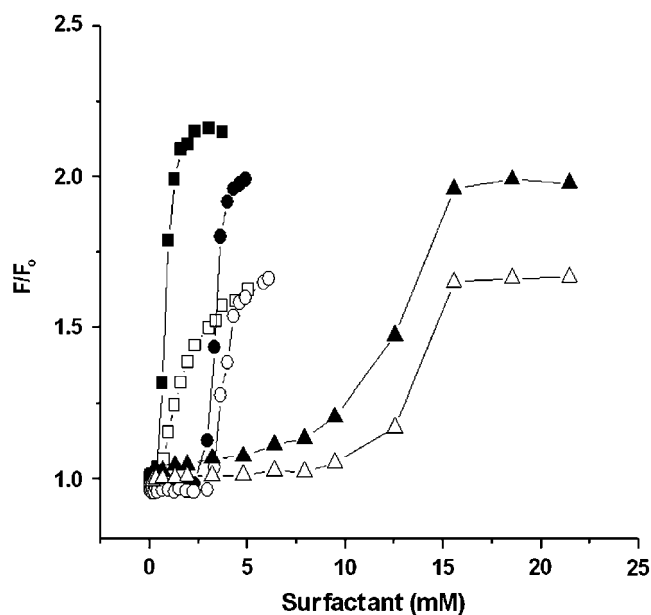


Fig. 3. Effect of surfactant concentration on the fluorescence intensity of St I and St II. CTAB: St I (■), St II (□); TTAB: St I (●), St II (○); DTAB: St I (▲), St II (△). Toxin concentration: 1 μ M. F/F_0 is the ratio of fluorescence intensities in the presence and absence of surfactant.

residues take place in a cooperative manner at concentrations below the surfactant CMC, particularly for St I. This conclusion applies irrespective of the surfactant acyl chain length. The fact that cooperative fluorescence changes appear prior to the CMC can be understood in terms of micelle formation induced by the macromolecule, as observed in other protein/surfactant systems (Santos et al., 2003). The high cooperativity could indicate that the presence of surfactant aggregates is required to produce significant changes in protein conformation. The fluorescence intensity of both proteins was not quenched by bromide ions since CTAB yielded results similar to those obtained with CTAC (data not shown). Furthermore, a moderate progressive red shift in $\lambda_{\max}$ (from 334 to 337 nm for St I and to 336 nm for St II) is seen, suggestive of more solvent-exposed Trp residues upon addition of surfactants.

In order to further characterize the exposure of Trp residues to the solvent, fluorescence quenching by acrylamide was evaluated. Fig. 4 shows that quenching of St II fluorescence is not affected by the presence of surfactants at concentrations equal to or higher than their CMC. On the other hand, quenching of St I fluorescence increases in the presence of surfactant, in particular in those with longer acyl chains. Fluorescence quenching data

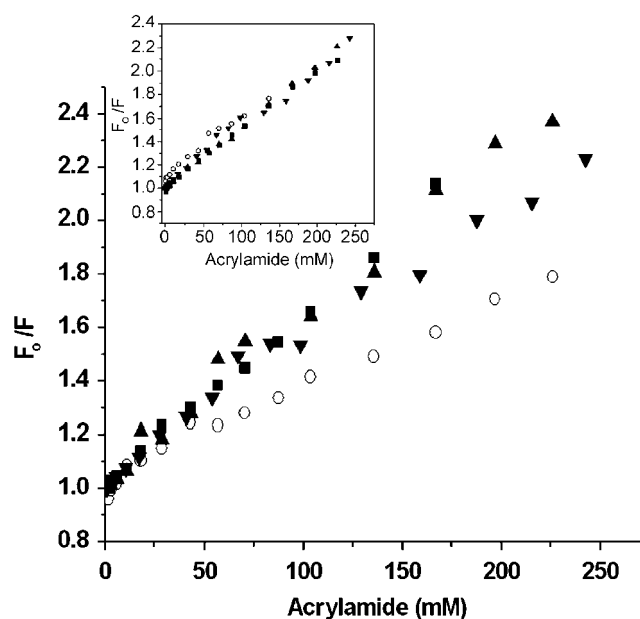


Fig. 4. Effect of surfactants on the quenching by acrylamide of St I intrinsic fluorescence. (○) Control, no surfactant; (■) 2 mM CTAB; (▲) 4 mM TTAB; (▼) 15 mM DTAB. The inset shows the results obtained with St II. Toxin concentration: 1 μ M. F_0/F is the ratio of fluorescence intensities in the absence and presence of acrylamide.

Table 2

Stern–Volmer (K_{sv}) constants obtained for acrylamide quenching of St I and St II fluorescence in the absence and presence of surfactants

Medium	Surfactant (mM)	K_{sv} (M^{-1}) St I	K_{sv} (M^{-1}) St II
Water	–	3.5	4.8
Water + CTAB	2	6.5	5.0
Water + TTAB	4	6.3	5.3
Water + DTAB	15	5.0	5.0

yielded nearly linear Stern–Volmer plots, allowing the determination of K_{sv} (Table 2) (Lakowicz, 1986).

3.3. Circular dichroism studies

In order to obtain insight into the influence of cationic surfactants and the relationship between their alkyl chain lengths on their ability to interact with and modify the toxins' conformation, CD spectra were acquired in the presence of the surfactants with the longest (CTAB) and shortest (DTAB) alkyl chains. To assess changes in the toxins' secondary structure, the spectra were obtained at concentrations below, near, and above the

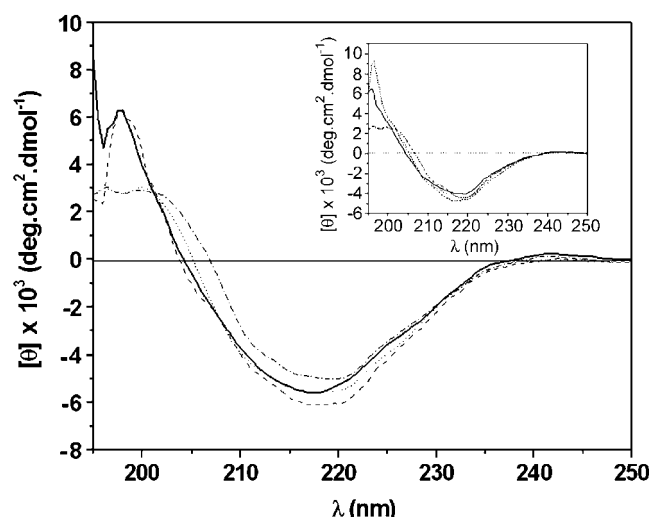


Fig. 5. Effect of CTAB on the far-UV-CD spectra of St I [CTAB], mM: 0 (—); 0.1 (----); 0.8 (· · · ·); 3.1 (---). The Inset shows the results obtained with St II. [Protein]: 3.4 μM.

surfactants' CMC. As previously reported, the far-UV-CD spectra of St II in solution show a broad negative band centered at 217 nm, indicative of a predominantly β -sheet structure (Alvarez et al., 2003; Martinez et al., 2001), in agreement with the three-dimensional structure determined by X-ray crystallography (Mancheño et al., 2003). Upon addition of detergent, the far-UV spectra of both St I and St II display small changes with an increase of negative ellipticity at 222 nm, giving rise to spectra suggestive of a tendency to acquire α -helical conformation. Fig. 5 illustrates the results obtained for CTAB. A similar behavior was observed for DTAB (data not shown). The high noise-to-signal ratio in the low-wavelength region (195–205 nm) at higher surfactant concentrations is probably related to the absorbance of bromide, since spectra using chloride as a counterion did not show this effect (data not shown). The above results are analogous to those obtained in previous studies that showed a small increase in St I and St II α -helical content, resulting from their binding to lipid bilayer systems (Alvarez et al., 2003; Menestrina et al., 1999).

To assess changes in protein tertiary structure as a result of the interaction with surfactants, near-UV CD spectra were obtained. St I and St II lack disulfide bonds (Huerta et al., 2001), thus the near-UV CD spectrum is essentially due to the contribution of aromatic residues and reflects the flexibility of their side chains and the general characteristics of the protein tertiary structure (Kelly and Price, 1997). Although a precise assignment of bands to residues from the near-UV-CD spectra is not

possible when the protein bears several aromatic groups, this approach can be useful to study changes of protein conformation under different experimental conditions (Alvarez et al., 1998, 2003; Martinez et al., 2001).

The effect of CTAB on the near-UV CD spectra of St I and St II is shown in Fig. 6. The most pronounced change is noticed in the negative peaks in the spectral region between 258 and 272 nm. A significant decrease in the intensity of these peaks takes place, especially above the detergent CMC. In the case of St II, a change in the relative intensity of the positive peaks at 277, 282, and 292 nm is also observed. Similar effects were obtained with the other detergent (data not shown). The data suggest that, upon interaction with cationic micelles, both

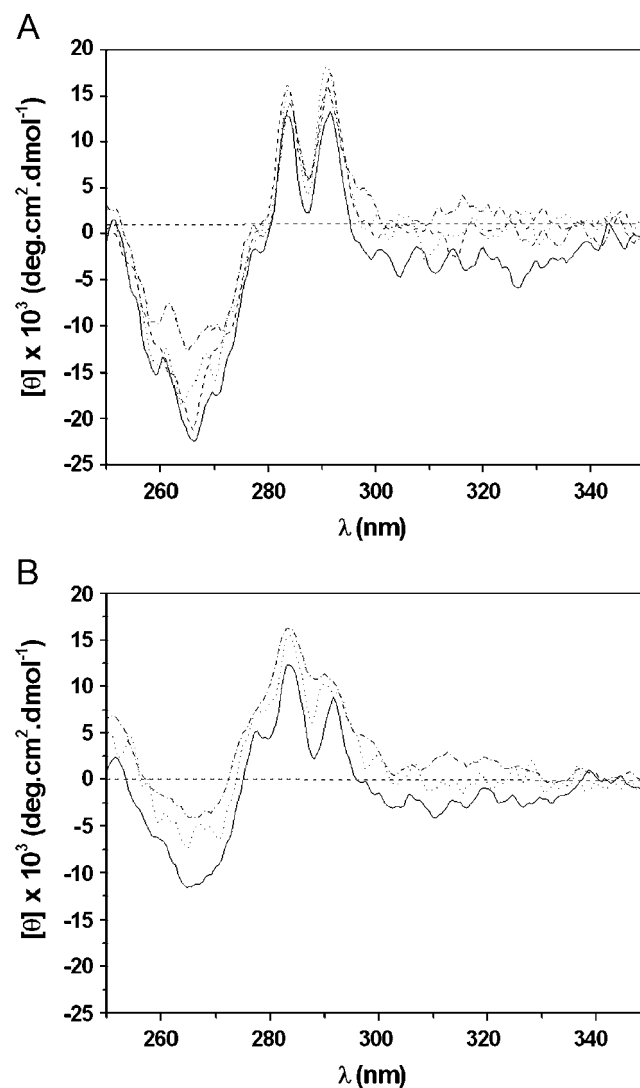


Fig. 6. Effect of CTAB on the near-UV-CD spectra of St I (A) and St II (B). [CTAB], mM: 0 (—); 0.1 (----); 0.8 (· · · ·); 3.1 (---). [Protein]: 15 μM.

St I and St II experience some modification of their aromatic residues' microenvironment without significant loss of tertiary and secondary structure.

4. Discussion

The main findings of the present work can be summarized thus:

- (i) Incubation of both St I and St II with cationic surfactants at concentrations close to their CMC produces changes in the toxins' structure that are reflected in their fluorescence and CD spectra.
- (ii) Toxins preincubated with surfactants (CTAB and TTAB), at concentrations close to their CMC, are more active than their controls in HA experiments carried out in the presence of low surfactant concentrations.
- (iii) Low CTAB and TTAB concentrations, added to the RBC ensemble prior to toxin addition, decrease St I HA.

Addition of the surfactants increases the fluorescence intensity and shifts the maximum emission fluorescence towards longer wavelengths. The data in Fig. 3 show that the effect of the surfactant is more pronounced for St I than for St II. This is evinced by the higher F/F_0 values, the lower surfactant concentrations required to reach a nearly constant F/F_0 value and the higher acrylamide-Ksv values (Table 2) for the former toxin. In particular, the differences in the latter two parameters increase when the alkyl chain length increases. This would indicate that hydrophobic interactions are more important for St I than for St II, a result compatible with the more hydrophobic character of the former toxin, as evaluated in hydrophobic binding experiments (Martinez et al., 2001).

The data shown in Table 2 agree with previous results showing that St II has a slightly more relaxed structure (Martinez et al., 2001). This less packed structure of St II could minimize the changes elicited in this protein by the interaction with the additives, rendering acrylamide-Ksv values essentially independent of the presence of surfactants. On the other hand, a more compact structure bearing more hydrophobic patches, such as that of St I (Martinez et al., 2001), should be more susceptible to the interaction with surfactants, in particular those more prone to form hydrophobic microdomains, i.e., those bearing longer alkyl chains. In addition,

the larger effect of the cationic surfactants on St I fluorescence could also be related to the greater negative charge present in the toxin's N-terminal region (Huerta et al., 2001) that would favor electrostatic interactions with the cationic surfactants. This could be particularly relevant, since this is one of the most flexible and solvent-exposed segments, as evinced by the protein's three-dimensional structure (Mancheño et al., 2003).

The red shift of protein $\lambda_{\max}$ on interaction with the detergents is suggestive of a greater solvent exposure of Trp residues. However, these results seem contradictory with the pronounced fluorescence increase elicited by the surfactants in both proteins (Fig. 3). A similar behavior has been observed in other systems (Alvarez et al., 2003; Gelamo and Tabak, 2000; Lanio et al., 2002) and has been explained in terms of a decrease in intramolecular protein quenching, associated with changes in the macromolecular conformation. This effect should be more noticeable for the more compact protein (St I), as suggested by the larger fluorescence increase observed for this isoform (Fig. 3).

Changes in the toxins sensed by Trp fluorescence intensity takes place with high cooperativity and at surfactant concentrations close to their CMC (Fig. 3). This indicates that the observed changes result mainly from toxin–micelle hydrophobic interaction and not from a non-cooperative surfactant monomer–toxin interaction. The fact that the onset of the effect take place at concentrations below the reported CMC can be related to the presence of buffer and the induction of aggregates by the template macromolecules (Santos et al., 2003).

CD spectra were also indicative of protein conformational changes upon interaction with the amphiphiles, albeit to a small extent. Far-UV and near-UV spectra evinced the occurrence of small changes in secondary (Fig. 5) as well as tertiary (Fig. 6) structures of both St I and St II. Significant interaction between β -lactoglobulin, a predominantly β -sheet protein, and cationic surfactants above their CMC has been reported (Viseu et al., 2004). However, in contrast with our results, these authors found a noticeable increase in α -helical content in the presence of micelles, with a significant change of the protein native structure. On the other hand, Wang and Lee (2006) recently reported three discrete conformational changes in BSA upon interaction with different concentrations of a cationic photoresponsive surfactant, characterized

by an α -helix $\rightarrow$ β -structure rearrangement, a decrease in the α -helix fraction in favor of unordered structures and a $\beta \rightarrow$ unordered transition adopting a highly elongated conformation in solution at low, intermediate, and higher surfactant concentrations, respectively. In our case, the fact that the toxin–micelle interaction takes place without significant loss of the toxin native structure could explain why the pore-forming ability of St I and St II is retained, and even increased, as assessed by their HA.

It is interesting to note that the effect of surfactant chain length depends on the property under analysis. In particular, maximum F/F_0 (Fig. 3), Ksv values (Fig. 4 and Table 2), and the conformation as followed by UV-CD (Figs. 5 and 6) show the same tendency, independent of the surfactant, while the HA is strongly conditioned by the detergent chain length. While CTAB and TTAB increase HA of both St I and St II, DTAB did not show any apparent effect (Fig. 1 and Table 1). In particular, it is noticeable that the increase in HA of both toxins upon addition of the longest alkyl chain cationic surfactants is due to changes in the protein and not due to a destabilizing effect of the surfactant on the RBC membrane.

Results summarized in Table 3 show that the surfactant concentration range and the driving forces involved in surfactant–protein interaction depend both on the surfactant characteristics (charge and hydrophobicity) and on the protein's

structural properties, such as isoelectric point, degree of coiling, and hydrophobic domains. The high isoelectric points of St I and St II ($pI > 9.0$, Lanio et al., 2001) imply that, at the working pH, there can be a favorable electrostatic interaction with anionic surfactants, explaining the non-cooperative effects observed for SDS surfactant at low concentrations (Lanio et al., 2003). On the other hand, when cationic surfactants are considered, the association is highly cooperative and evinced only at concentrations near the surfactant CMC, a result compatible with a predominantly hydrophobic interaction. The types of effects described for St I and St II in Table 3 cannot then be extrapolated to other proteins. In this regard, it is noticeable that the changes described for the interaction of cationic surfactants with β -lactoglobulin ($pI < 5.5$; Viseu et al., 2004) resemble more those measured for St I and St II in the presence of SDS (Lanio et al., 2003) than those observed in the present work.

In conclusion, the increase in sticholysins HA observed with CTAB and TTAB would indicate that association with these micelles could induce changes in the toxins that, while keeping their native structure, favor the acquisition of a more lytic competent structure. The apparent lack of correspondence between the changes detected by fluorescence and UV-CD studies for the three surfactants and the invariant HA for DTAB could be explained by a reversible binding of sticholysins to this less hydrophobic surfactant when compared with CTAB and TTAB.

Table 3
Effect of surfactant head group charge on St I and St II spectroscopic properties

Surfactant	Toxin	F/F_0	Ksv (M^{-1})	Conformational changes ^a	HA
SDS (Lanio et al., 2003)	St I	0.5	10.8	Large increase in α -helical content	Decreased, partially recovered at higher SDS concentrations
	St II	0.5	8.1	Total loss of tertiary structure	
HPS (Lanio et al., 2002)	St I	–	4.5	Negligible changes in secondary structure	Unchanged
	St II	1.8	7.4	Progressive loss of tertiary structure	
CTAB (present work)	St I	2.0	6.5	Small changes in secondary structure and modification of tertiary structure	Increased
	St II	1.65	5.0		
None	St I	–	3.5 ± 0.3	–	–
	St II	–	4.8 ± 0.4		

Surfactants were used at concentrations above their CMC.

^aObtained by far- and near-UV-CD measurements.

Acknowledgments

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Anexo 20

Combinatorial Synthesis and Directed Evolution Applied to the Production of α -Helix Forming Antimicrobial Peptides Analogues

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Abstract: Antimicrobial peptides (AMPs) are effector molecules of innate immune systems found in different groups of organisms, including microorganisms, plants, insects, amphibians and humans. These peptides exhibit several structural motifs but the most abundant AMPs assume an amphipathic α -helical structure. The α -helix forming antimicrobial peptides are excellent candidates for protein engineering leading to an optimization of their biological activity and target specificity. Nowadays several approaches are available and this review deals with the use of combinatorial synthesis and directed evolution in order to provide a high-throughput source of antimicrobial peptides analogues with enhanced lytic activity and specificity.

Keywords: Antimicrobial peptides, amphipathic α -helix, combinatorial synthesis, directed evolution, high-throughput.

ANIMAL ANTIMICROBIAL PEPTIDES

The emergence of bacterial strains presenting resistance to the currently available antibiotics is a driving force, accelerating the search and development of antimicrobial compounds with new mechanisms of action [1,2]. Within this context, antimicrobial peptides (AMPs) seem to be a remarkable alternative as a new source of therapeutic drugs. AMPs are ubiquitous, with members found in all kingdoms, including fungi and plants [3-8]. The antimicrobial peptides are key elements in a highly effective defense system against microorganisms, being responsible for the protection against infections mainly in invertebrates, such as insects [9]. In general, AMPs present deleterious effects on biological membranes and these activities are usually observed in *in vitro* assays against Gram-positive and Gram-negative bacteria, fungi and protozoa.

Nearly 800 AMPs have been isolated and fully characterized from different biological sources [their sequences are available at public access databases: SwissProt and TrEMBL (<http://www.expasy.org/sprot/sprot-top.html>), AMSDd (<http://www.bbcm.univ.trieste.it/~tossi/pag1.htm>) and APD (<http://aps.unmc.edu/AP/main.html>)]. Most of the described AMPs are basic peptides, presenting amphipathic spatial structures that are organized in α -helix, β -hairpin-like beta-sheet, β -sheet or α -helix/ β -sheet mixed structures.

Amphipathic α -helical antimicrobial peptides represent a well-studied large group among AMPs [10]. These peptides usually exhibit a random structure but, when in contact with biological membranes, fold into an amphipathic α -helix with the hydrophobic residues found along one side and the polar

side chains along the opposite side. Such organization allows interactions with lipoproteic membranes, especially those found in microorganisms which are largely composed of hydroxylated phospholipids (phosphatidylglycerol, cardiolipin and phosphatidylserine) which are more electronegative than the membranes of animals and plants (rich in zwitterionic lipids such as phosphatidylcholine, phosphatidylethanolamine and sphingomyelin) [11-13]. Three major mechanisms have been proposed to be responsible for membrane permeation by α -helical antimicrobial peptides: 1) barrel-stave, 2) toroidal-pore and 3) carpet mechanisms [14-16]. Such molecules were submitted to selective pressures during the evolutionary process, which generated best-fit structures adapted to the conditions of an environment rich in pathogenic microorganisms.

Our knowledge about the structure-activity relationships of α -helical antimicrobial peptides remains to be fully elucidated, but we are now capable of producing antimicrobial analogues that are more efficient than the natural ones. There are several examples in the literature where the development of antimicrobial peptides analogues allowed improvements in biological activity and specificity (Table 1). Since our understanding of the mechanisms of action of these molecules is still incomplete, rational design of more active analogues is compromised. Nowadays several methodologies are available to overcome these limitations. Combinatorial synthesis and directed evolution are emerging as important approaches in the field of protein engineering since these strategies allow the use of previous knowledge about the active motifs and the production of libraries of closely related structures that could be selected in high-throughput screenings.

COMBINATORIAL SYNTHESIS

To provide the development of new antimicrobial peptides, two approaches have been used. The first is the search

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Table 1. Antimicrobial and Hemolytic Activities of α -Helix Forming Antimicrobial Peptides and their Analogues

Peptide (source) [ref.]	Amino Acid Sequences	<i>E. coli</i> MIC (μ M)	<i>S. aureus</i> MIC (μ M)	HCS0 (μ M)
Pseudin 2 (frog) [17]	GLNALKKVFQGIHEAIKLNHHVQ	20	160	360
Pseudin 2 analogue K3,14 [17]	GLKALKKVFQGIHKAIKLNHHVQ	5	40	350
Pseudin 2 analogue K3,10,14 [17]	GLKALKKVFQGIHKAIKLNHHVQ	5	20	330
Pseudin 2 analogue F19 [17]	GLNALKKVFQGIHEAIKLFNNHHVQ	10	160	430
LL37 (human) [18]	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES	3	ND	ND
LL37 analogue P60.4-Ac [18]	Ac-IGKEFKRIVERIKRFLRELVRPLR-NH ₂	2	ND	ND
HP (2-20) (bacteria) [19]	AKKVFKRLEKLFISKIQNDK	12.5	12.5	>100
HP (2-20) analogue W19 [19]	AKKVFKRLEKLFISKIWNK	3.12	6.25	>100
HP (2-20) analogue W17 [19]	AKKVFKRLEKLFISKIWNK	3.12	6.25	>100
HP (2-20) analogue W17,19 [19]	AKKVFKRLEKLFISKIWNK	1.56	0.78	>100
Mellitin (honey bee) [20]	GIGAVLKVLTTGLPALISWIKRKRQ-NH ₂	4	0.26	1.8
Mellitin analogue Mel 12-26 [20]	GLPALISWIKRKRQ-NH ₂	49.5	3.1	>1000
Mellitin analogue Mel 12-26, G14,15 [20]	GLPALISWIKRKRGG-NH ₂	47.4	1.6	>1000
Mellitin analogue Mel 12-25 [20]	GLPALISWIKRKRQ-NH ₂	6.2	1.6	72
Mellitin analogue Mel 12-25, L14 [20]	GLPALISWIKRKR-NH ₂	6.9	1.6	>1000
Mellitin analogue Mel 12-24 [20]	GLPALISWIKRKR-NH ₂	27	0.84	>1000
Pleurocidin (fish) [21]	GWGSFFKKAHVGKHHVGAALTHYL	1.48	5.9	>36
Pleurocidin analogue A1,3 [21]	AWASFFKKAHVGKHHVGAALTHYL	5.8	2.9	>36
IsCT (scorpion) [22]	ILGKIWEGIKSLF-NH ₂	4	2	30
IsCT analogue A6 [22]	ILGKIAEGIKSLF-NH ₂	64	>64	>100
IsCT analogue I.6 [22]	ILGKILEGIKSLF-NH ₂	16	4	>100
IsCT analogue K7 [22]	ILGKIWKGIKSLF-NH ₂	2	1	100
IsCT analogue I.6, K11 [22]	ILGKILKGIKSLF-NH ₂	2	2	>100
IsCT analogue K7, P8, K11 [22]	ILGKIWKPIKKLF-NH ₂	2	2	>100
IsCT analogue K3, K7, P8, K11 [23]	ILKKIWKPIKKLF-NH ₂	2	1	>200
IsCT analogue K3, K7, P8, K11 [23]	ILKKIWKPIKKLF-NH ₂	2	1	>200

P, L-Pro; p, D-Pro
ND, not determined.

for new molecules of the innate defense system from host organisms [24,25]. Alternatively, antimicrobial peptides can be developed and optimized by combinatorial synthesis. Combinatorial chemistry is a synthetic chemical process that originates libraries of compounds faster and more efficiently. This methodology involves the design, synthesis and screening of peptides. A synthetic library can be prepared by two different ways: "parallel synthesis" where the products are generated in different reaction vessels and are available individually, or alternatively, the compounds can be obtained

as pool mixtures by "one-pot synthesis" (Fig. 1). Several comprehensive reviews about synthetic combinatorial libraries can be found in the literature [26-30].

The great number of possible combinations in the synthesis of AMPs, in general 10-60 residues long and using the 20 natural amino acids generating 20^{10} at 20^{60} combinations respectively, urges reduction in the numbers of sequences to be screened. Consequently, a scaffold of synthetic combinatorial libraries is defining one or more positions in the peptide sequence or a motif which will decrease the number of dif-

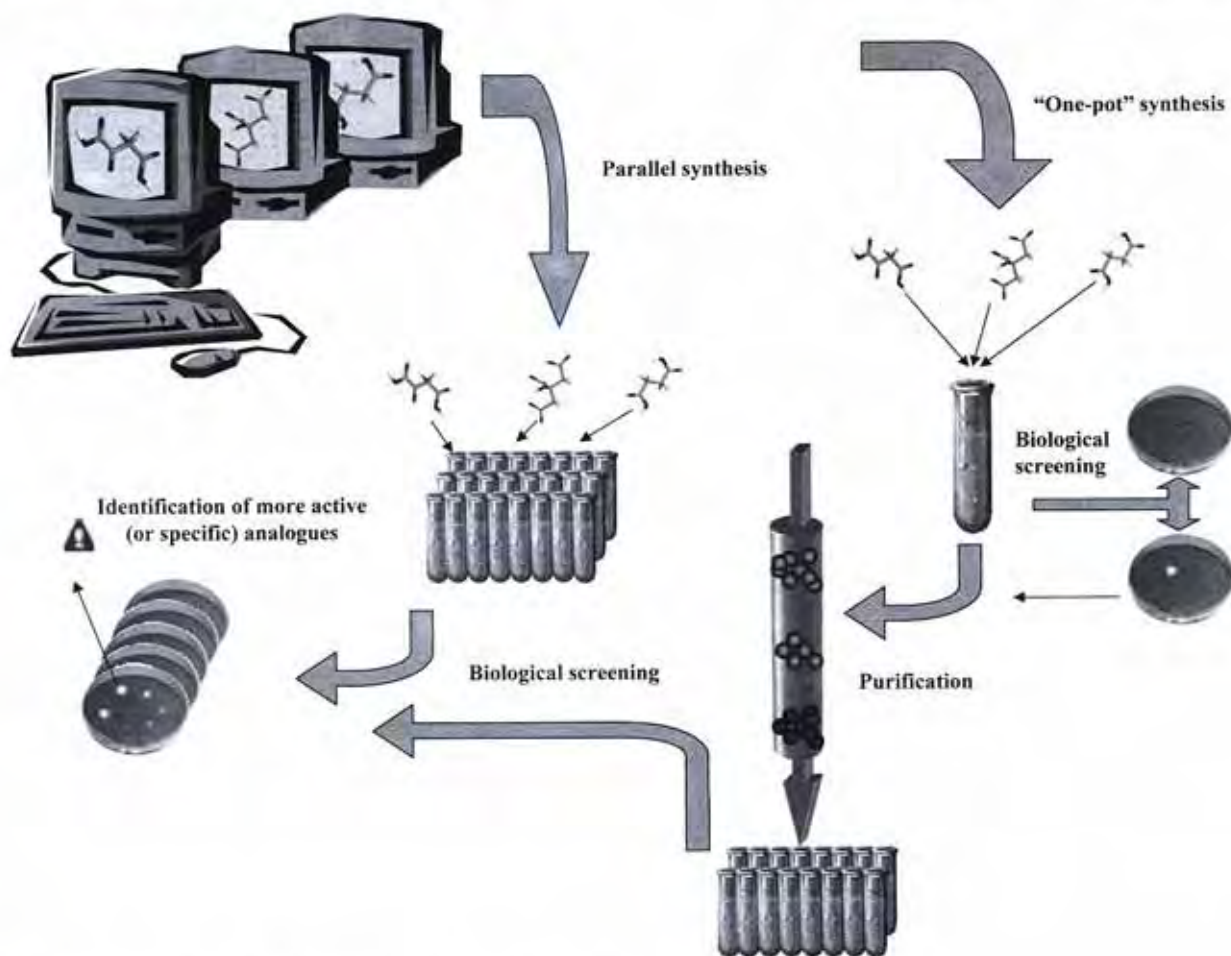


Fig. (1). Schematic workflow of combinatorial synthesis to generate libraries of peptide analogues.

ferent compounds to be produced and analyzed. Among the methods for compound selection, the most currently used is the *in silico*/virtual screening [31,32]. This method involves a variety of computational techniques in order to reduce and optimize the numbers of possible combinations based mainly on structure similarity of the molecule [33] or the molecular target [34]. A desirable goal of this methodology is to obtain molecules by rational drug design and to diminish the number of sequences to be screened by traditional methods, i.e., after obtaining the library, the identification of bioactive molecules demands assays designed to detect the biological activity of the new compounds against a target microorganism.

In the field of the production of AMPs analogues, the studies developed by Richard A. Houghten and Sylvie E. Blondelle are important to notice. They have generated sets of antimicrobial peptides analogues based on non-support-bound mixture-based peptide synthetic combinatorial libraries (SCLs) since the beginning of the 90's [35-38]. Using such approach, SCLs were built around lead antimicrobial peptides that have already been characterized and a limited number of positions were randomized. The active mixtures were submitted to a deconvolution process in which sets of individual peptides were selected to be synthesized and their biological activity tested against target microorganisms. Recently, Boggiano *et al.* identified several anti-HIV and an-

timicrobial peptides derived from SCLs [39], highlighting the important role of combinatorial synthesis for the development of new anti-infectious agents.

Kundu *et al.* also described the identification of potent antifungal peptides from the screening of combinatorial peptides libraries. These studies allowed the optimization of a lead molecule (a hexapeptide His-D-Trp-D-Phe-Phe-D-Phe-Lys-NH₂ related to the growth hormone releasing hexapeptide, GHRP-6) against pathogenic fungi [40-42].

DIRECTED EVOLUTION

Genetic forms that do not assign adaptive advantages to the organisms are not selected in the evolutionary process and consequently they are not transmitted to the next generations. The amazing advances in genetic engineering made available a technique that enables us to obtain these variants in the laboratory. Such method, named directed evolution or *in vitro* evolution, is a powerful technique capable of generating libraries of engineered proteins (or peptides) allowing the improvement of their biological properties in a high-throughput fashion.

During directed evolution, random mutations are produced in the gene that encodes the target protein by using error-prone PCR, which is easily accomplished by adding a small amount of Mn²⁺ and biased concentrations of dNTPs.

Such modified copies can be inserted into vectors to express those genes and the resulting clones are selected according to their biological activities (Fig. 2). Directed evolution also allows *in vitro* recombination, broadening even more the genetic variability. Favorable variants from a certain gene are selected and submitted to recombination in order to select forms that accumulate favorable mutations. One of the most used techniques is DNA shuffling, where parental DNA is digested with DNase I generating fragments of different sizes that are then submitted to a new PCR turn in which the same fragments generated by digestion are used as initiators, producing genetic recombinants. The genetic variants produced *in vitro* are again introduced in organisms for the selection of the desired function [43–45].

Directed evolution of proteins is a relatively new field of research and yet to be fully explored. Such technique has been successfully employed in the development of industrial enzymes, enabling improvements in their structural and functional properties, such as stability and catalytic efficiency. Khan *et al.* described enhancement of thermostability of a glutamate dehydrogenase from *Bacillus subtilis* by ran-

dom mutagenesis. The mutant enzyme Q144R has a T_m value of 61°C in contrast to the wild-type, 41°C and a high k_{cat} for amination reaction at 37°C, 1.3 times higher than that of the wild-type enzyme [46]. Alcade *et al.* created a mutant library of fungal laccases by error-prone PCR and the mutant 6C9 displayed about 3.5-fold higher activities than parent type in the presence of organic solvents [47]. Other excellent example of the straightness of this technique is the study conducted by Wang *et al.* Using an error-prone PCR approach they enhanced the alkali-tolerant ability of endoglucanase III (EG III) from *Trichoderma reesei* and also concluded that residue N321 might be one of the key residues involved in determining the optimal pH of EG III from *T. reesei* [48].

The use of such methods to develop new antibacterials is yet incipient, although quite promising. Recently Rao *et al.* described the use of the “codon-shuffling” method [49] for the synthesis and selection of new AMPs with α -helix as the predominant secondary structure [50]. The “codon shuffling” method entails *in vitro* assembly of hexamer of DNA duplexes that represent all 20 amino acids as codon pairs (“di-

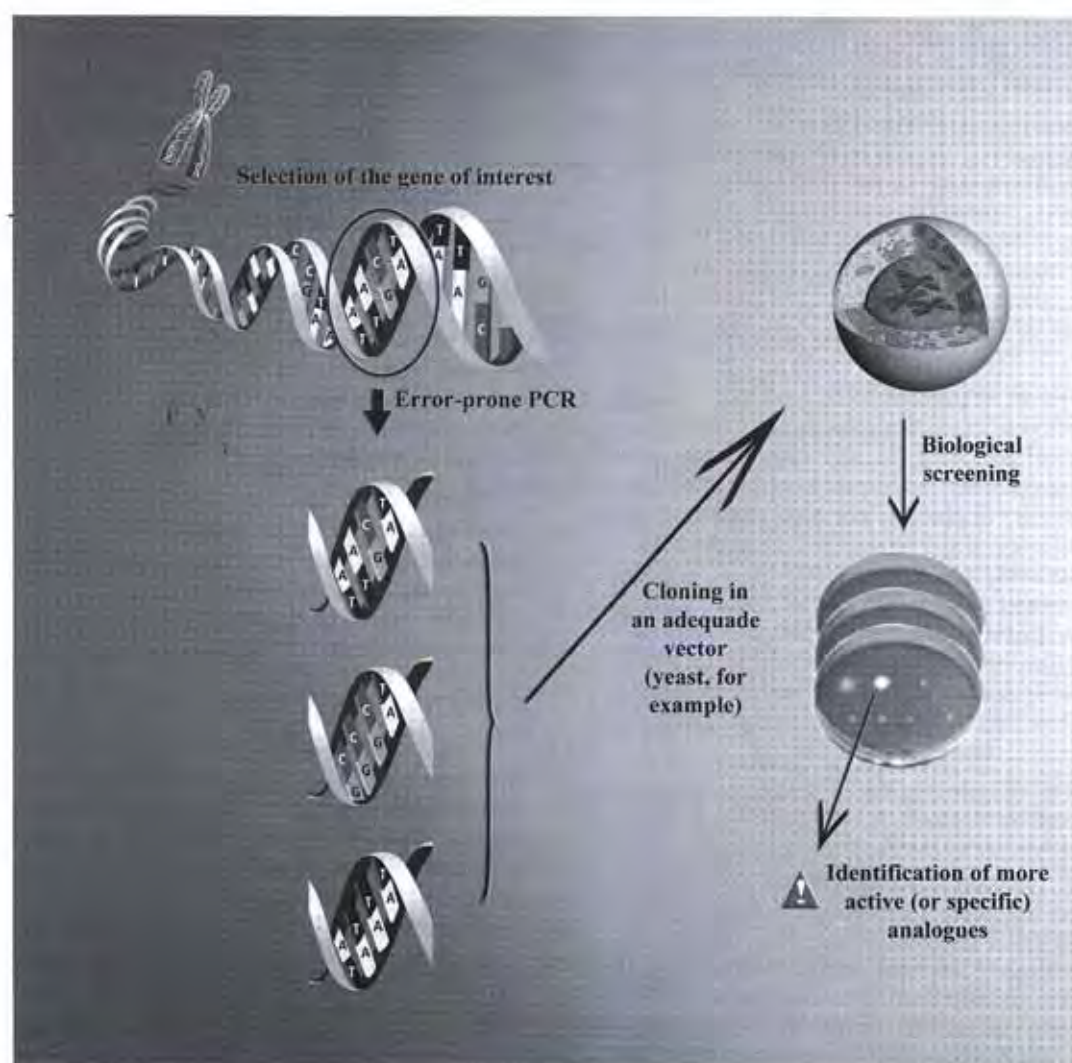


Fig. (2). Schematic workflow of directed evolution by error-prone PCR to generate libraries of peptide analogues.

condons"). These "dicodons" are introduced (and shuffled randomly) in an inactive gene scaffold and the ligation mixture is used to transform *Escherichia coli*. The application of this technique allows the selection of proteins and not only peptides: three unique translation products were obtained which exhibit remarkable differences in the protein length and pI (EQAMP1, 92 aa and pI 8.6; EQAMP2, 116 aa and pI 6.6; and EQAMP3, 172 aa and pI 6.5). One major advantage of the "codon shuffling" is that skewed libraries can be generated, such as the production of an extremely positive charged AMP (SKAMP1) from a mixture enriched with the dicodons Lys-Leu and Arg-Thr [50].

CONCLUDING REMARKS

In general, one major limitation of the combinatorial approaches applied to improvement of the majority of bioactive peptides is the need for high-throughput screening methods, making virtually impossible, with the current technology, to test the biological activity of all produced derivatives. In the case of antimicrobial peptides analogues such limitation can be overcome using susceptibility microdilution assays as described by the National Committee for Clinical Laboratory Standards [51,52]. Such simple methods allow for the testing of biological activity of a large number of samples in a short period of time and provide reliable data to determine bioactivities differences of the modified peptides. Another high-throughput screening assay applied to the selection of AMPs was described by Rauesh *et al.* [53] and this method consists in the release of Tb²⁺ from lipid bilayer vesicles after incubation in a multiwell plate.

Because of the urgent necessity for novel anti-infectious agents, the traditional methods for isolation and characterization of antimicrobial compounds from natural sources are revealed insufficient and the generation of active analogues using combinatorial synthesis and directed evolution is an expanding field that could feed the pharmaceutical industry with new drugs in a short period of time.

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