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Pressure dependence of side chain ^{13}C chemical shifts in model peptides Ac-Gly-Gly-Xxx-Ala-NH$_2$

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Abstract For evaluating the pressure responses of folded as well as intrinsically unfolded proteins detectable by NMR spectroscopy the availability of data from well-defined model systems is indispensable. In this work we report the pressure dependence of ^{13}C chemical shifts of the side chain atoms in the protected tetrapeptides Ac-Gly-Gly-Xxx-Ala-NH$_2$ (Xxx, one of the 20 canonical amino acids). Contrary to expectation the chemical shifts of a number of nuclei have a nonlinear dependence on pressure in the range from 0.1 to 200 MPa. The size of the polynomial pressure coefficients B_1 and B_2 is dependent on the type of atom and amino acid studied. For H^N, N and C$^\alpha$ the first order pressure coefficient B_1 is also correlated to the chemical shift at atmospheric pressure. The first and second order pressure coefficients of a given type of carbon atom show significant linear correlations suggesting that the NMR observable pressure effects in the different amino acids have at least partly the same physical cause. In line with this observation the magnitude of the second order coefficients of nuclei being direct neighbors in the chemical structure also are weakly correlated. The downfield shifts of the methyl resonances suggest that gauche conformers of the side chains are not preferred with pressure. The valine and leucine methyl groups in the model peptides were assigned using stereospecifically ^{13}C enriched amino acids with the pro-R carbons downfield shifted relative to the pro-S carbons.

Introduction

A detailed knowledge of chemical shifts of unstructured model peptides is mandatory when chemical shifts of folded proteins have to be interpreted or chemical shift changes after a possible denaturation have to be discussed. This is also substantial for the interpretation of chemical shifts of intrinsically disordered proteins (IDPs) that may still show some residual structural propensity. Therefore, random coil model peptides were studied extensively in the past in the NMR community. Most of the studies focus on ^{1}H chemical shifts but only a few of them present heteronuclear chemical shift data (Richarz and Wüthrich 1978; Bundi and Wüthrich 1979; Jimenez et al. 1986; Braun et al. 1994; Thanabal et al. 1994; Wishart et al. 1995b; Merutka et al. 1995; Plaxco et al. 1997; Schwarzinger et al. 2000, 2001; Arnold et al. 2002; Kjaergaard et al. 2011b; Kjaergaard and Poulsen 2011; Koehler et al. 2012; Platzer et al. 2014; Beck Erlach et al. 2016). So far the most complete experimental datasets for ^{13}C nuclei are from Richarz and Wüthrich (1978) and Wishart et al. (1995b). An alternative approach to determine random coil chemical shifts is the evaluation of chemical shift databases for proteins such as the BioMagResBank (BMRB). Random coil-like chemical shifts also can be obtained as an average of unstructured regions in folded proteins (Wishart

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and Sykes 1992, 1994; Wang and Jardetzky 2002; Zhang et al. 2003; De Simone et al. 2009), denatured proteins (Peti et al. 2001) or from IDPs (Tamiola et al. 2010). The influence of several factors such as solvent composition, temperature and more recently hydrostatic pressure on the random coil shifts was evaluated. When comparing random coil chemical shifts, not only the type of the random coil model peptide including the neighboring amino acids but also these external factors cannot be neglected. As an example the backbone H^α shifts of the protected tetrapeptide Ac-Gly-Gly-Xxx-Ala-NH$_2$ (with Xxx being one of the 20 canonic proteinogenic amino acids) show standard deviations to published data (Beck Erlach et al. 2016) in the range between 0.02 ppm (Kjaergaard et al. 2011b) and 0.06 ppm (Wang and Jardetzky 2002). For C^α nuclei the range of the standard deviations is between 0.09 ppm (Tamiola et al. 2010) and 0.62 ppm (Wang and Jardetzky 2002), for the C′-nuclei it is between 0.09 ppm (Kjaergaard et al. 2011b) and 0.55 ppm (Richarz and Wüthrich 1978).

In recent years the analysis of the pressure response of peptides and proteins by high pressure NMR spectroscopy led to the characterization of the free energy landscape of proteins [for reviews see e.g. (Akasaka 2006; Kitahara et al. 2013)], supplementing the well-developed NMR methodology based on relaxation dispersion [for reviews see e.g. (Halle and Denisov 2001; Neudecker et al. 2009)] or chemical exchange saturation transfer experiments [for reviews see e.g. (van Zijl and Yadev 2011; Wu et al. 2016)]. An important property of high pressure NMR spectroscopy is its ability to detect rare "excited" states of proteins (Kalbitzer et al. 2009) that can be used to develop new types of allosteric inhibitors of proteins involved in signal transduction, called intrinsic allosteric inhibitors (Rosnizeck et al. 2010, 2012; Kalbitzer et al. 2013).

Even peptides assumed to be in a random coil like state show rather strong pressure responses of their chemical shifts as originally reported for the protons of the tetrapeptide Gly-Gly-Xxx-Ala by Arnold et al. (2002). These chemical shifts δ can be sufficiently well described by a second degree polynomial as a function of pressure P (Eq. 1).

$$\delta = \delta_0 + B_1\left(P - P_0\right) + B_2\left(P - P_0\right)^2 \qquad (1)$$

The coefficient δ_0 describes the chemical shift at atmospheric pressure P_0 (0.1 MPa). The pressure coefficient B_1 describes the linear part of the chemical shift change with pressure, whereas the pressure coefficient B_2 describes the nonlinearity and therefore the curvature of the pressure dependence. A dataset recorded at 800 MHz was published for all backbone atoms (H^N, N, H^α, C^α, C′) of the protected tetrapeptide Ac-Gly-Gly-Xxx-Ala-NH$_2$ (Koehler et al. 2012; Beck Erlach et al. 2016). It turned out that for most residues also the heteronuclear pressure induced chemical shift

changes are nonlinear. In this paper we will present the pressure dependence of the ^{13}C chemical shifts of the side chain atoms of the amino acids Xxx in the protected tetrapeptides of the type Ac-Gly-Gly-Xxx-Ala-NH$_2$.

With these pressure coefficients the random coil chemical shifts at any pressure can be predicted for the 20 different amino acids. An important application is the structural characterization of disordered regions in folded proteins or in IDPs. Here, the data can be used to distinguish completely disordered regions from partly structured regions by their pressure response (Munte et al. 2013; Roche et al. 2013). In completely disordered regions the difference between the observed and the predicted pressure response should be invariant for all pressures. For the interpretation of the pressure response of folded proteins the random coil data can be used to separate "trivial" pressure effects from effects caused by structural transitions. By correction of the experimental data with the random coil pressure effects more meaningful data for the thermodynamic analysis of a given multistate equilibrium can be obtained [see e.g. (Kachel et al. 2006)].

Materials and methods

Amino acids and synthesis of peptides

The synthesis of the tetrapeptide Ac-Gly-Gly-Xxx-Ala-NH$_2$, where Xxx stands for one of the 20 canonical amino acids, was already described in detail earlier (Koehler et al. 2012; Beck Erlach et al. 2016). Only the amino acid Xxx at position 3 in the tetrapeptide Ac-Gly-Gly-Xxx-Ala-NH$_2$ was uniformly ^{13}C and ^{15}N enriched. The purity of the peptides was confirmed by ESI-MS (Bruker, Billerica, MA, USA) and RP-HPLC (Waters, Milford, MA, USA).

The uniformly ^{15}N and ^{13}C enriched amino acids required for the synthesis of the tetrapeptides as well as the N-terminal protection group Fmoc chloride (9H-fluoren-9-yl methyl carbonochloridate) and Fmoc-OSu (1-{[(9H-fluoren-9-ylmethoxy)carbonyl]oxy}-2,5-pyrrolidinedione) were purchased from Sigma Aldrich (St. Louis, MO, USA). The isotope enrichment is larger than 98%. All other chemicals were purchased from Merck (Darmstadt, Germany).

Block labeled (BL) valine and BL-leucine were produced by microbial fermentation using a mixture of [U-98% ^{13}C]-glucose (15 w/w%) and unlabeled glucose (85 w/w%) (Kainosho 1997; Kainosho and Güntert 2009). For the synthesis of the tetrapeptides Ac-Gly-Gly-(BL-Val)-Ala-NH$_2$ and Ac-Gly-Gly-(BL-Leu)-Ala-NH$_2$ the α-amino group of the amino acid was protected with Fmoc-OSu to obtain the Fmoc-Val-OH and Fmoc-Leu-OH based on the procedure described by Rietschoten and co-workers (1983) with the following modifications: The Fmoc-OSu and amino acids were solved in methanol:H$_2$O (1:1) and the reaction was incubated at

60 °C for 3 h. The products were extracted with acetone, sodium sulphate was added, and afterwards the product was dried for crystallization of Fmoc-amino acids.

Sample preparation

2.5 μmol of the peptide were dissolved in 500 μL of a buffer containing 20 mM perdeuterated Tris-d$_{11}$ (Tris(hydroxymethyl-d3)amino-d2-methane) and 0.5 mM DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) with a H$_2$O:D$_2$O ratio of 90:10 leading to a concentration of the peptide of 5 mM. The pH value was adjusted to 6.7 using a Hamilton Spintrode attached to a Beckman Coulter pH meter. The histidine containing tetrapeptide was also measured at pH 4.0. The pH values have not been corrected for the deuterium isotope effect.

NMR spectroscopy

Most of the experiments were performed on an 800 MHz Bruker Avance spectrometer (Bruker, Billerica, MA, USA) with a room temperature probe head (QXI). The corresponding ^{1}H and ^{13}C frequencies were 800.2 and 201.2 MHz, respectively. The experiments were performed at 283 K with a temperature calibration carried out after each sample change by measuring the difference of the proton resonance of the hydroxyl and the methyl group in 100% methanol as described by Raiford et al. (1979).

^{1}H NMR spectra were directly referenced to the methyl resonances of internal DSS, ^{15}N and ^{13}C signals were indirectly referenced to DSS using Ξ-values (Wishart et al. 1995a; Markley et al. 1998) of 0.101329118 (^{15}N/^{1}H) and 0.25144953 (^{13}C/^{1}H). Atom labels were named according to IUPAC recommendations (Markley et al. 1998).

^{1}H and ^{13}C chemical shifts were obtained from highly resolved one-dimensional spectra with a typical digital resolution of the time domain data of 0.04 Hz (^{1}H) and 0.16 Hz (^{13}C). A Lorentzian-to-Gaussian transformation was applied to the FID to obtain signals as narrow as possible.

Data acquisition and processing was performed with Bruker TopSpin 3.2 PL6. For peak picking the software AUREMOL (Gronwald and Kalbitzer 2004; http://www.auremol.de) was used. Data evaluation and fitting was done with the software package R (R Core Team 2017).

High pressure system

The high pressure system, especially the autoclave holding the ceramic cell was described earlier in detail by Koehler et al. (2012). Pressure was applied to the NMR sample via pressurized fluids (methylcyclohexane or water) contained in high pressure lines. For generating the pressure a manually operated piston compressor and an air-to-liquid pressure

intensifier (Barocycler®, HUB440, Pressure BioSciences Inc., South Easton, MA, USA), which is controlled by the spectrometer, were used. The ceramic cell was purchased from Daedalus Innovations LLC (Aston, PA, USA) with a maximum pressure limit of 250 MPa. For safety reasons pressure was only applied up to 200 MPa. The autoclave holding the ceramic cell is similar to the original autoclave (Peterson and Wand 2005) provided by Daedalus Innovations LLC but has an integrated safety valve, similar to the security valve described by Beck Erlach et al. (2010).

Data evaluation

For all 20 model peptides a pressure series from 1 to 200 MPa was performed using pressure steps of 20 MPa. The obtained pressure dependent chemical shifts δ were fitted as a function of pressure P with Eq. 1.

Results and discussion

Assignment of resonance lines

The assignments of most ^{13}C-resonances could be done on the basis of the already published assignments from Richarz and Wüthrich (1978) of the tetrapeptide Gly-Gly-Xxx-Ala and Wishart et al. (1995b) of the hexapeptide Ac-Gly-Gly-Xxx-Ala-Gly-Gly-NH$_2$ using one-dimensional ^{13}C spectra and an additional analysis of multiplet patterns. In case of ambiguities [^{1}H,^{13}C]-HSQC spectra were recorded to use the additional information of ^{1}H chemical shifts. Stereochemical assignments of the valine and leucine methyl groups in proteins can be obtained by biosynthetic fractional ^{13}C labeling (Neri et al. 1989). However, these data cannot be directly transferred to our random coil model peptides. Since to our knowledge data for random coil peptides are not available, we used fractionally ^{13}C labeled amino acids Val and Leu to synthesize Ac-Gly-Gly-(BL-Val)-Ala-NH$_2$ and Ac-Gly-Gly-(BL-Leu)-Ala-NH$_2$. Assignments were performed by comparing the [^{1}H,^{13}C]-HSQC spectra of uniformly labeled tetrapeptides with either BL free amino acids or tetrapeptides that contained the specifically ^{13}C BL amino acids Val and Leu (Fig. 1). Due to the biosynthetic pathways for these amino acids, the pro-R methyl groups (C$^{\gamma 1}$ in Val and C$^{\delta 1}$ in Leu) are simultaneously ^{13}C labeled at their adjacent carbons and therefore appear as a doublet in the ^{13}C dimension ($^1J_{C-C}$). On the other hand, the pro-S methyl groups (C$^{\gamma 2}$ in Val and C$^{\delta 2}$ in Leu) labeled with ^{13}C appear as a singlet in the ^{13}C dimension (Kainosho 1997; Kainosho and Güntert 2009; Miyanoiri et al. 2013, 2016; Kainosho et al. 2006).

The specifically ^{13}C labeled amino acids were measured under the same conditions as the uniformly labeled tetrapeptides. The leucine cross peak positions in the BL free amino
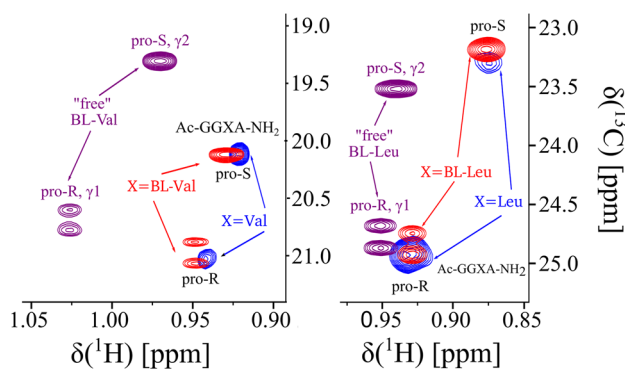
Fig. 1 Stereospecific assignment of leucine and valine methyl resonances. (Left) part of a [^{1}H,^{13}C]-HSQC spectra of biosynthetically ^{13}C block labeled valine, Ac-Gly-Gly-Val-Ala-NH$_2$ and Ac-Gly-Gly-(BL-Val)-Ala-NH$_2$, (right) part of a [^{1}H,^{13}C]-HSQC spectra of biosynthetically ^{13}C block labeled leucine, Ac-Gly-Gly-Leu-Ala-NH$_2$ and Ac-Gly-Gly-(BL-Leu)-Ala-NH$_2$. For experimental conditions see "Materials and methods"

acid are very close to those in the tetrapeptide (Fig. 1) indicating that these assignments can be transferred to the tetrapeptide. The valine data are less convincing but nevertheless it seems reasonable that the downfield shifted peak corresponds to pro-R Val. To verify these putative stereospecific assignment, it was necessary to prepare tetrapeptides with the BL amino acids Val and Leu. The chemical shift patterns of these BL tetrapeptides are in almost perfect agreement with the not BL tetrapeptides (Fig. 1). The small deviation of the signal positions may be explained by the low resolution of the spectrum of the not BL tetrapeptide. For Ac-Gly-Gly-(BL-Val)-Ala-NH$_2$, the (H$^{\gamma1}$)$_3$ and the C$^{\gamma1}$ atoms give a cross peak at (0.95, 20.97 ppm), (H$^{\gamma2}$)$_3$ and the C$^{\gamma2}$ atoms at (0.93, 20.12 ppm). The (H$^{\delta1}$)$_3$, C$^{\delta1}$ and the (H$^{\delta2}$)$_3$, C$^{\delta2}$ cross peaks of Ac-Gly-Gly-(BL-Leu)-Ala-NH$_2$ are observed at (0.93, 24.83 ppm) and (0.87, 23.18 ppm), respectively. These stereospecific assignments are used in the following (Tables 1, 2, 3). Our stereospecific assignments using BL tetrapeptides confirm the assignments obtained for the pentapeptides Ac-Gly-Gly-Xxx-Gly-Gly-NH$_2$ and Ac-Gln-Gln-Xxx-Gln-Gln-NH$_2$ (Xxx is Val or Leu) by an interpretation of the γ-gauche effect by Kjaergaard et al. (2011a).

Random coil chemical shift values

By applying pressure to the tetrapeptides and fitting the resulting pressure dependences of the chemical shift to Eq. 1, we also obtained a complete dataset of ^{13}C random coil chemical shift values for the side chains of the amino acids Xxx in the model peptides Ac-Gly-Gly-Xxx-Ala-NH$_2$.

The complete set of random coil chemical shift values δ_0 at 0.1 MPa is summarized in Table 1. The given chemical shifts are obtained by a fit of the 11 pressure points recorded

in the range from 0.1 to 200 MPa and therefore have a very high accuracy (average fit error 0.008 ppm). A comparison with already available datasets is presented in Fig. 2. If required the data were corrected for different ^{13}C referencing methods as proposed by Wishart and coauthors (Wishart and Sykes 1994; Wishart et al. 1995a). To adapt the data from Richarz and Wüthrich (1978) to our referencing system with DSS, a value of 1.5 ppm was added to the published data (TMS vs. DSS). As proposed by Wishart and Sykes (1994) this value also includes a correction for dioxane shift used as primary reference by Richarz and Wüthrich (1978). For the data of Thanabal et al. (1994) a value of 0.15 ppm was subtracted from the published data (TSP vs. DSS). Figure 2 shows the mean deviation for all atom groups in the published datasets and also includes C$^\alpha$ and C′ shifts analyzed earlier (Beck Erlach et al. 2016). The mean deviation over the amino acids is depicted by the black thick line inside the colored bar, whereas the size of the bar corresponds to the standard deviation. A small standard deviation is an indication for an offset that can be explained by temperature or other experimental differences, whereas a large standard deviation indicates a rather inhomogeneous behavior with regard to our data, maybe due to the differences between the model compounds themselves.

Overall the best agreement was found for the dataset of Wishart et al. (1995b), with an averaged mean deviation of 0.16 ppm, followed by Kjaergaard et al. (2011b) and Kjaergaard and Poulsen (2011) with an averaged mean deviation of 0.17 ppm for both peptides (Ac-Gly-Gly-Xxx-Gly-Gly-NH$_2$ and Ac-Gln-Gln-Xxx-Gln-Gln-NH$_2$). One has to note that the sample size for the datasets is quite different and therefore the averaged mean values have to be interpreted carefully. The biggest deviation to our dataset was found for Schwarzinger et al. (2000) with an averaged mean value of 0.5 ppm. This is most probably the consequence of the very low pH of 2.3 and the use of 8 M urea as a denaturant. The low pH probably has a large influence on negatively charged side chains but not on the peptide N- and C-termini since they were protected. For the non-experimental datasets the chemical shifts obtained from IDPs by Tamiola et al. (2010) show the best agreement with an average mean deviation of 0.26 ppm.

Pressure dependence of ^{13}C chemical shifts

The pressure dependence of the C$^\alpha$ and C′ chemical shifts has been already published elsewhere (Beck Erlach et al. 2016), here we will describe the pressure dependence of all carbon side chain atoms. As described before for the backbone atoms of the model peptide, the chemical shift dependence of most of the atoms is clearly nonlinear and requires a fit with a second degree polynomial. Some typical examples are shown in Fig. 3. They clearly differ for different atoms

Table 1 Chemical shift values δ_0 of the side chain carbon atoms at ambient pressure in the peptide Ac-Gly-Gly-Xxx-Ala-NH$_2$

Amino acid Xxx	δ_0 [ppm]									
	C^β	C^γ		C^δ		C^ε		C^ζ/C^η		
Ala	19.07									
Arg	30.73		26.98		43.27				ζ	159.57
Asn	38.87		177.33							
Asp	41.14		180.37							
Cys$_{red}$	28.12									
Cys$_{ox}$	40.93									
Gln	29.37		33.73		180.75					
Glu	29.99		36.13		184.27					
His (pH 6.7)	30.30		133.48	$\delta 2$	119.92	$\varepsilon 1$	137.76			
His (pH 4.0)	29.03		131.07	$\delta 2$	120.16	$\varepsilon 1$	136.36			
Ile	38.77	$\gamma 1$	27.11	$\delta 1$	12.95					
		$\gamma 2$	17.40							
Leu[a]	42.27		26.91	$\delta 1$	24.91					
				$\delta 2$	23.24					
Lys	33.03		24.65		29.06		42.10			
Met	32.76		31.92				16.88			
Phe	39.55		138.74	$\delta 1, \delta 2$	131.94	$\varepsilon 1, \varepsilon 2$	131.49		ζ	129.97
Pro$_{trans}$	32.04		27.18		49.78					
Pro$_{cis}$	34.64		24.75		50.33					
Ser	63.77									
Thr	69.81	$\gamma 2$	21.49							
Trp	29.39		111.18	$\delta 1$	127.42	$\varepsilon 2$	138.87	$\zeta 2$	114.67	
				$\delta 2$	129.44	$\varepsilon 3$	122.16	$\zeta 3$	124.77	
								$\eta 2$	120.95	
Tyr	38.74		130.40	$\delta 1, \delta 2$	133.30	$\varepsilon 1, \varepsilon 2$	118.27	ζ	157.30	
Val[b]	32.85	$\gamma 1$	21.05							
		$\gamma 2$	20.13							

5 mM of Ac-Gly-Gly-Xxx-Ala-NH$_2$, 20 mM perdeuterated Tris-d$_{11}$, 0.5 mM DSS, H$_2$O:D$_2$O ratio of 90:10, pH 6.7, temperature T_0 283 K, pressure P_0 0.1 MPa. The amino acids Xxx were uniformly ^{13}C and ^{15}N enriched (for detailed information see "Materials and methods"). The digits shown are significant with regard to the standard error calculated from the second degree fit (Eq. 1) of the data

[a] The proton chemical shifts of the corresponding (H$^{\delta 1}$)$_3$ and (H$^{\delta 2}$)$_3$ atoms are 0.928 and 0.874 ppm, respectively. The stereospecific assignment was taken from Ac-Gly-Gly-Leu-Ala-NH$_2$ containing stereospecifically labeled ^{13}C BL-leucine

[b] The proton chemical shifts of the corresponding (H$^{\gamma 1}$)$_3$ and (H$^{\gamma 2}$)$_3$ atoms are 0.945 and 0.931 ppm, respectively. The stereospecific assignment was taken from Ac-Gly-Gly-Val-Ala-NH$_2$ containing stereospecifically labeled ^{13}C BL-valine

as well as different amino acid types. The data were fitted with a second degree polynomial with the first order pressure coefficient B_1 and the second order pressure coefficient B_2. The pressure coefficients B_1 and B_2 derived for the side chain carbon atoms are summarized in Tables 2 and 3 and depicted graphically in Fig. 4.

Some of the residues Xxx in the peptide have ionizable side chains. Since pK values can be influenced by pressure they could change their protonation state. In addition the pH value of the sample may somewhat change with pressure by a change of the side chain pK of Tris-buffer itself and thus, indirectly, the ionization state of the side chains may vary.

These factors may change the observed chemical shifts by changing the protonation state of the respective side chains with pressure.

All measurements were performed with samples whose pH values were calibrated to pH 6.7 at 0.1 MPa and 283 K. With a pK_a of 7.0 (Richarz and Wüthrich 1978) only histidine has a pK_a value close to the actual pH and its protonation state should be sensitive to pressure. At pH 6.7 the H$^{\delta 2}$ and H$^{\varepsilon 1}$ of histidine show a downfield shift of 1.63 and 1.50 ppm/GPa (Table 2) that could be the consequence of a drop in pH or an increase of the pK_a value with pressure. For getting more information on this effect, we measured for

Table 2 First order pressure coefficients B_1 of the side chain carbon atoms in the peptide Ac-Gly-Gly-Xxx-Ala-NH$_2$

Amino acid Xxx	B_1 [ppm/GPa]							
	C$^\beta$	C$^\gamma$	C$^\delta$		C$^\varepsilon$		C$^\zeta$/C$^\eta$	
Ala	1.95							
Arg	−0.33	−0.51		−0.04			ζ	−0.83
Asn	0.51	0.54						
Asp	0.09	1.42						
Cys$_{red}$	0.87							
Cys$_{ox}$	−0.68							
Gln	−0.40	0.29		0.34				
Glu	−0.21	−0.23		1.10				
His (pH 6.7)	−0.05	−1.21	δ2	1.63	ε1	1.50		
His (pH 4.0)	−0.09	−0.87	δ2	0.82	ε1	1.33		
Ile	−0.99	γ1 −0.18	δ1	2.26				
		γ2 1.99						
Leu[a]	−0.84	−0.64	δ1	1.94				
			δ2	2.40				
Lys	−0.61	−0.49		−0.59		−0.17		
Met	−0.38	−0.53				2.07		
Phe	−0.32	−0.51	δ1, δ2	0.68	ε1, ε2	0.69	ζ	0.70
Pro$_{trans}$	0.65	0.73		0.68				
Pro$_{cis}$	0.52	0.90		0.84				
Ser	−0.06							
Thr	−0.50	γ2 1.72						
Trp	0.14	−0.47	δ1	1.42	ε2	−0.78	ζ2	1.32
			δ2	0.16	ε3	0.46	ζ3	0.51
							η2	1.06
Tyr	−0.28	−0.60	δ1, δ2	0.61	ε1, ε2	1.20	ζ	−0.68
Val[a]	−0.72	γ1 1.84						
		γ2 1.90						
Mean	−0.08	0.25		0.95		0.79	ζ	0.20

5 mM of Ac-Gly-Gly-Xxx-Ala-NH$_2$, 20 mM perdeuterated Tris-d$_{11}$, 0.5 mM DSS, H$_2$O:D$_2$O ratio of 90:10, pH 6.7, temperature T_0 283 K, pressure P_0 0.1 MPa. The amino acids Xxx were uniformly ^{13}C and ^{15}N enriched (for detailed information see "Materials and methods"). The digits shown are significant with regard to the standard error calculated from the second degree fit (Eq. 1) of the data

[a] Stereochemical assignment see Table 1

the histidine containing tetrapeptide also a dataset at pH 4.0 far away from the histidine pK_a value. Here the first order pressure coefficient is smaller but the sign is unchanged. At 283 K Tris-HCl has a pK_a value of 8.35 (Beck Erlach et al. 2016) which decreased by <0.01 units at 200 MPa. The corresponding resonance position can be used as a pH sensor for possible pH changes. Experimentally, the observed chemical shift changes of the –CHD and the –CH$_2$ signals of Tris-d$_{11}$ with pressure are very small (0 to −0.011 ppm) indicating a slight increase of pH with pressure in some samples. Since pressure should favor the charged state in the equilibrium (Gross and Jaenicke 1994), the histidine pK_a value is expected to increase with pressure.

With respect to their pressure response the most homogeneous group is represented by the methyl groups, where the first order coefficients of all residues are positive, leading to a downfield shift of theses resonances with pressure. In contrast, all second order coefficients are negative leading to a saturation-like behavior of the chemical shifts at higher pressures. The average carbon B_1 and B_2 values of the methyl resonances are 2.01 ppm/GPa and −1.55 ppm/GPa^2, respectively (Table 4).

The pressure response of the methylene groups is not uniform, most of them are characterized by an upfield shift with pressure ($B_1 < 0$). Exceptions are the C$^\beta$ resonances of asparagine, aspartic acid, cysteine, and tryptophan. Another exception is proline where all methylene signals are shifted downfield with pressure. In glutamine only the C$^\gamma$-resonance is shifted downfield with pressure (Table 2). The methylene resonances of many residues show a saturation-like behavior

Table 3 Second order pressure coefficients B_2 of the side chain carbon atoms in the peptide Ac-Gly-Gly-Xxx-Ala-NH$_2$

Amino acid Xxx	B_2 [ppm/GPa^2]				
	C$^\beta$	C$^\gamma$	C$^\delta$	C$^\epsilon$	C$^\zeta$/C$^\eta$
Ala	−1.49				
Arg	−0.58	−0.12	−0.46		ζ 0.52
Asn	−0.90	−0.29			
Asp	−0.63	−1.39			
Cys$_{red}$	−0.86				
Cys$_{ox}$	−0.03				
Gln	−0.33	−0.50	−0.20		
Glu	−0.73	0.06	−1.03		
His (pH 6.7)	0.14	1.61	δ2 −2.76	ε1 −1.88	
His (pH 4.0)	−0.09	−0.83	δ2 −0.45	ε1 −1.42	
Ile	0.16	γ1 −0.22	δ1 −1.26		
		γ2 −1.01			
Leu[a]	0.35	0.41	δ1 −2.02		
			δ2 −2.70		
Lys	−0.25	−0.12	−0.02	−0.33	
Met	−0.59	0.04		−1.34	
Phe	−0.36	0.56	δ1, δ2 −0.25	ε1, ε2 −0.23	ζ −0.22
Pro$_{trans}$	−0.63	−0.69	−0.27		
Pro$_{cis}$	−0.58	−0.78	−0.99		
Ser	−0.34				
Thr	0.02	γ2 −1.32			
Trp	−0.81	0.22	δ1 −0.75	ε2 0.43	ζ2 −0.80
			δ2 −0.03	ε3 −0.18	ζ3 −0.15
					η2 −0.82
Tyr	−0.35	0.32	δ1, δ2 −0.25	ε1, ε2 −0.86	ζ 0.31
Val[a]	0.09	γ1 −1.25			
		γ2 −1.58			
Mean	−0.40	γ −0.34	δ −0.90	ε −0.73	ζ −0.07

5 mM of Ac-Gly-Gly-Xxx-Ala-NH$_2$, 20 mM perdeuterated Tris-d$_{11}$, 0.5 mM DSS, H$_2$O:D$_2$O ratio of 90:10, pH 6.7, temperature T_0 283 K, pressure P_0 0.1 MPa. The amino acids Xxx were uniformly ^{13}C and ^{15}N enriched (for detailed information see "Materials and methods"). The digits shown are significant with regard to the standard error calculated from the second degree fit (Eq. 1) of the data

[a] Stereochemical assignment see Table 1

as e.g. the hydrophobic amino acids leucine, isoleucine and valine. However, other residues such as lysine show an increased shift dependence at higher pressures.

The methine resonances are all shifted upfield with pressure ($B_1 < 0$) and show an asymptotic behavior at higher pressures ($B_2 > 0$). The C-resonances from asparagine, aspartic acid, glutamine, and glutamic acid all show a downfield shift with pressure and a saturation-like behavior (Table 4). All quaternary carbons of the ring systems show an upfield shift with pressure ($B_1 < 0$), whereas the other ring carbons shift downfield with pressure ($B_1 > 0$) with exception of the C$^\zeta$-resonance of tyrosine (Fig. 4). The largest first and second order coefficients of side chain carbons are found for methyl groups. The most extreme B_1- and B_2-values are found for C$^{\delta 2}$ of leucine with 2.4 ppm/GPa and

−2.7 ppm/GPa^2, respectively. These values are also much larger than the most extreme B_1 and B_2 values observed for the C$^\alpha$ and C′ atoms (Beck Erlach et al. 2016) (B_1, glutamate C$^\alpha$ −1.2 ppm/GPa, glycine C′ 0.9 ppm/GPa; B_2, cystine C$^\alpha$ −3.0 ppm/GPa^2, histidine C′ −1.4 ppm/GPa^2). The corresponding average values of B_1 for C$^\alpha$ and C′ are −0.41 and 0.59 ppm/GPa, respectively, and 0.12 and −0.67 ppm/GPa^2 for B_2, respectively (Table 4).

In folded proteins the analysis of backbone chemical shifts is a well-established method to predict secondary structures. A downfield shift of H$^\alpha$ >0.1 ppm relative to the random coil value, an upfield shift of C$^\alpha$-resonances >0.7 ppm and of C′-resonances >0.5 ppm, and a downfield shift of C$^\beta$ >0.7 is typical for a β-strand structure (Wishart and Sykes 1992, 1994). For helical structures the inverse
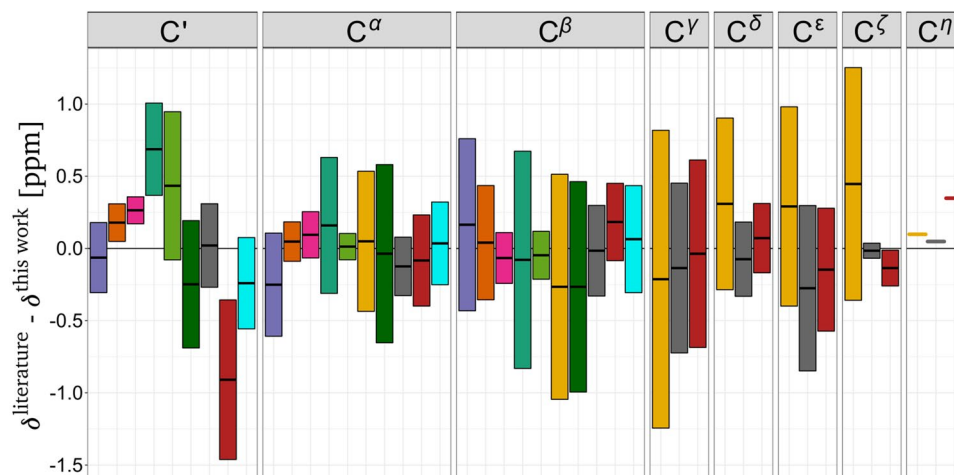
Fig. 2 Comparison of random coil chemical shift values of this study with already published datasets. The black bar indicates the mean deviation of the different datasets to our values. The size of the bars represent the standard deviation $\pm \sigma$. The datasets are color coded as listed in the following: De Simone et al. (2009) (violet), Kjaer-gaard and Poulsen (2011) (orange), Kjaergaard et al. (2011b) (pink), Schwarzinger et al. (2000) (blue green), Tamiola et al. (2010) (light green), Thanabal et al. (1994) (yellow); Wang and Jardetzky (2002) (dark green), Wishart et al. (1995b) (gray), Richarz and Wüthrich (1978) (dark red), Zhang et al. (2003) (light blue)
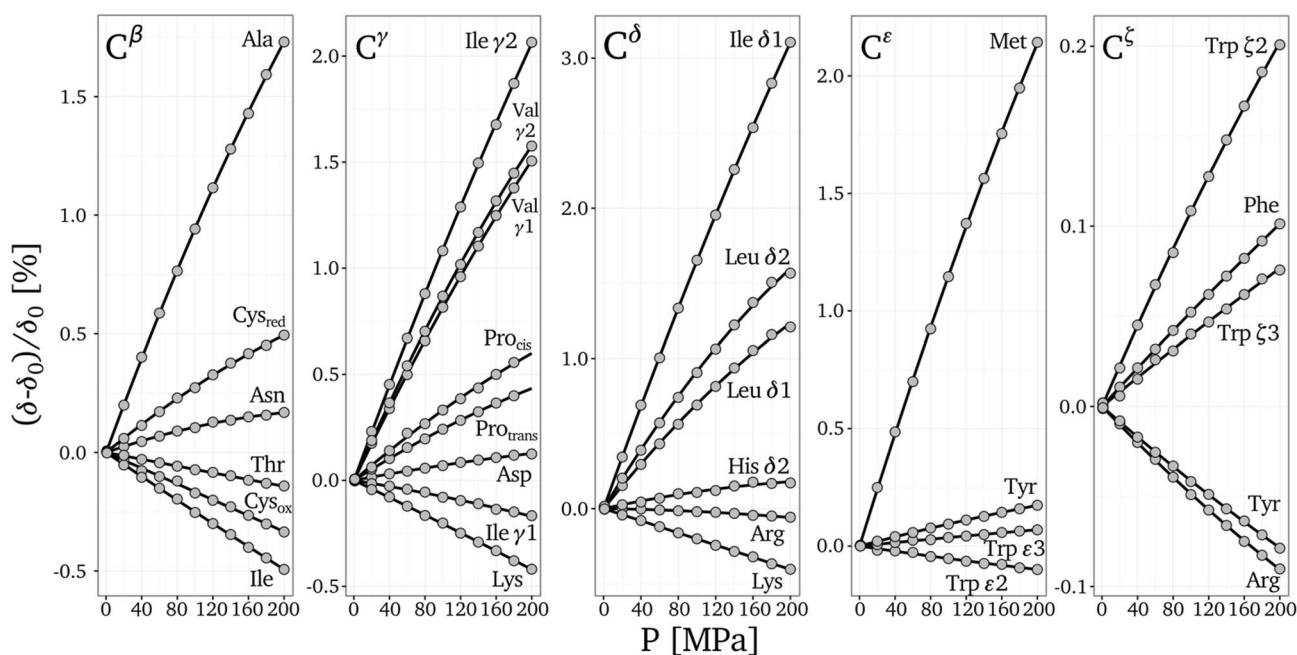

Fig. 3 Pressure dependence of side chain chemical shifts for different amino acids Xxx in Ac-Gly-Gly-Xxx-Ala-NH$_2$. Examples of different pressure dependent chemical shift changes for the side chain car-bon atoms. For experimental conditions see "Materials and methods". The curves (black line) describing the data points (grey circles) were calculated with Eq. 1 using the parameters given in Tables 1, 2 and 3

relation of shifts applies. A graphical overview of the pressure coefficients for the side chains of all amino acids is given in Fig. 4. When pressure would enhance the α-helical propensity in the tetrapeptides, an upfield shift for H$^\alpha$ and C$^\beta$ and a downfield shift of C$^\alpha$ and C′-resonances would be expected (Wang and Jardetzky 2002). As reported by Beck

Erlach et al. (2016) with a few exceptions, C′-resonances in our tetrapeptides shift downfield with pressure whereas H$^\alpha$ and C$^\alpha$ resonances shift upfield. As shown here also most of the C$^\beta$-resonances shift upfield with pressure. The observed downfield shift of the C$^\alpha$-resonances indicates, together with the earlier results, that pressure most likely does not

Fig. 4 First order pressure coefficient B_1 and second order pressure coefficient B_2 of the side chain carbon atoms. This figure summarizes the pressure coefficient B_1 (upper part) and the pressure coefficient B_2 (lower part) for the different amino acids and different side chain atoms. The side chain atoms are color coded: C^β (blue), C^γ (green), C^δ (brown), C^ε (yellow), C^ζ (violet), C^η (grey)
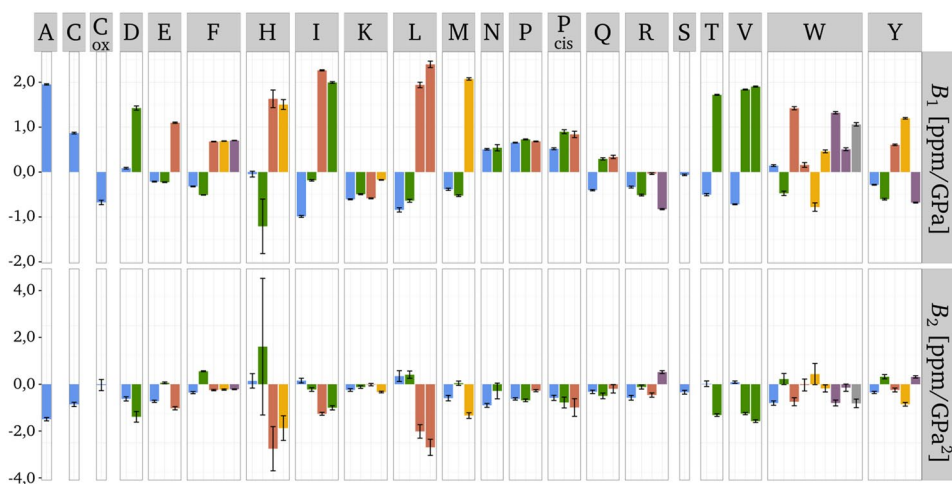
Table 4 Mean B_1, B_2, and B_2/B_1 values for different chemical groups

Atom	B_1 [ppm/GPa]	B_2 [ppm/GPa^2]	B_2/B_1 [1/GPa]
H^N	0.52 (0.13)	−0.41 (0.22)	−0.73 (0.32)
H^N (NMA)[a]	0.11	0.02	0.18
N	2.95 (0.40)	−2.34 (0.75)	−0.79 (0.20)
N (NMA)[a]	5.31	−5.80	−1.09
H^α	−0.04 (0.05)	0.02 (0.09)	−0.41 (2.15)
C^α	−0.41 (0.38)	0.12 (0.42)	−0.11 (0.83)
C'	0.59 (0.18)	−0.67 (0.24)	−1.18 (0.35)
C' (NMA)[a]	0.48	−0.65	−1.35
C^β–H_2	−0.14 (0.51)	−0.42 (0.37)	−0.29 (3.14)
C–H	−0.71 (0.20)	0.17 (0.17)	−0.24 (0.27)
C–H_2	−0.02 (0.52)	−0.25 (0.23)	1.06 (3.87)
C–H_3	2.01 (0.21)	−1.55 (0.51)	−0.77 (0.21)
C=O_{amide}	0.44 (0.14)	−0.24 (0.07)	−0.56 (0.03)
C=$O_{carboxy}$	1.26 (0.23)	−1.21 (0.26)	−0.96 (0.03)
C_{quart}	−0.43 (0.34)	0.23 (0.23)	−0.48 (0.38)
C_{arom}	0.40 (0.88)	−0.30 (0.93)	−0.65 (0.41)

5 mM of Ac-Gly-Gly-Xxx-Ala-NH_2, 20 mM perdeuterated Tris-d_{11}, 0.5 mM DSS, H_2O:D_2O ratio of 90:10, pH 6.7, temperature T_0 283 K, pressure P_0 0.1 MPa. C–H_2 includes C^β–H_2; C_{quart}, quarternary carbons; C_{arom}, all carbons of the His, Tyr, Phe, and Trp ring systems except quaternary carbons. Pro_{cis}, cystine, and His at low pH was omitted from the calculations. Values in brackets give the corresponding standard deviations σ

[a] Data from *N*-methyl acetate (NMA) (Frach et al. 2016)

enhance the propensity for α-helical secondary structure. In the IDP α-synuclein there is a slight increase of the polyproline II structure with pressure as derived from the decrease of the $^3J_{HN-H\alpha}$-coupling constant (Roche et al. 2013). The tendency of the average H^N, H^α, N, C' and C^α shift changes with pressure in α-synuclein is similar to those observed in our model peptides. This is also true when looking at the C^β shifts available in this study. This suggests that similar structural changes are induced in the model peptide and the unfolded protein.

The B_2/B_1 ratio of side chain atoms

In case that the chemical shift changes can be approximately described by a two-state model the ratio B_2/B_1 is proportional to the ratio of the differences of the compressibility factors $\Delta\beta'^0$ and of the partial molar volumes ΔV^0 of the two states (Beck Erlach et al. 2014). More generally, one can consider it as a measure for conformational states with a similar pressure dependence of free energies (note that this relation has only been derived for a two-state model with the condition $|\Delta G^0| \ll 2RT$). A negative value of B_2/B_1 would mean a positive value of the compressibility as it should be expected. We have calculated the ratio of B_2/B_1 for the backbone atoms and the carbon atoms of different groups of side chain atoms (Table 4). It turns out that for all these atoms and groups of atoms except the methylenes the ratios B_2/B_1 are negative and have similar magnitudes. The most negative values are found for the carbon atoms of the backbone carbonyl and the side chain carboxyl groups. The average values are −1.18 and −0.96 GPa^{-1}, respectively (Table 4). This suggests a common mechanism for this behavior. The positive average value of the methylene groups is not really significant since the corresponding standard deviation of 3.87 GPa^{-1} is larger than the mean value and indicates that there is a considerable number of methylene groups that also show negative B_2/B_1 values.

Apart from conformational changes a plausible mechanism would also include the pressure response of the adjacent water shell. At least for the rigid peptide bond model *N*-methyl acetamide (NMA) the chemical shift changes with pressure can be explained by polarization effects from the water shell that changes its structure with pressure (Frach et al. 2016). In NMA the ratio B_2/B_1 is 0.2, −1.1

and -1.4 GPa^{-1} for H^N, N and C′, respectively, The B_2/B_1 ratios of N and C′ in the tetrapeptides are with -0.79 and -1.18 GPa^{-1} rather close to the corresponding values found in the peptide bond model NMA. The pressure dependence of the amide proton shifts in NMA is with a B_1 value of 0.11 ppm/GPa substantially smaller than the pressure dependence of the amide proton shifts in the tetrapeptides with an average B_1 value of 0.52 ppm/GPa (Table 4). The same is true for the second order coefficient B_2 that almost vanishes in NMA with a value of 0.02 ppm/GPa^2 and shows a large negative mean value of -0.41 ppm/GPa^2 in the tetrapeptides.

Correlations between pressure coefficients and chemical shift at ambient pressure

We had already described that the B_1 and B_2 values of the backbone atoms H^N, N, H^α, C^α, and C′ are significantly correlated with Pearson correlation coefficients between -0.51 and -0.80 (Beck Erlach et al. 2016). For a given position in the side chain the B_1 and B_2 values in most amino acids are quite well correlated (Fig. 5). The special type of the side chain (aliphatic, charged, aromatic) seems to be of secondary importance. Some characteristic outliers are the C^α of glycine, *cis*-proline and cystine, and the amide nitrogens of amino acids with long side chains (leucine, phenylalanine, tyrosine, and histidine). Since at least concerning the secondary structure prediction of proteins the C^β shifts are also important, it is interesting to test if a similar correlation is found. In fact, it is -0.83 (Fig. 5). What has not been tested before is a possible correlation between the chemical shifts

$δ_0$ at atmospheric pressure and the pressure coefficients. The $δ_0$ of H^N, N, and C^α are correlated to their own B_1 values with correlation coefficients of -0.6, -0.6, -0.7, respectively (Fig. 6). A surprising exception are the C^ζ atoms of aromatic residues and the guanidine group of arginine where $δ_0$, B_1, and B_2 are strongly correlated. Here a common feature is their participation in π-systems. However, care has to be taken with the strong correlations of C^ζ as there is only a limited amount of data points available (n = 5).

Correlation between pressure responses of chemical shifts of atoms in different positions

The correlation analysis shows that not only the B_1 and B_2 values of a given atom in a residue are more or less strongly correlated (see above), but that also correlations of the pressure response between different atoms in the same amino acid exists (Fig. 6). This was already reported for the backbone atoms (Beck Erlach et al. 2016). Most pronounced were the correlations between B_2 of H^N with B_2 of N (0.67) and H^α (-0.51), between B_2 of N with B_2 of H^α (-0.58), C^α (0.48) and C′ (0.58), between B_2 of H^α with B_2 of C^α (-0.62) and C^δ (0.77). These correlations are stronger for the second order coefficients than for the first order coefficients and are also largest for atoms that are direct neighbors in the chemical structure. However, also significant correlations exist between the first order coefficient B_1 of N with B_1 of C^α (0.54) and C′ (0.52), and between B_1 of C^α with B_1 of C′ (0.61).

A surprising fact that to our knowledge was not reported in literature is a correlation of the chemical shifts
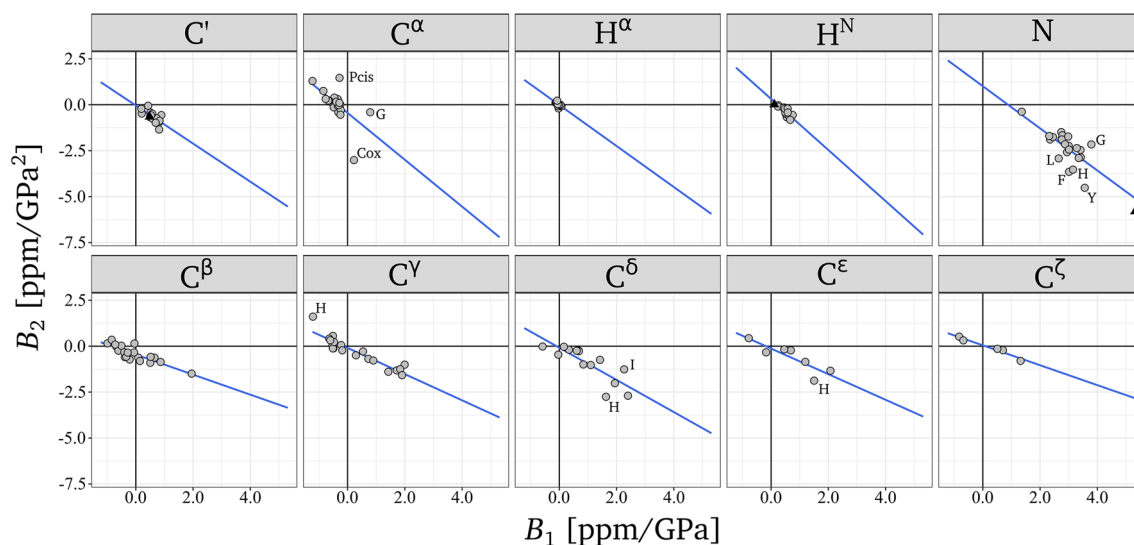
Fig. 5 Correlations between pressure coefficients B_1 and B_2 for all backbone atoms and side chain carbon atoms. Pearson correlation coefficients r are r(C′) = -0.72, r(C^α) = -0.75, r(H^α) = -0.65, r(H^N) = -0.80, r(N) = -0.54, r(C^β) = -0.83, r(C^γ) = -0.85, r(C^δ) = -0.82, r(C^ε) = -0.88, r(C^ζ) = -0.98. The filled triangles represent data from NMA (Frach et al. 2016). Outliers are labeled with the corresponding amino acid

Fig. 6 Correlation coefficients between δ_0, B_1 and B_2 values for the backbone atoms and side chain carbon atoms. This table summarizes the significant (p value <0.05) Pearson correlation coefficients for different nuclei. The upper right half depicts the value of the correlation coefficients as circles, where a bigger circle indicates a stronger correlation. Blue circles represent positive correlation coefficients ($r > 0$), whereas red circles represent negative correlation coefficients ($r < 0$). The lower left half shows the actual values of the correlation coefficient rounded to 1 digit after the decimal point

	C^α B_1	B_2	δ_0	C^β B_1	B_2	δ_0	C^δ B_1	B_2	δ_0	C^ε B_1	B_2	δ_0	C^γ B_1	B_2	δ_0	C' B_1	B_2	δ_0	C^ζ B_1	B_2	δ_0	H^α B_1	B_2	δ_0	H^N B_1	B_2	δ_0	N B_1	B_2	δ_0
C^α B_1																														
B_2	-0.8																													
δ_0	-0.7																													
C^β B_1																														
B_2				-0.8																										
δ_0																														
C^δ B_1																														
B_2	0.9	-0.8		0.8			-0.8																							
δ_0																														
C^ε B_1																														
B_2																														
δ_0			1.0			1.0			1.0																					
C^γ B_1				-0.6																										
B_2																														
δ_0												1.0																		
C' B_1	0.6						-0.8	0.9					-0.7																	
B_2								-0.9						-0.7																
δ_0															-0.7															
C^ζ B_1										1.0																				
B_2										1.0	-1.0		1.0						-1.0											
δ_0												-1.0							-1.0	1.0										
H^α B_1																			-0.6											
B_2	-0.6						0.8															-0.7								
δ_0															0.9															
H^N B_1			0.7			0.7																0.5								
B_2						-0.5																-0.5	-0.5	-0.8						
δ_0			-0.5												-0.6									-0.5	-0.6	0.5				
N B_1	0.5	-0.5	-0.5													0.5			-0.6											
B_2	0.5															0.6						-0.6				0.7		-0.5		
δ_0			0.5			-0.5			-1.0									0.7										-0.6		
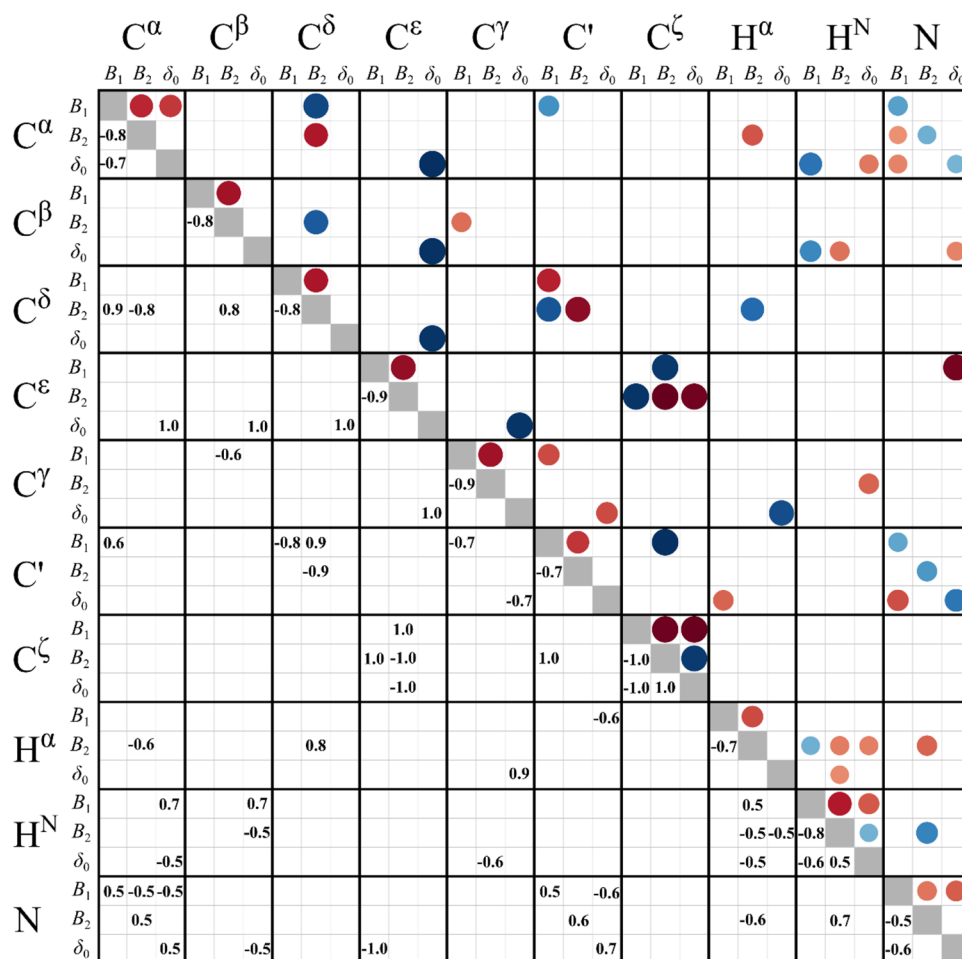
in random coil peptides at ambient pressure. δ_0 of H^N is correlated to δ_0 of C^α (−0.52), δ_0 of N is correlated to δ_0 of C^α (0.54) and C' (0.73), δ_0 of H^α is correlated to δ_0 of H^β (0.50), the δ_0 of H^β is correlated to δ_0 of C^β and C' (0.68, −0.82).

There is also a correlation between the chemical shifts of one atom and the pressure response of the atoms in close neighborhood (Fig. 6). δ_0 of H^N is correlated to B_2 of H^α (−0.50), H^β (0.52), and C^β (0.62), δ_0 of C^α is correlated to B_1 of H^N (0.74) and B_2 of N (−0.50), δ_0 of H^β is correlated to B_1 of H^N (0.62) and N (0.54) and B_2 of H^N (−0.56), δ_0 of C^β is correlated to B_1 of H^N (0.65) B_1 and B_2 of H^β (−0.65; −0.56), δ_0 of C' is correlated to B_1 of N (−0.64) and H^α (−0.59). Interestingly, the chemical shifts δ_0 of the amide proton and nitrogen show a negative correlation to the corresponding first order pressure coefficient B_1 (H^N: −0.62, N: −0.6). This is also true for C^ζ with a strong negative correlation of −0.98 between its chemical shift δ_0 and B_1. Figure 6 shows also some very strong correlations for the pressure coefficients of C^ζ and C^ε. The limited number of data points for these correlations may overemphasize the magnitude of the correlation coefficient. However, a common feature

is again that these carbon atoms are dominantly located in π-systems of aromatic side chains.

Origin of the pressure dependent chemical shift changes

Apart from using the random coil data for identifying processes occurring in the manifold of structured polypeptides, they can be used to understand chemical shift effects and possible structural transitions in so-called unstructured peptides. It can be expected that the conformational ensemble is shifted when pressure is applied. This effect will influence the observed average chemical shift and will be dependent on the amino acid in position 3 as it is observed also experimentally. This pressure dependent shift of the conformational equilibrium could be further quantified by measuring the pressure dependent changes of J-couplings and NOEs and correlate them with molecular dynamics calculations at different pressures. However, such an extensive study is not in the scope of this paper but is planned for the future. Nevertheless, we have already some evidence for such a pressure dependence of J-couplings in our random

coil peptides indicating a possible conformational response for the amide $^1J_{NH-N}$-couplings Koehler et al. (2014). In the IDPs α-synuclein the $^3J_{HN-Hα}$-coupling constants are pressure dependent and also indicate a shift of the conformational equilibrium with pressure (Roche et al. 2013).

A recently often discussed mechanism that influences the ^{13}C chemical shifts of side chains is the γ-gauche effect (Mackenzie et al 1996; London et al. 2008; Mulder 2009; Hansen et al. 2010; Kjaergaard et al. 2011a; Hansen and Kay 2011) since selective ^{13}C labeling of methyl groups is often used for structural characterization of large proteins. The γ-gauche effect is characterized by an upfield shift of ^{13}C signals due to a heavy atom in γ-position in gauche conformation coupled to the corresponding χ angle. The chemical shift difference of the two methyl groups in leucine and valine residues can be used to determine the average conformation of the side chains (London et al. 2008; Mulder 2009). Pressure leads to a downfield shift of all methyl groups and if this downfield shift would be due to the γ-gauche effect, it would mean a general decrease of the gauche conformation in all amino acids with methyl groups with pressure (Ala, Thr, Ile, Leu, Met, Val) and thermodynamically a larger volume of this conformation. A shift of the conformational ensemble to structures with smaller partial volumes could also explain part of the nonlinearity of the pressure response observed.

When aromatic residues are present, ring current shifts are to be expected to influence the chemical shifts of the environment (Perkins and Wüthrich 1979; Haigh and Mallion 1979). Small changes of the average ring orientation will strongly influence all resonances in the environment. However, there is also a direct pressure effect on the atoms of the ring systems themselves, visible as a strong correlation of $δ_0$, B_1, and B_2 values of the different carbons in the ring system (Fig. 6). The pressure effects on histidine are also strongly influenced by the protonation/deprotonation of the nitrogen atoms (note that in the deprotonated state an additional equilibrium exists between an isomer with the $N^{δ1}$ protonated and one with the $N^{ε2}$ protonated). Since pK values are pressure dependent, the largest effects are expected at pH values close to the pK as it is indeed observed (Tables 2, 3). For the positively charged species the pressure coefficients at pH 4.0 can be considered as representative.

Apart from this "classical" pressure response of the conformational ensemble additional pressure related effects have to be discussed. Direct compression effects such as changes of bond lengths and bond angles would also lead to chemical shift changes (Wilton et al. 2009). Wilton et al. (2009) calculated the average chemical shifts for two folded proteins, protein G and barnase. Here the average first order chemical shift coefficients are −0.9, −0.85, −0.45, and 1.2 ppm/GPa for $C^α$, CH, CH_2 and CH_3 resonances, respectively. Except of CH_2 resonances they are quite close to the average B_1 values in the tetrapeptides of −0.41, −0.71, −0.02 and 2.01 ppm/GPa (Table 4). This indicates, that for these groups the random-coil pressure effects determine strongly the average pressure response in folded proteins whereas for the methylene groups a clear influence of the three-dimensional structure on the average pressure response is found. A more detailed analysis would have to take into account the amino acid composition of these proteins.

To date not much data are available on model compounds. A quantum chemistry and infrared spectroscopy based high pressure study on the rigid small molecule trimethylamine N-oxide (TMAO) predicts that significant changes of geometry do not occur in the pressure range up to 200 MPa but are to be expected at very high pressures in the GPa-range (Imoto et al. 2016; Hölzl et al. 2016). Part of the observed random coil backbone shifts with pressure can be explained from experimental studies and quantum chemical calculations of the peptide bond model NMA (Frach et al. 2016; Kurrmann 2015). In this rigid molecule conformational dynamics except of a *cis/trans* isomerization and rotation of the methyl groups are not possible that probably additionally modify the pressure response in the tetrapeptides. Bond length variations or distortions as well as changes of force constants should not occur in NMA in the pressure range studied here and therefore should not significantly influence chemical shifts (S. M. Kast, personal communication). In this regard NMA data were not analyzed yet but data from another small molecules. Nevertheless, at least qualitatively chemical shifts of the backbone atoms H^N, N, and C′ of NMA show similar trends as in the tetrapeptides (Table 4). Also the two methyl ^{13}C resonances qualitatively behave analogously to the observations of the tetrapeptides. They show a nonlinear downfield shift with first order pressure coefficients of 2.8 and 3.0 ppm/GPa (Kurrmann 2015), quite similar to the values found for methyl groups in the tetrapeptides that vary between 1.72 ppm/GPa (Thr) and 2.40 ppm/GPa (Leu). The pressure effects observed for the methyl groups may also be due to a conformational equilibrium including a γ-gauche effect; however, only a *cis–trans* equilibrium of the peptide bond is possible here.

The quantum chemical calculations that include the electric field of the water shell reproduce the sign of the coefficients as well as the nonlinearity of the pressure response of N and C′ satisfactorily (Frach et al. 2016). It is clear from that study that more than the directly hydrogen bonded water molecules is required for explaining the shifts. Experimentally and theoretically the pressure response of the H^N in NMA does not have a significant second order contribution in the pressure range up to 200 MPa (Frach et al. 2016). Therefore, the pressure dependence of the backbone chemical shifts in random coil peptides and IDPs can be explained at least partly by the pressure response of the water shell around the proteins that in turn modifies the electron

distribution in the target molecules and thus the magnetic field at the nucleus and the resulting chemical shifts. However, the observed nonlinearity of amide proton shifts in the model peptides requires an additional factor that is not contained in the peptide bond model NMA. The same is true also for the side chain carbons as discussed above.

Conclusions

In summary, not only almost all backbone nuclei of the random coil model peptides show a pressure dependence of chemical shifts that can be fitted only by a nonlinear polynomial satisfactorily but this is also true for the side chain atoms. Probably, such an effect could also have been observed for residues showing no clear deviation from linearity up to 200 MPa, when higher pressures would had been available experimentally. Nonlinear pressure effects are taken as indicators of larger structural fluctuations of folded proteins as they are to be expected close to cavities inside the protein or in protein–protein interaction sites [see e.g. Li et al. (1998), Kalbitzer et al. (2000), Akasaka et al. (2006)]. They would occur in a pressure dependent multistate equilibrium with dynamics that are fast on the NMR time scale. From thermodynamic principles, all conformational transitions including localized transitions in a protein must first be handled as occurring in the whole protein unless one can show that they are completely uncoupled. Even then, spectroscopically they may be observed in regions further away since ring current effects can significantly influence the chemical shifts of atoms in a distance of more than 1 nm [the resonance line of a nucleus located above the center of a phenylalanine ring is shifted by -0.01 ppm in a distance of 1.76 nm (Hausser and Kalbitzer 1989)].

To distinguish more "trivial" pressure effects occurring in unstructured peptides from effects characterizing a specific transition in a folded protein, the pressure response of chemical shifts in random coil model peptides have to be known. For main chain and side chain protons they were reported earlier by Arnold et al. (2002), for backbone ^{13}C and ^{15}N atoms by Koehler et al. (2012) and Beck Erlach et al. (2016). Here, the pressure responses of side chain ^{13}C shifts are reported to complete the picture. Qualitatively, a pressure response caused by a structural transition of a folded or partly structured protein can be identified by chemical shift changes significantly different to those observed in a "random coil" peptide. When after subtracting the random coil pressure response from the pressure response of the native protein still significant changes are observed, it indicates a pressure dependent shift of a conformational equilibrium and the presence of some residual structure even when at normal pressures chemical shifts close to random coil values are detected (Kachel et al. 2006).

There is also a number of experimental and theoretical studies of high pressure on the secondary structure propensity. In most cases, α-helical structures are predicted to be stabilized by pressure. The chemical shift analysis of the H^{α}, C^{α}, C' and C^{β} resonances does not predict a clear preference of a specific secondary structure with pressure for any amino acid 3 (see above). The obtained pressure responses are not consistent with any pattern predicted for α-helices or β-strands.

References

Akasaka K (2006) Probing conformational fluctuation of proteins by pressure perturbation. Chem Rev 106:1814–1835

Arnold MR, Kremer W, Lüdemann HD, Kalbitzer HR (2002) ^{1}H-NMR parameters of common amino acid residues measured in aqueous solutions of the linear tetrapeptides Gly-Gly-X-Ala at pressures between 0.1 and 200 MPa. Biophys Chem 96:129–140

Beck Erlach M, Munte CE, Kremer W, Hartl R, Rochelt D, Niesner D, Kalbitzer HR (2010) Ceramic cells for high pressure NMR spectroscopy on proteins. J Magn Reson 204:196–199

Beck Erlach M, Koehler J, Moeser B, Horinek D, Kremer W, Kalbitzer HR (2014) Relationship between nonlinear pressure-induced chemical shift changes and thermodynamic parameters. J Phys Chem B 118:5681–5690

Beck Erlach M, Koehler J, Crusca E Jr, Kremer W, Munte CE, Kalbitzer HR (2016) Pressure dependence of backbone chemical shifts in the model peptides Ac-Gly-Gly-Xxx-Ala-NH$_2$. J Biomol NMR 65:65–77

Braun D, Wider G, Wüthrich K (1994) Sequence-corrected ^{15}N random coil chemical shifts. J Am Chem Soc 116:8466–8469

Bundi A, Wüthrich K (1979) ^{1}H-NMR parameters of the common amino acid residues measured in aqueous solutions of the linear tetrapeptides H-Gly-Gly-X-L-Ala-OH. Biopolymers 18:285–297

De Simone A, Cavalli A, Hsu S.T.D., Vranken W, Vendruscolo M (2009) Accurate random coil chemical shifts from an analysis of loop regions in native states of proteins. J Am Chem Soc 131:16332–16333

Frach R, Kibies P, Böttcher S, Pongratz T, Strohfeldt S, Kurrmann S, Koehler J, Hofmann M, Kremer W, Kalbitzer HR, Reiser O, Horinek D, Kast SM (2016) The chemical shift baseline for high-pressure NMR spectroscopy of proteins. Angew Chem Int Ed 55:8757–8760

Gronwald W, Kalbitzer HR (2004) Automated structure determination of proteins by NMR spectroscopy. Prog Nucl Magn Reson Spectrosc 44:33–96

Gross M, Jaenicke R (1994) Proteins under pressure. The influence of high hydrostatic pressure on structure, function and assembly of proteins and protein complexes. Eur J Biochem 221:617–630

Haigh CW, Mallion RB (1979) Ring current theories in nuclear magnetic resonance. Prog Nucl Magn Reson Spectrosc 13:303–344

Halle B, Denisov VP (2001) Magnetic relaxation dispersion studies of biomolecular solutions. Methods Enzymol 338:178–201

Hansen DF, Kay LE Determining valine side-chain rotamer conformations in proteins from methyl 13C chemical shifts: application to the 360 kDa half-proteasome. J Am Chem Soc, 8272–8281 (2011)

Hansen DF, Neudecker P, Kay LE (2010) Determination of isoleucine side-chain conformations in ground and excited states of proteins from chemical shifts. J Am Chem Soc 132:7589–7591

Hausser KH, Kalbitzer HR (1989) NMR für Mediziner und Biologen. Strukturbestimmung, Bildgebung, In-Vivo-Spektroskopie. Springer, Heidelberg

Hölzl C, Kibies P, Imoto S, Frach R, Suladze S, Winter R, Marx D, Horinek D, Kast SM (2016) Design principles for high–pressure force fields: aqueous TMAO solutions from ambient to kilobar pressures. J Chem Phys 144:144104

Imoto S, Kibies P, Rosin C, Winter R, Kast SM, Marx D (2016) Toward extreme biophysics: deciphering the infrared response of biomolecular solutions at high pressures. Angew Chem Int Ed 55:9534–9538

Jimenez MA, Nieto JL, Rico M, Santoro J, Herranz J, Bermejo FJ (1986) A study of the NH NMR signals of Gly-Gly-X-Ala tetrapeptides in H_2O at low temperature. J Mol Struct 143:435–438

Kachel N, Kremer W, Zahn R, Kalbitzer HR (2006) Observation of intermediate states of the human prion protein by high pressure NMR spectroscopy. BMC Struct Biol 16:6

Kainosho M (1997) Isotope labelling of macromolecules for structural determinations. Nat Struct Biol 4:858–861

Kainosho M, Güntert P (2009) SAIL—stereo-array isotope labeling. Q Rev Biophys 42:247–300

Kainosho M, Torizawa T, Iwashita Y, Terauchi T, Mei Ono A, Güntert P (2006) Optimal isotope labelling for NMR protein structure determination. Nature 440:52–57

Kalbitzer HR, Görler A, Li H, Dubovskii P, Hengstenberg W, Kowolik C, Yamada H, Akasaka K (2000) ^{15}N and ^1H NMR study of histidine containing protein (HPr) from *Staphylococcus carnosus* at high pressure. Prot Sci 9:693–703

Kalbitzer HR, Spoerner M, Ganser P, Hosza C, Kremer W (2009) Fundamental link between folding states and functional states of proteins. J Am Chem Soc 131:16714–16719

Kalbitzer HR, Rosnizeck IC, Munte CE, Puthenpurackal Narayanan S, Kropf V, Spoerner M (2013) Intrinsic allosteric inhibition of signaling proteins by targeting rare interaction states detected by high-pressure NMR spectroscopy. Angew Chem Int Ed 52:14242–14246

Kitahara R, Hata K, Li H, Williamson MP, Akasaka K (2013) Pressure-induced chemical shifts as probes for conformational fluctuations in proteins. Prog Nucl Magn Reson Spectrosc 71:35–58

Kjaergaard M, Poulsen FM (2011) Sequence correction of random coil chemical shifts: correlation between neighbor correction factors and changes in the Ramachandran distribution. J Biomol NMR 50:157–165

Kjaergaard M, Iesmantavicius V, Poulsen FM (2011a) The interplay between transient α-helix formation and side chain rotamer distributions in disordered proteins probed by methyl chemical shifts. Prot Sci 20:2023–2034

Kjaergaard M, Brander S, Poulsen FM (2011b) Random coil chemical shift for intrinsically disordered proteins: effects of temperature and pH. J Biomol NMR 49:139–149

Koehler J, Beck Erlach M, Crusca E, Kremer W, Munte CE, Kalbitzer HR (2012) Pressure dependence of ^{15}N chemical shifts in model peptides Ac-Gly-Gly-X-Ala-NH_2. Materials 5:1774–1786

Koehler J, Beck Erlach M, Crusca E Jr, Kremer W, Munte CE, Meier A, Kalbitzer HR (2014) Pressure response of amide one-bond *J*-couplings in model peptides and proteins. J Biomol NMR 60:45–50

Kurrmann S (2015) Hochdruck-NMR-Spektroskopie an Trimethylamin-N-Oxid und N-Methyl-Acetamid. Bachelor thesis, University of Regensburg

Li H, Yamada H, Akasaka K (1998) Effect of pressure on individual hydrogen bonds in proteins. Basic pancreatic trypsin inhibitor. Biochemistry 37:1167–1173

London RE, Wingad BD, Mueller GA (2008) Dependence of amino acid side chain 13C shifts on dihedral angle: application to conformational analysis. J Am Chem Soc 130:11097–11105

MacKenzie KR, Prestegard JA, Engelman DM (1996) Leucine side-chain rotamers in a glycophorin A transmembrane peptide as revealed by three-bond carbon-carbon couplings and 13C chemical shifts. J Biomol NMR 7:256–260

Markley JL, Bax A, Arata Y, Hilbers CW, Kaptein R, Sykes BD, Wright PE, Wüthrich K (1998) Recommendations for the presentation of NMR structures of proteins and nucleic acids. Pure Appl Chem 70:117–142

Merutka G, Dyson HJ, Wright PE (1995) 'Random coil' ^1H chemical shifts obtained as a function of temperature and trifluoroethanol concentration for the peptide series GGXGG. J Biomol NMR 5:14–24

Miyanoiri Y, Takeda M, Okuma K, Ono AM, Terauchi T, Kainosho M (2013) Differential isotope-labeling for leu and val residues in a protein by *E. coli* cellular expression using stereo-specifically methyl labeled amino acids. J Biomol NMR 57:237–249

Miyanoiri Y, Ishida Y, Takeda M, Terauchi T, Inouye M, Kainosho M (2016) Highly efficient residue-selective labeling with isotope-labeled ile, leu, and val using a new auxotrophic *E. coli* strain. J Biomol NMR 65:109–119

Mulder FAA (2009) Leucine side-chain conformation and dynamics in proteins from C-13 NMR chemical shifts. ChemBioChem 10:1477–1479

Munte CE, Beck Erlach M, Kremer W, Koehler J, Kalbitzer HR (2013) Distinct conformational states of the alzheimer β-amyloid peptide can be detected by high pressure nmr spectroscopy. Angew Chem Int Ed 52:8943–8947

Neri D, Sys.persky T, Otting G, Senn H, Wüthrich K (1989) Stereospecific nuclear magnetic resonance assignments of methyl groups of valine and leucine in the DNA-binding domain of the 434 repressor by biosynthetically directed fractional 13C labelling. BioChemistry 28:7510–7516

Neudecker P, Lundstroem P, Kay LE (2009) Relaxation dispersion NMR spectroscopy as a tool for detailed studies of protein folding. Biophys J 96:2045–2054

Perkins SJ, Wüthrich K (1979) Ring current effects in the conformation dependent nmr chemical shifts of aliphatic protons in the basic pancreatic trypsin inhibitor. Biochim Biophys Acta 576:409–423

Peterson RW, Wand AJ (2005) Self-contained high pressure cell, apparatus, and procedure for the preparation of encapsulated proteins dissolved in low viscosity fluids for nuclear magnetic resonance spectroscopy. Rev Sci Instrum 76:094101

Peti W, Smith LJ, Redfield C, Schwalbe H (2001) Chemical shifts in denatured proteins: resonance assignments for denatured ubiquitin and comparisons with other denatured proteins. J Biomol NMR 19:153–165

Platzer G, Okon M, McIntosh LP (2014) pH-dependent random coil ^1H, ^{13}C, and ^{15}N chemical shifts of the ionizable amino acids: a guide for protein pK_a measurements. J Biomol NMR 60:109–129

Plaxco KW, Morton CJ, Grimshaw SB, Jones JA, Pitkeathly M, Campbell ID, Dobson CM (1997) The effects of guanidine hydrochloride on the 'random coil' conformations and NMR chemical shifts of the peptide series GGXGG. J Biomol NMR 10:221–230

R Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Raiford DS, Fisk CL, Becker ED (1979) Calibration of methanol and ethylene glycol nuclear magnetic resonance thermometers. Anal Chem 51:2050–2051

Richarz R, Wüthrich K (1978) Carbon-13 NMR chemical shifts of the common amino acid residues measured in aqueous solutions of the linear tetrapeptides H-Gly-Gly-X-L-Ala-OH. Biopolymers 17:2133–2141

Roche J, Ying J, Maltsev AS, Bax A (2013) Impact of hydrostatic pressure on an intrinsically disordered protein: a high-pressure NMR study of α-synuclein. ChemBioChem 14:1754–1761

Rosnizeck IC, Graf T, Spoerner M, Tränkle J, Filchtinski D, Herrmann C, Gremer L, Vetter IR, Wittinghofer A, König B, Kalbitzer HR (2010) Stabilizing a weak binding state for effectors in the human ras-protein by cyclen complexes. Angew Chem Int Ed 49:3830–3833

Rosnizeck IC, Spoerner M, Harsch T, Kreitner S, Filchtinski D, Herrmann C, Engel D, König B, Kalbitzer HR (2012) Metal–Bis(2-picolyl)amine complexes as state 1(T) inhibitors of activated ras protein. Angew Chem Int Ed 51:10647–10651

Schwarzinger S, Kroon GJ, Foss TR, Wright PE, Dyson HJ (2000) Random coil chemical shifts in acidic 8M urea: implementation of random Coil shift data in NMRView. J Biomol NMR 18:43–48

Schwarzinger S, Kroon GJ, Foss TR, Chung J, Wright PE, Dyson HJ (2001) Sequence-dependent correction of random coil NMR chemical shifts. J Am Chem Soc 123:2970–2978

Tamiola K, Acar B, Mulder FAA (2010) Sequence-specific random coil chemical shifts of intrinsically disordered proteins. J Am Chem Soc 132:18000–18003

Tessier M, Albericio F, Pedroso E, Grandas A, Eritja R, Giralt E, Granier C, van Rietschoten J (1983) Amino-acids condensations in the preparation of Nα-9-fluorenylrnethyloxycarbonylamino-acids with 9-fluorenylmethylchloroformate. Chem Biol Drug Des 22:125–128

Thanabal V, Omecinsky DO, Reily MD, Cody WL (1994) The ^{13}C chemical shifts of amino acids in aqueous solution containing organic solvents: application to the secondary structure characterization of peptides in aqueous trifluoroethanol solution. J Biomol NMR 4:47–59

Van Zijl PCM, Yadev NN (2011) Chemical exchange saturation transfer (CEST): what is in a name and what isn't? Magn Reson Med 65:927–948

Wang Y, Jardetzky O (2002) Probability-based protein secondary structure identification using combined NMR chemical-shift data. Protein Sci 11:852–861

Wilton DJ, Kitahara R, Akasaka K, Williamson MP (2009) Pressure-dependent 13C chemical shifts in proteins: origins and applications. J Biomol NMR 44:25–33

Wishart DS, Sykes BD (1992) The chemical shift index: a fast and simple method for the assignment of protein secondary structure through NMR spectroscopy. J Biomol NMR 31:1647–1651

Wishart DS, Sykes BD (1994) The ^{13}C chemical-shift index: a simple method for the identification of protein secondary structure Using ^{13}C chemical-shift data. J Biomol NMR 4:171–180

Wishart DS,Bigam CG, Yao J, Abildgaard F, Dyson HJ, Oldfield E, Markley JL, Sykes BD (1995a) ^{1}H, ^{13}C and ^{15}N chemical shift referencing in biomolecular NMR. J Biomol NMR 6:135–140

Wishart DS, Bigam CG, Holm A, Hodges RS, Sykes BD (1995b) ^{1}H, ^{13}C and ^{15}N random coil NMR chemical shifts of the common amino acids. I. investigations of nearest-neighbor effects. J Biomol NMR 5:67–82

Wu B, Warnock G, Zaiss M, Lin C, Chen M, Zhou Z, Mu L, Nanz D, Tuura R, Delso G (2016) An overview of CEST MRI for non-MR physicists. EJNMMI Phys 3:19

Zhang H, Neal S, Wishart DS (2003) RefDB a database of uniformly referenced protein chemical shifts. J Biomol NMR 25:173–195