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Effects of Dimerization on the Structure and Biological Activity of Antimicrobial Peptide Ctx-Ha

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It is well known that cationic antimicrobial peptides (cAMPs) are potential microbicidal agents for the increasing problem of antimicrobial resistance. However, the physicochemical properties of each peptide need to be optimized for clinical use. To evaluate the effects of dimerization on the structure and biological activity of the antimicrobial peptide Ctx-Ha, we have synthesized the monomeric and three dimeric (Lys-branched) forms of the Ctx-Ha peptide by solid-phase peptide synthesis using a combination of 9-fluorenylmethyloxycarbonyl (Fmoc) and *t*-butoxycarbonyl (Boc) chemical approaches. The antimicrobial activity assay showed that dimerization decreases the ability of the peptide to inhibit growth of bacteria or fungi; however, the dimeric analogs displayed a higher level of bactericidal activity. In addition, a dramatic increase (50 times) in hemolytic activity was achieved with these analogs. Permeabilization studies showed that the rate of carboxyfluorescein release was higher for the dimeric peptides than for the monomeric peptide, especially in vesicles that contained sphingomyelin. Despite different biological activities, the secondary structure and pore diameter were not significantly altered by dimerization. In contrast to the case for other dimeric cAMPs, we have shown that dimerization selectively decreases the antimicrobial activity of this peptide and increases the hemolytic activity. The results also show that the interaction between dimeric peptides and the cell wall could be responsible for the decrease of the antimicrobial activity of these peptides.

Due to the rapid development of bacterial resistance to conventional antibiotics, there is currently an extensive search for alternative antimicrobial agents (15, 29). Cationic antimicrobial peptides (cAMPs) appear to be an interesting alternative because they are not hindered by resistance mechanisms, which are placing conventional antibiotics in jeopardy (25). cAMPs are part of the innate defense systems of several organisms, including animals, plants, insects, and microorganisms, and exhibit a broad spectrum of antimicrobial activity against bacteria, fungi, and viruses (30). In addition, cAMPs have even been shown to have anticancer activity (19, 24). cAMPs are composed of 10 to 45 amino acids that are rich in hydrophobic and basic amino acid residues and can adopt an amphipathic α-helical secondary structure within the cell membrane. Although this mechanism is not completely understood, it is generally accepted that these peptides interact with the surface of the membrane, penetrate the lipid bilayer, promote the leakage of cytoplasmic components, and result in cell death.

Several mechanisms have been proposed to explain the membrane permeabilization induced by cAMPs. In the "barrel-stave" model, membrane lysis is performed by pore formation formed by amphipathic cAMP units where the exposed hydrophobic regions are aligned with the lipid core region of the bilayer and the hydrophilic peptide regions form the interior face of the pore. In a second model, which is named the "toroidal pore" model, the peptides promote bending of the bilayer, which forms a pore. In this process, the peptide is tightly bound to the polar lipid groups. Lastly, in the "carpet-like" model, the peptides are electrostatically attracted to the anionic phospholipid head groups and cover the membrane like a carpet. They remain parallel to the cellular surface, and when a threshold concentration of peptide is reached, the peptides rotate and reorient themselves toward the hydrophobic core of the membrane to induce the disintegration and/or micellization of the membrane (3, 6, 41).

Although cAMPs are evaluated as interesting novel therapeutic agents for clinical purposes, including as single antimicrobial agents and as synergistic agents with existing antibiotics, a number of problems must be solved before they can be effectively used. Thus, selected strategies have been performed to improve peptide stability, efficacy, delivery, and selectivity (10, 21, 27, 32, 34, 38, 45).

As the plasma membrane is the target for cAMPs, selectivity is important and is responsible for their cytotoxicity to human cells. Therefore, various studies have attempted to determine the parameters that are responsible for this toxicity. It has been determined that high helicity, hydrophobicity, and amphipathicity are correlated with an increase in eukaryotic cell toxicity (5). We have also shown that the N-terminal region is important for the hemolytic activity of synthetic peptides containing sequences from the N termini of sticholysins (7) and for selectivity of the cAMPs (10). Therefore, efforts have been undertaken to improve the selectivity of cAMPs, including sequence modification to optimize their physicochemical parameters (18, 27, 38, 43).

Recent studies have shown that aggregation/oligomerization of cAMPs is another parameter that is important for pore formation, regardless of the mechanism of cytotoxicity (28, 33). Therefore, dimerization is a new parameter that needs to be studied. The studies of dimeric forms of select bioactive peptide sequences have shown pharmacotechnical advantages, such as enhanced antimicrobial potency, solubility, and resistance to proteases (13, 26, 32, 44). These issues make dimeric cAMPs promising candidates for

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the development of novel antibiotic agents. However, the effects of dimerization on antimicrobial activity are unclear because dimeric versions of some cAMPs have been toxic (6, 40, 45).

In this study, we analyzed the effects of dimerization and the length/polarity of the spacer unit on the structure and biological activity of the cAMP Ctx-Ha (ceratotoxin-like peptide from *Hypsiboas albopunctatus*; Gly-Trp-Leu-Asp-Val-Ala-Lys-Lys-Ile-Gly-Lys-Ala-Ala-Phe-Asn-Val-Ala-Lys-Asn-Phe-Leu-$CONH_2$) and three homodimeric forms that were Lys branched. Ctx-Ha is a natural antimicrobial peptide, isolated from frog skin, with potent antimicrobial and hemolytic activities. This peptide is cationic and has an amphipathic α-helical structure (4). The rationale for using homodimers of Ctx-Ha is that dimerization could lead to an increased reaction rate and antimicrobial activity.

MATERIALS AND METHODS

Chemicals and microorganisms. Only analytical-grade reagents from commercial suppliers were used throughout this work, and all solutions were prepared with Milli-Q water (Millipore reagent water system). Solvents for chromatographic procedures were of high-pressure liquid chromatography (HPLC) grade and were from several sources. All 9-fluorenylmethyloxycarbonyl (Fmoc) amino acids and resins were purchased from Synbiosci and Novabiochem. Solvents and reagents for peptide synthesis were from Sigma-Aldrich Co. and Fluka. Dichloromethane (DCM) and dimethylformamide (DMF) were purchased from Hexis Cientifica. All lipids were purchased from Avanti Polar Lipids. The microorganisms used in this study were bacterial strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 and yeast strain *Candida albicans* ATCC 18804.

Peptide synthesis. All peptide syntheses were manually performed using a solid-phase peptide synthesis (SPPS) protocol (14). The monomeric peptide (MON) and dimeric peptide without spacer (DSE) were synthesized using the standard 9-fluorenylmethyloxycarbonyl (Fmoc) strategy (10) on Rink amide 4-methylbenzhydrylamine (MBHA) resin. For the dimeric peptides, Fmoc-Lys(Fmoc)-OH was used as a linker unit for the synthesis of the chains. Dimeric peptides containing spacers were synthesized using a combination of the Fmoc and *t*-butoxycarbonyl (Boc) chemical approaches on benzhydrylamine (BHA) resin. In these syntheses, Fmoc-Lys(Boc)-OH was coupled to resin, and after deprotection with 30% trifluoroacetic acid (TFA)-dichloromethane (DCM) (2 and 30 min), the Fmoc-8-amino-3,6-dioxaoctanoic and Fmoc-8-amino-octanoic acid groups were introduced to obtain dimeric forms with a polar spacer (DEP) and an apolar spacer (DEA), respectively.

The cleavage of Rink MBHA resin and removal of the side chain protecting groups were simultaneously performed with 95% TFA, 2.5% water, and 2.5% *p*-cresol. The cleavage of BHA resin was performed with 73.5% TFA, 12.5% thioanisole, 9% trifluoromethanesulfonic acid, and 5% *m*-cresol (22).

Crude peptides were purified by semipreparative HPLC on a Shimatzu system (Japan) using a reverse-phase C_{18} column. The peptide homogeneity was determined by analytical HPLC and analyzed using electrospray mass spectrometry.

Antimicrobial assay. The MIC and minimum microbicidal concentration (MMC) were determined following the recommendations of the Clinical and Laboratory Standards Institute (8, 9).

Kinetics of bactericidal activity. The kinetics of bactericidal activity of the peptides were assessed using *E. coli* at a concentration of two times the MIC for each peptide. These assays were performed using the experimental procedures described by Eckert et al. (12). Briefly, the initial density of the cultures was approximately 1×10^5 CFU/ml. The peptides were added to the cell suspension and incubated at 25°C. At different times (0, 10, 20, 30, 40, 60, and 80 min), 20 μl of cell suspension was removed and diluted 100-fold, and the volume was adjusted to 1,000 μl. This solution was spread on LB agar plates, and colonies were counted after overnight incubation at 37°C. Growth controls were performed in the absence of peptides from samples at the starting and final time points.

Vesicle permeabilization. The release of carboxyfluorescein (CF) from vesicles was measured by the fluorescence intensity at a wavelength of 520 nm (492-nm excitation wavelength) after the addition of 0.01 to 0.02 μmol $liter^{-1}$ of peptide. Data were acquired using a fluorescence spectrophotometer (Cary Eclipse; Varian). The experiments were performed at 25°C. Determination of the value for 100% of release was achieved using Triton X-100. The final value was the percentage of release of CF after the addition of peptides into the system, obtained using the equation percent release = $(F_{fin} - F_{in})/(F_{max} - F_{in}) \times 100$, where F_{in} and F_{fin} represent the initial and final fluorescence values before and after the addition of peptides, respectively, and F_{max} represents the fluorescence after the addition of Triton-X. All experiments were performed in triplicate.

Two kinds of large unilamellar vesicles (LUVs) were used. One LUV contained 80% 1,2-dipalmitoyl-3-phosphocholine (DPPC), 5% 1,2-dipalmitoyl-3-phosphatidic acid (DPPA), and 15% 1,2-dipalmitoylphosphatidylethanolamine (DPPE), and the other LUV contained 80% DPPC, 5% DPPA, and 15% sphingomyelin (SM). Both LUVs were prepared by mixing the appropriate amounts of lipid in a 4:1 chloroform-methanol mixture in a round-bottom flask. The solvent was rapidly evaporated using nitrogen gas. The lipid biofilm was placed under a vacuum overnight and hydrated at 60°C with 80 mmol $liter^{-1}$ of CF in Tris (0.01 mol $liter^{-1}$, pH 7.4) and NaCl (0.15 mol $liter^{-1}$) to give a final lipid concentration of 15 mmol $liter^{-1}$. This suspension was extruded 40 times through two stacked Nucleopore polycarbonate filters (100-nm pore size) using an extruder system from Avanti Polar Lipids at approximately 40°C. Vesicles were separated from nonencapsulated CF by gel filtration on a Sephadex G-50 column using Tris (0.01 mol $liter^{-1}$, pH 7.4) and NaCl (0.15 mol $liter^{-1}$) for elution.

Hemolysis assay. Hemolysis assays were performed using the experimental procedure described by Castro et al. (3). Briefly, before use, freshly prepared human red blood cells (RBCs) were washed three times with 0.01 M Tris-HCl (pH 7.4) containing 0.15 M NaCl (Tris-saline). A suspension of 1% (vol/vol) erythrocytes was made with packed red blood cells resuspended in Tris-saline. Synthetic peptides were dissolved in Tris-saline at an initial concentration of 128 mM and were serially diluted in the same buffer to determine the concentration that causes 50% hemolysis (HC_{50}). As a positive control (100% lysis), a 1% (vol/vol) Triton X-100 solution was used. After incubation for 1 h at 37°C, the samples were centrifuged at $3,000 \times g$ for 5 min. Aliquots of 100 μl of the supernatant were transferred to 96-well microplates, and the absorbance was determined at 405 nm. The assay was performed in triplicate.

Pore size determination. The sizes of the pores formed by the synthesized peptides in red blood cells (RBCs) were estimated using the "osmotic protection" method based on polyethylene glycols (PEGs) of increasing size. RBC solution containing 30 mmol $liter^{-1}$ of osmotic protector was mixed with the peptides at twice the respective HC_{50}. The percentage of hemolysis in the presence of different PEGs was determined as described for the hemolysis assay. The following osmotic protectants were used: PEG 200 (mean hydrated diameter, ≈0.9 nm), PEG 300 (≈1.2 nm), PEG 400 (≈1.4 nm), PEG 600 (≈1.5 nm), PEG 900 (≈1.7 nm), PEG 1000 (≈1.8 nm), PEG 1500 (≈2.4 nm), PEG 3000 (≈3.0 nm), and PEG 4000 (≈ 3.8 nm) (2, 42). The assay was performed in triplicate.

CD spectra. Circular dichroism (CD) spectra were obtained at between 190 and 250 nm with a JASCO J-715 CD spectrophotometer on nitrogen flush in 1-mm-path-length quartz cuvettes at room temperature. To investigate the conformational changes caused by membrane environments, Milli-Q water and 10 mmol $liter^{-1}$ of lysophosphatidylcholines (LPC) were used. The peptide concentration was 60 μmol $liter^{-1}$. CD spectra were typically recorded as an average of six scans, which were obtained in millidegrees and converted to molar ellipticity (θ) (in degrees cm^2 $dmol^{-1}$).

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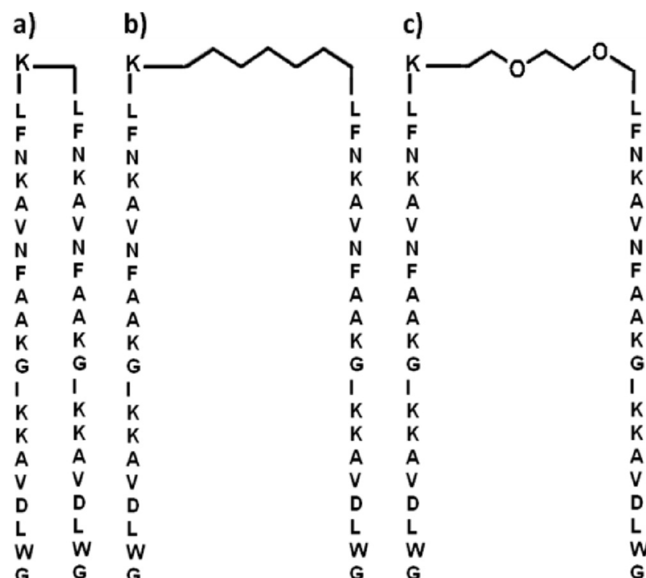

FIG 1 DSE (a), DEA (b), and DEP (c). Spacer groups used in DEA and DEP are represented by the carbon backbone. Amino acids are represented in one-letter code.

Statistics. Values were reported as the averages from at least three independent experiments. The coefficient of variation (CV) was defined as the standard deviation of a set of measurements divided by the mean. To determine significance, analyses of variance (ANOVA) (GraphPad Software, San Diego, CA) were done. Significance was accepted at a P value of <0.05.

RESULTS AND DISCUSSION

Peptide design. One of the principal problems that restrict the use of cAMPs as antibiotics is their toxicity, which is because cAMPs target the cell membrane. Thus, many cAMPs cannot discriminate between eukaryotic and prokaryotic cells. Several efforts have been made to improve the cellular selectivity of cAMPs, including the optimization of physicochemical parameters (5, 18, 27, 38, 43). It is believed that high hydrophobicity and helicity are correlated with this selectivity (6, 38). In addition, the self-association (oligomerization) of cAMPs in an aqueous environment is also known to be important for controlling the selectivity of the cAMPs (6, 44). This self-association can be mimicked using synthetic dimers. Several studies have shown that dimerization of cAMPs improves the desirable antibiotic-like characteristics, such as *in vitro* and *in vivo* antimicrobial activity, solubility, serum stability, and activity at a high salt concentration (13, 23, 36, 39). However, other work has shown that the dimerization decreases the antimicrobial activity of cAMPs (40, 45). Thus, the use of dimeric cAMPs remains controversial. To evaluate the effects of the length and polarity of the spacer used in the dimerization of Ctx-Ha, we synthesized the monomeric peptide and three different dimeric forms (Lys branched with different spacers) using SPPS methodology and determined their antimicrobial properties (11, 16, 23, 39). The properties of the spacer could affect the interaction with the head groups of the membrane phospholipids or cell wall, which would change the biological activities of the peptides. DSE has only a Lys residue between the two chain of Ctx-Ha, and it is smaller than DEP and DEA. DEP has the 8-amino-3,6-dioxaoctanoic acid group and a Lys residue in its structure, and it is more polar than DEA. DEA dimer contained the Lys residue and the 8-amino-octanoic acid group between the peptide chains. Thus, DEP has ethoxy groups (polar groups) in the structure, and DEA contains methyl groups (apolar groups); however, these two spacers are the same size (Fig. 1). MON and DSE were obtained using the conventional Fmoc/*t*-butyl (tBu) protocol. In the synthesis of DSE, the first amino acid coupled to resin was the trifunctional amino acid Fmoc-Lys(Fmoc)-OH. After coupling and deprotection with 20% piperidine in DMF, two reactive amino ends that allowed for the generation of the dimeric peptide were produced (35). DEP and DEA peptides were synthesized using a combination of Fmoc and Boc chemical approaches. In these syntheses we used the BHA resin, since the peptide-resin cleavage does not occur when using TFA. Initially, the trifunctional amino acid Fmoc-Lys(Boc)-OH was coupled to resin, and the partial deprotection of this residue was achieved using 30% TFA-DCM. This mixture deprotected only the Boc group, which released the ε-amino group for coupling. The spacer, Fmoc-8-amino-3,6-dioxaoctanoic acid or Fmoc-8-amino-octanoic acid, was coupled, and the Fmoc protector was eliminated using 20% piperidine in DMF. This process produced two reactive amino group ends (the α-amino group of Lys and the amino groups of the spacers), which allowed for the synthesis of the dimeric peptide. Analysis by reverse-phase chromatography and mass spectrometry (Table 1) confirmed the success of the syntheses and purifications (up to 98% purity).

Biological activity. Table 2 shows the biological activities of the synthesized peptides. The antimicrobial assay showed that dimerization of Ctx-Ha, except for DEP against *E. coli*, did not improve the ability of the peptide to inhibit the growth of bacteria or fungi, and the MIC values for the dimers were similar to or greater than those for MON (Table 2). DSE showed the same activity against *E. coli* and *C. albicans*, but it was less active against *S. aureus* than MON. In addition, DEA was the worst peptide for killing bacteria and fungi. The same conclusions can be extended to the MMC values. These results are in agreement with recent studies that have shown that dimerization does not always lead to improved antimicrobial activity (40, 45).

In addition, HC_{50} values were determined to evaluate the toxicities of the peptides (Table 2). The hemolysis test has been the most commonly used procedure to assess and characterize the toxicities of cAMPs because it is a sensitive assay to evaluate eukaryotic membrane damage. No significant differences were observed between the dimers. However, the hemolytic activities of the dimeric forms were 50 times greater than that of the monomer, which shows that dimerization significantly enhanced peptide activity against human red blood cells. These results are similar to those from other studies, which have reported no

TABLE 1 Peptide synthesis results

Peptide	Resin	Yield (%)[a]	Retention time (min)	Mass (Da)	
				Observed[b]	Calculated
MON	Rink MBHA	84	18.1	2,289.6	2,289.8
DSE	BHA	11	19.6	4,690.5	4,690.6
DEP	BHA	6	19.4	4,836.0	4,835.8
DEA	BHA	16	19.6	4,832.0	4,831.8

[a] Determined by reverse-phase HPLC of the crude product.
[b] Determined from mass spectra of the purified peptides.

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TABLE 2 Biological activities of the peptides[a]

Peptide	MIC (μmol liter^{-1})			MMC (μmol liter^{-1})			HC_{50} (μmol liter^{-1}), mean ± SD
	E. coli	*S. aureus*	*C. albicans*	*E. coli*	*S. aureus*	*C. albicans*	
MON	3.4	1.7	54.6	3.4	6.8	<100	37.3 ± 1.4
DSE	3.3	8.9	53.3	13.3	13.3	53.3	0.6 ± 0.1*
DEP	1.6*	3.2	51.7	3.2	12.9	51.7	0.7 ± 0.1*
DEA	6.5	12.9	100	12.9	25.9	<100	0.8 ± 0.1*

[a] *, Statistically significant difference (decrease) compared to result for MON ($P < 0.05$ by ANOVA; $n = 4$); SD, standard deviation. The CV (standard deviation of a set of measurements divided by the mean) was lower than 20% for MIC and MMC.

improvement in antimicrobial activity and higher eukaryotic cell toxicity due to peptide dimerization (6, 40, 45). As described by Jiang et al. (20), self-associated peptides may be inhibited from passing through the capsules and cell walls of prokaryotic cells to reach the membrane. However, peptide self-association was directly related to increased hemolytic activity of human red blood cells, because there is no capsule or cell wall to prevent access to the cytoplasmic membrane. In addition, the decrease in antimicrobial activity was higher for peptides that contained the apolar spacer. The lower activity of these peptides could be indicative of specific apolar interactions between the peptide and cell wall.

Vesicle permeabilization. The ability of the synthesized peptides to permeabilize vesicles was measured by the release of CF. The results are presented in Fig. 2. For both vesicle types, the kinetic release profiles for the dimers were characterized by an initial rapid efflux and instantaneous release of the dye, while the monomeric form at the same concentration (0.01 μmol liter^{-1}) showed reduced and slower permeabilization, characterized by a sustained release of the fluorophore. In addition, the spacers did not affect the permeabilization capacities of the peptide dimers. These findings are summarized in Table 3. This test also showed that doubling the monomer concentration was not sufficient to reach the permeabilization capacity of the dimers and confirmed that the proximity of the chains is an important factor in the activity of the peptide (Fig. 2, MON 0.2). The results of the permeabilization studies correlated with the hemolytic activities and showed that the percentage of CF release and HC_{50} values were larger for the dimers than for the monomer, especially in vesicles that contained sphingomyelin (Table 3). In addition, these experiments were carried out in buffer solution without the effects of proteases (which are responsible for peptide hydrolysis). Thus, these data indicated that the increase of permeabilization is due mainly to dimerization. The vesicle permeabilization results are further evidence that the interaction with the cell wall is the cause of the decreased activity of the dimers. In vesicles, without a cell wall, the dimers have a higher permeabilization capacity than the monomer.

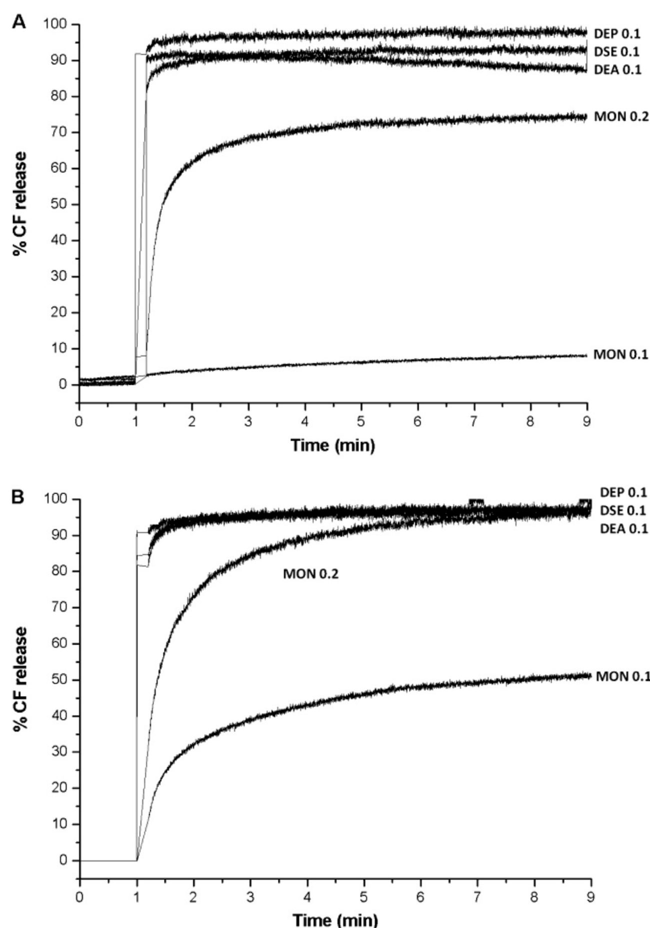

FIG 2 Release of CF from DPPA-DPPC-sphingomyelin (SM) (A) and DPPA-DPPC-DPPE (B) vesicles by the peptides at the indicated concentrations.

Kinetics of bactericidal activity. In an attempt to understand the differences between the biological activities and permeabilization profiles of the dimeric and monomeric peptides of Ctx-Ha, the kinetics of the bactericidal activity against *E. coli* were investigated (Fig. 3). In these experiments, a concentration of two times the MIC was used for each peptide, that is, 6.8 μmol liter^{-1} for MON and DSE, 3.2 μmol liter^{-1} for DEP, and 13 μmol liter^{-1} for DEA. As shown in Fig. 3, the time needed to reduce the initial amount of bacteria to 10% was approximately 10 min for the

TABLE 3 Permeabilization of vesicles by the peptides[a]

Peptide/concn (μmol liter^{-1})	DPPA-DPPC-SM		DPPA-DPPC-DPPE	
	% Max perm	T_{50} (min)	% Max perm	T_{50} (min)
MON/0.01	6	NR	46	4.11
MON/0.02	74	0.5	97	0.32
DSE/0.01	97*	0.08*	96*	0.11*
DEP/0.01	95*	0.08*	98*	0.12*
DEA/0.01	95*	0.08*	93*	0.11*

[a] % Max perm, maximum permeabilization percentage; T_{50}, time required to reach 50% of maximum permeabilization percentage; NR, 50% of the maximum permeabilization percentage was not reached; *, statistically significant difference compared to result for MON at 0.01 μmol liter^{-1} ($P < 0.05$ by ANOVA; $n = 3$).

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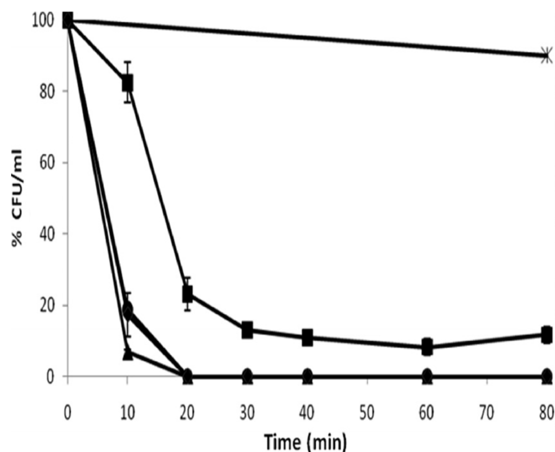

FIG 3 Kinetics of the bactericidal activities of the peptides against *E. coli.* Results are shown for incubation in the absence of peptide (×) and in the presence of MON (■), DSE (▲), DEP (●), or DEA (♦) at twice the respective MIC. The results are the means from at least two sets of independent experiments.

dimers and at least 60 min for the monomer. The results showed that the dimeric analogs of Ctx-Ha displayed faster bactericidal activity than monomer, which is in agreement with previous studies using other cAMPs (17, 23, 39, 44). Vesicle permeabilization studies and the kinetics of bactericidal activity confirm that aggregation/oligomerization, before or after membrane interaction, is necessary to form water-filled pores and kill the cell and indicate that the initial interactions among the peptides are an essential step in pore formation (28, 33). Thus, the time required to approximate the number of molecules necessary for pore formation was lower for the dimers because the peptide chain was previously paired due to its dimerization state. This may explain the differences in the kinetics of bactericidal activity observed with monomeric and dimeric versions of Ctx-Ha.

Pore size determination. To better evaluate whether the differences in biological activity between the monomeric and dimeric forms were related to the mechanism of action, the osmotic protection assay was performed to determine the sizes of the pores formed by MON and DSE in RBCs (Fig. 4). While PEG 200, PEG 300, and PEG 400 showed no appreciable effect on the hemolytic activity of the peptides, a decrease was observed when larger PEGs were used. Therefore, this assay suggested that both tested peptides as well as DEP and DEA formed pores in the RBCs with a diameter in the range of 1.4 to 1.5 nm. These data indicated that the mechanism of action is the same for dimers and monomer. The data for biological activity, permeabilization, kinetics of bactericidal activity, and pore size suggested that the interaction with the cell wall is the main factor to explain the reduced antimicrobial activity of dimers.

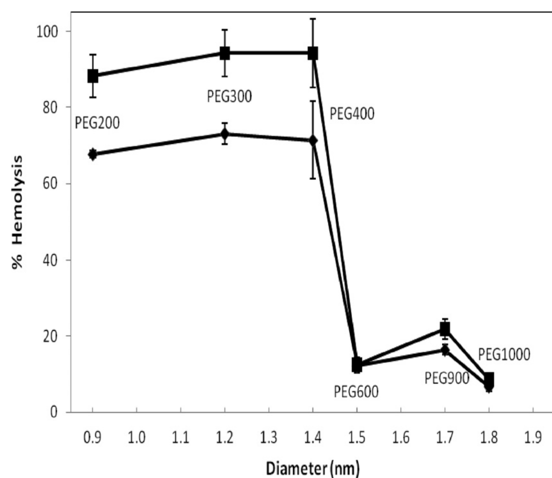

FIG 4 Inhibitory effects of osmotic protectants on hemolysis induced by MON (♦) and DSE (■) at 80 and 1.2 μmol liter^{-1}, respectively. Each point represents the mean result from three independent experiments, and the error bars indicate standard deviations.

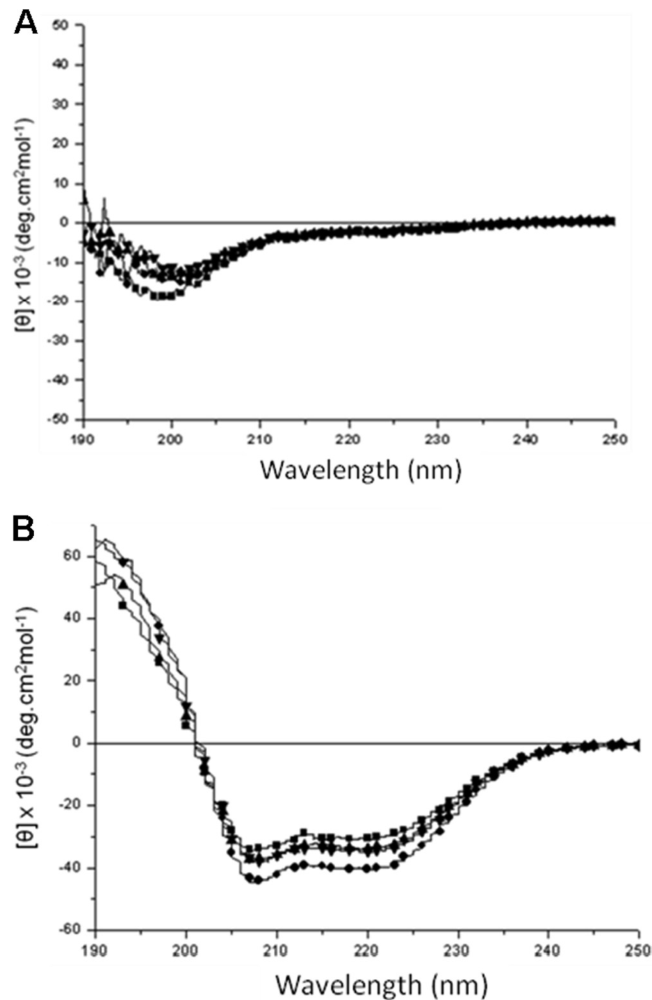

FIG 5 CD spectra of MON (■), DSE (♦), DEP (▲), and DEA (▼) in aqueous solution (A) and LPC (1 mmol liter^{-1}) micelles (B). In each experiment, the peptide concentration was 60 μmol liter^{-1}.

CD spectroscopy. To evaluate whether the reduced antimicrobial activity is related to any changes in the structure of the peptide promoted by dimerization, CD spectroscopy was utilized to investigate the secondary structures of the peptides. In aqueous solutions, all peptides displayed typical spectra for disordered structures (Fig. 5A), with a minimal dichroic value at 206 nm. However, in the presence of LPC micelles, the spectra displayed the typical appearance for α-helical structures (Fig. 5B), with min-

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imal dichroic values at 208 and 222 nm and a maximum near 190 nm. In both environments, all peptides had similar CD spectra, which indicates that the structures of the dimeric peptides were similar to that of the monomer. Thus, any change in structure is not responsible for the different antimicrobial and hemolytic activities.

The analysis of the data as a whole indicates that the decreased antimicrobial activities of the dimeric peptides are due to interactions with the cell walls of bacteria and fungi. These findings are supported by recent studies that have shown that inhibition of antimicrobial activity is due to the interaction between AMPs and cell wall carbohydrates (31, 37). The natural sequestration of antimicrobial agents by polysaccharides present in the cell wall also may render ineffective the activities of cAMPs, such as LL-37 and β-defensins (1).

Conclusions. The results show that dimerization of Ctx-Ha selectively affected the biological activity of this peptide by increasing the hemolytic activity and decreasing the antimicrobial activity. The difference in the biological activities of the monomer and dimeric peptides could not be explained by different mechanisms of action, secondary structure, or proximity of the peptide chains. In addition, this study presents evidence that the interaction between the peptide and the cell wall is important to antimicrobial peptide activity. This interaction must be considered in designing cAMPs with improved therapeutic applications. The results showed that the effects of dimerization on antimicrobial activity are unclear and may be sequence dependent.

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