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Probing the "Charge Cluster Mechanism" in Amphipathic Helical Cationic Antimicrobial Peptides[†]

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ABSTRACT: Clustering of anionic lipids away from zwitterionic ones by cationic antimicrobial agents has recently been established as a mechanism of action of natural small, flexible peptides as well as non-natural synthetic peptide mimics. One of the largest classes of antimicrobial peptides consists of peptides that form cationic amphipathic helices on membranes and whose toxic action is dependent on the formation of pores in the membrane or through the "carpet" mechanism. We have evaluated the role of anionic lipid clustering for five of these peptides, i.e., MSI-78, MSI-103, MSI-469, MSI-843, and MSI-1254, with different sequences and properties. We determined whether these amphipathic helical cationic antimicrobial peptides cluster anionic lipids from zwitterionic ones and if this property is related to the species specificity of their toxicity. All five of these peptides were capable of lipid clustering, in contrast to the well-studied amphipathic helical antimicrobial peptide, magainin 2, which does not. We ascribe this difference to the lower density of positive charges in magainin 2. Peptides that efficiently cluster anionic lipids generally have a ratio of MIC for *Staphylococcus aureus* to that for *Escherichia coli* of >1. The addition of an N-terminal octyl chain did not preclude anionic charge clustering, although the ratio of MIC for *S. aureus* to that for *E. coli* was somewhat lowered. In most Gram-positive bacteria, there is a predominance of anionic lipids in the cytoplasmic membrane. In Gram-negative bacteria, however, clustering of anionic lipids away from zwitterionic ones is emerging as an important contributing mechanism of bacterial toxicity for some antimicrobial agents.

There is much current interest in increasing the therapeutic efficacy of antimicrobial peptides (*1*−*3*). A large variety of antimicrobial agents have been designed for this purpose (*4*), and one of the major groups comprises the cationic, linear peptides that can form amphipathic helices in a membrane environment, the amphipathic helical cationic antimicrobial peptides (AHCAPs).[1] Some of these antimicrobial peptides are also of interest because of their anticancer activities (*5*). This study deals with five different AHCAPs, whose sequences and overall charge at neutral pH are given in Table 1.

Some AHCAPs have been shown to damage bacterial membranes by forming pores that can lead to membrane depolarization and even the loss of soluble molecules from the cytoplasm of the cell. A well-studied example of a peptide working by this mechanism is provided by magainin 2 (*6*). Among the peptides used in this study, MSI-78, an analogue of magainin 2, has also been suggested to act by forming a toroidal-type pore (*7*) as has MSI-103, based on the location of the peptide in a bilayer (*8*). It is thus common for amphipathic helical peptides to form pores in membranes. The formation of these pores is coupled with the self-association of many of these peptides in a highly cooperative manner (*9*), resulting in a high local density of positive charges that could cluster anionic lipids. The phenomenon of clustering of anionic lipids away from zwitterionic ones has been shown to contribute to the action of a synthetic antimicrobial agent, an oligo-acyl-lysine (*10*), as well as several small, flexible cationic peptides (*11*−*14*). In this study, we determined if this phenomenon extends to AHCAPs and what factors modulate the extent of charge clustering. Because a large number of antimicrobial peptides are capable of forming amphipathic helices, it is important to understand what contribution anionic−zwitterionic lipid clustering makes to their mechanism of action. This property does not negate pore formation; for many amphipathic helical peptides, both mechanisms may contribute to the microbial toxicity to varying extents. In addition, we have demonstrated that the AHCAPs used in this study can also segregate two different classes of anionic lipids as evidenced by the formation of crystalline phases in PG-containing bilayers. However, in the case of two anionic headgroups, separation into domains does not relate to the antimicrobial potency of the peptides and is therefore not a major mechanism of bacterial toxicity (*15*). In the design of novel antimicrobial agents, those favoring charge clustering over other mechanisms, like pore formation, may provide some advantage.

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[1]Abbreviations: AHCAPs, amphipathic helical cationic antimicrobial peptides; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; LPS, lipopolysaccharide; LTA, lipoteichoic acid; ONPG, *o*-nitrophenyl 3-D-galactoside; TSB, tryptic soy broth; SUVs, small unilamellar vesicles; MLVs, multilamellar vesicles; LUVs, large unilamellar vesicles; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; DOPG, 1,2-dioleoylphosphatidylglycerol; DMPG, 1,2-dimyristoylphosphatidylglycerol; TOCL, tetraoleoyl cardiolipin; TMCL, tetramyristoyl cardiolipin; PG, phosphatidylglycerol; CL, cardiolipin; ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry; PLL, poly-L-lysine; PLA, poly-L-arginine; PLO, poly-L-ornithine; Dab, diaminobutyric acid.

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Table 1: Sequences of Antimicrobial Peptides Studied and Comparison with Magainin 2

peptide name	sequence[a]	overall charge at neutral pH
magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	+3.5
MSI-78 (Pexiganan)	GIGKFLKKAKKFGKAFVKILKK-NH_2	+10
MSI-103	KIAGKIAKIAGKIAKIAGKIA-NH_2	+7
MSI-469	octyl-KIAGKIAKIAGKIAKIAGKIA-NH_2	+6
MSI-843	octyl-OOLLOOLOOL-NH_2	+6
MSI-1254	octyl-XXLLXXLXXL-NH_2	+6

[a]O is ornithine; X is 2,4-diaminobutyric acid (Dab).

They would be less toxic to host mammalian cells that do not contain exposed anionic lipids, although they would still be toxic to bacteria as well as to mammalian cancer cells and apoptotic cells. Furthermore, lipid clustering agents would not cause the rapid release of the internal contents in bacteria but would rather keep them intact to be cleared by phagocytic cells.

Many methods have been used to demonstrate the clustering of anionic lipids in anionic−zwitterionic mixtures by antimicrobial agents, including DSC (*10*, *11*, *14*, *16*), ^{31}P MAS/NMR (*10*, *11*), FTIR (*14*), ^{2}H NMR (*13*, *17*), freeze fracture electron microscopy (*12*), atomic force microscopy (*18*), and polarized total internal reflection fluorescence microscopy (*18*). Among these methods, one that is simple to implement, is flexible in being applied to a wide variety of systems, and does not require the use of labels is DSC (*19*). Of course, the lipid mixtures used in the DSC studies are simpler than the large variety of lipid molecular species found in biological membranes. Nevertheless, in the case of agents that were proposed to cluster anionic lipid on the basis of DSC results, previous studies using NMR (*10*, *11*), freeze fracture electron microscopy (*12*), and AFM (*18*) and FTIR (*14*) confirmed that this was indeed the phenomenon taking place. Furthermore, this phenomenon was shown to be predictive of the bacterial species specificity of antimicrobial agents (*10*, *20*, *21*). This is a clear demonstration that the model system studies are relevant to the behavior of the more complex biological systems.

There is a consequence to the bacterial species selectivity whether pore formation or anionic lipid clustering is the major mechanism of action. In the case of pore formation, for a given amount of membrane-bound peptide, there should be little dependence of toxicity on the relative amounts of anionic versus zwitterionic or uncharged lipid in the bacterial membrane (*15*), although there would be a dependence on the elastic energy of membrane thinning (*9*). In contrast, with lipid clustering, bacteria that are largely devoid of neutral or zwitterionic lipids on the extracellular leaflet of the cell membrane will be more resistant to these agents (*20*, *21*).

Lipopeptides have been extensively studied for the impact of the hydrocarbon chain on peptide structure as well as on biological activity (*22*−*25*). Here, we address the issue of the consequences of adding an acyl chain at the amino terminus of a peptide for anionic lipid clustering. Peptides MSI-469, MSI-843, and MSI-1254 are all lipopeptides with an N-terminal octyl group. NMR studies have shown that MSI-843 binds to the surface of a lipid bilayer with its N-terminal octyl chain deeply embedded in the membrane, the peptide interacting electrostatically and inflicting transient defects in acyl chain packing (*26*).

This study provides several new insights regarding differences in the specific properties of these AHCAPs, relating to their interaction with Gram-negative bacterial membranes and their ability to cluster anionic lipids away from zwitterionic ones. A peptide and its corresponding lipopeptide with an octyl chain at the amino terminus (MSI-103 and MSI-469), as well as lipopeptides containing cationic amino acids that differ by only one methylene group in their side chain (MSI-843 and MSI-1254), were examined both in bacterial membranes and in mimetic systems with respect to their properties and their contribution to the charge cluster mechanism.

EXPERIMENTAL PROCEDURES

Materials. Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Lipopolysaccharide (LPS) O111:B4 from *Escherichia coli* and lipoteichoic acid (LTA) from *Staphylococcus aureus* were purchased from Sigma Chemical Co. as well as *o*-nitrophenyl 3-D-galactoside (ONPG) and the poly-amino acids.

Antimicrobial Activity. The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) were determined by the microbroth dilution method, in Mueller Hinton broth, to conform to American Clinical Laboratory Standards. All peptide stock solutions had concentrations of 2 mg/mL in phosphate buffer, and serial dilutions were made in culture medium in a 96-well sterile polypropylene untreated microplate. Bacteria were grown overnight at 37 °C. Bacteria were added to each well of a 96-well sterile polypropylene untreated microplate to a final density of 5×10^5 colony-forming units (CFU)/mL. Plates were incubated at 37 °C for 18 h, and then the absorbance at 600 nm was determined. The lowest concentration that reduced bacterial growth by >90% was taken as the MIC.

To determine the MBC, aliquots from wells containing peptides at concentrations 2- and 4-fold higher than the MIC were plated in agar and incubated for 18 h at 37 °C. The lowest peptide concentration that prevented colony formation was taken as the MBC.

Simultaneous E. coli Inner and Outer Membrane Permeabilization. The mutant *E. coli* ML-35p was employed in this assay. This engineered strain is constitutive for cytoplasmic β-galactosidase, lacks *lac* permease, and expresses a plasmid-encoded periplasmic β-lactamase. Two chromogenic reporter molecules, nitrocefin and ONPG, were used to monitor permeabilization of outer and inner membranes, respectively, in a single assay (*27*−*29*).

Bacteria were grown in tryptic soy broth (TSB) from a single colony, overnight, at 37 °C. After three washings in phosphate buffer (pH 7.4) (10 mM phosphate buffer and 0.1 M NaCl), the bacterial culture was diluted to 10^6 CFU/mL in incubation buffer [phosphate buffer (pH 7.4) containing 300 μg/mL TSB] and added to all wells in a nontreated polystyrene microplate, together with increasing concentrations of peptide (0−100 μg/mL) in duplicate, which were later averaged. The solutions of peptide were also made in phosphate buffer. Each well also contained 30 μM nitrocefin in phosphate buffer and 2.5 mM ONPG in phosphate buffer. Absorbance was followed simultaneously at the accessible wavelengths of the instrument of 490 nm and 450 nm for 60 min, taking readings every 2 min at 37 °C, in a SpectraMax Pro microplate reader equipped with MaxSoft Plus (Molecular Devices, Sunnyvale, CA), with shaking. Seven different concentrations of each peptide were followed for 30 min, taking absorbance points every 2 min, to construct permeabilization curves.

Preparation of Phospholipid Vesicles. We made lipid films by dissolving appropriate amounts of lipids in a 2:1 (v/v) chloroform/methanol mixture followed by solvent evaporation under nitrogen gas to deposit the lipid as a film on the walls of a tube.

Final traces of solvent were removed in a vacuum chamber attached to a liquid nitrogen trap for 3−4 h. Dried films were kept under argon gas at −20 °C until they were used. Films were hydrated with buffer and vortexed extensively to make multilamellar vesicles (MLVs). The suspension was sonicated to clarity under argon in a bath-type sonicator at room temperature to make SUVs.

CD. CD spectra were recorded from 260 to 200 nm on an AVIV model 215 spectropolarimeter. A quartz cell with a 0.1 cm path length was placed in a thermally controlled cell holder. The machine was equipped with a Peltier junction thermal device and a Thermo Neslab M25 circulating bath. The sample temperature was maintained at 25 °C in all wavelength scans. Spectra were recorded with a wavelength step of 1 nm and an averaging time of 3 s for each data point, with a 30 s equilibration time between points. The CD spectra of peptide solutions in 5 mM HEPES buffer (pH 7.4), with 1 mM EDTA and 140 mM NaCl, were recorded in the absence or presence of small unilamellar vesicles (SUVs) of 75:25 POPE/TOCL or of 80:15:5 POPE/POPG/TOCL at a lipid:peptide ratio of 80. Solvent baselines with or without SUVs were subtracted from the spectra of samples with peptide, as appropriate.

ITC. Titrations were performed in a VP-ITC instrument (MicroCal Inc., Northampton, MA), at 30 °C. Peptide solutions were placed in the syringe in 10 mM HEPES buffer and 0.14 M NaCl (pH 7.4). LPS or LTA at a concentration of 400 μg/mL in 10 mM HEPES and 0.14 M NaCl (pH 7.4) was titrated with 5 μL injections of peptide for LPS, or 10 μL injections of peptide for LTA. A quantitative thermodynamic analysis of the LPS and LTA titrations was not conducted because of the high degree of heterogeneity of the sugar chains in LPS and LTA. Data were analyzed with Origin version 7.0.

DSC. Measurements were taken in a Nano II differential scanning calorimeter (Calorimetry Sciences Corp., Linden, UT). The method was adapted from that reported by Epand et al. (*30*). 75:25 POPE/TOCL MLVs were prepared by extensive vortexing to hydrate the films with 20 mM Pipes buffer (pH 7.4), 1 mM EDTA, and 140 mM NaCl. Peptides were incorporated into the lipid by hydration of the lipid films with a solution of the peptide or polymer in buffer, at room temperature. The lipid suspension was placed in the calorimeter cell, brought to 0 °C, and scanned for several cycles of heating and cooling at a rate of 1 °C/min. When 60:40 POPE/TMCL mixtures were used, heating was conducted in the range of 24−38 °C, at different scan rates from 0.5 to 0.125 °C/min. Curves were plotted with Origin version 7.0 and analyzed with the fitting program DA-2 provided by MicroCal Inc. (Northampton, MA).

RESULTS

MIC and MBC. We determined the MIC and MBC for the peptides used in this study against *E. coli* and *S. aureus* (Table 2). MIC values for three of these peptides are available from the literature, but for comparison of the relative activities of this group of peptides, the values determined in this study have an advantage in that they were all determined at the same time with the same procedure, bacterial strains, and materials.

The barrier of the outer membrane of Gram-negative bacteria can be made more permeable with EDTA that acts by removing Mg^{2+} ions from LPS. Adding EDTA to the medium used with *E. coli* resulted in a reduction of the MIC for MSI-843 (Table 2). EDTA had an impact on the MBC of MSI-843, turning it bactericidal from initially bacteriostatic. The MIC of MSI-1254 was also lowered, but the MBC remained at its initial value (Table 2). The fact that EDTA lowered the MIC of both MSI-843 and MSI-1254 indicates that EDTA allowed more peptide to traverse the LPS layer.

Table 2: Antimicrobial Action of Peptides

	S. aureus ATCC 29213		*E. coli* K12	
peptide	MIC (μg/mL)	MBC (μg/mL)	MIC (μg/mL)	MBC (μg/mL)
MSI-78	12.5	25	6.3	12.5
MSI-103	25	50	1.6	1.6
MSI-469	12.5	25	3.1	25−50
MSI-843	12.5	12.5	3.1	25−50
MSI-843 with EDTA	−	−	1.6	1.6
MSI-1254	12.5	12.5	12.5	12.5
MSI-1254 with EDTA	−	−	3.1	12.5

Simultaneous Inner and Outer Membrane E. coli Permeabilization. Peptides MSI-78, MSI-103, and MSI-469 are efficient permeabilizers of both inner and outer membranes of *E. coli* ML-35p (Figure 1). MSI-843 is inefficient but is able to permeabilize the outer membrane of *E. coli* and to reach the cytoplasmic membrane. MSI-1254 did not permeabilize either membrane (Figure 1).

DSC with 75:25 POPE/TOCL Mixtures. Changes in the phase transition properties of a lipid mixture caused by an added component indicate binding of this component to the lipid. In selecting a binary mixture to mimic the cytoplasmic membrane of Gram-negative bacteria, we have chosen a 75:25 POPE/TOCL mixture, because the lipids are well-mixed and show a single phase transition at 13−15 °C (*10*). Mixtures with three major lipid components, PE, PG, and CL, which are present in some Gram-negative bacteria, are not as suitable for DSC studies because in general they exhibit broader and/or complex phase transition behavior in mimetic systems. In addition, comparable two-component systems in which the anionic lipid is DOPG are generally demixed and not appropriate for zwitterionic−anionic lipid clustering studies (*10*). When the anionic lipid is POPG in similar two- or three-component systems, the transition is sharper, but the transition temperature of the mixtures becomes too close to that of pure POPE to be suitable for the study of changes caused by lipid phase separation induced by peptides.

Pure POPE has a gel to liquid crystalline phase transition temperature of 25 °C, while for TOCL, the phase transition is below 0 °C. Binding of peptides to TOCL causes the phase transition temperature of the mixture to shift toward a higher temperature as a result of formation of TOCL-depleted domains. That the changes observed in DSC are due to clustering of anionic lipid has been shown previously by NMR (*11*), freeze fracture electron microscopy (*12*), and pTIRF-AFM (*17*).

Magainin 2 has very little effect on the phase transition properties of 75:25 POPE/TOCL mixtures (Figure 2) and is the only peptide of this group that does not induce rearrangement of these lipid species into domains. Interestingly, MSI-78, which has a sequence related to magainin 2 but contains several additional cationic residues (Table 1), is effective in inducing phase separation. This is indicated by the observed increase in the transition temperature in both heating and cooling curves, consistent with the enrichment of a domain with POPE. The other four peptides used in this work behave like MSI-78. Although the curves show reasonable reversibility, splitting of the phase transition is more prominent in the cooling than in the heating curve. There can be several reasons for this; i.e., small reversible changes in the

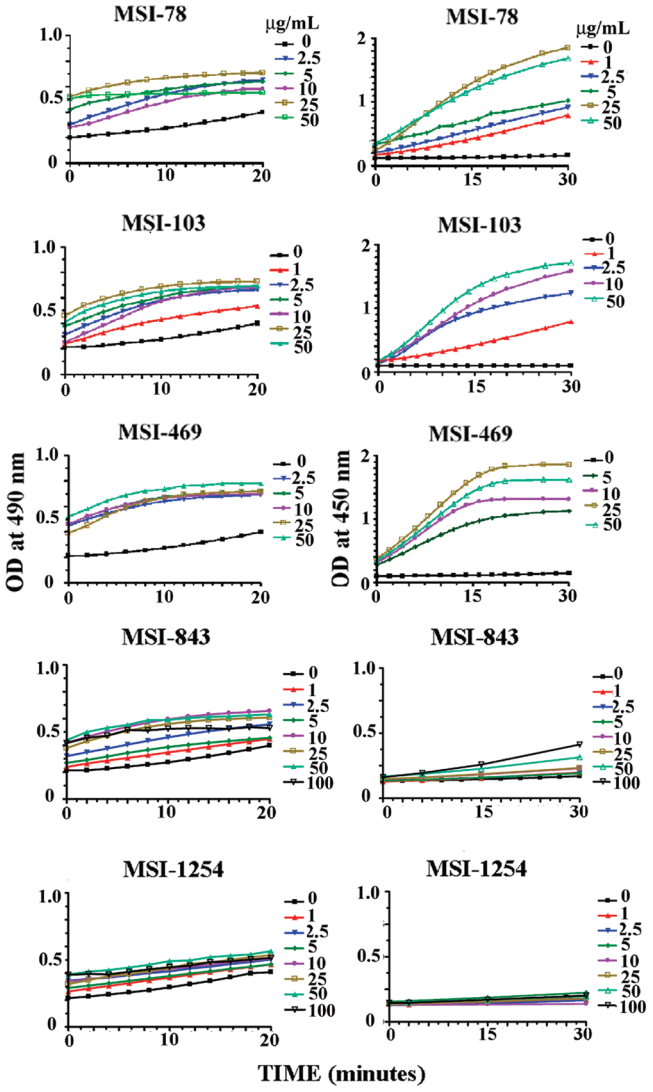

FIGURE 1: Outer and inner membrane permeabilization of *E. coli* ML-35p as a function of time caused by each of the five peptides used in this work at increasing peptide concentrations as indicated and at 37 °C. Hydrolysis of nitrocefin by β-lactamase was followed by absorbance at 490 nm (left) as a measure of outer membrane permeability. Reaction of ONPG with β-galactosidase was measured by absorbance at 450 nm as a measure of inner membrane permeability (right). Note that the ONPG data for MSI-843 and MSI-1254 are plotted using an expanded scale to more easily compare the action of these two peptides.

structure of the peptides can occur in the heating range used, or there can be small differences in the time required for the equilibration between the free and bound peptide as a function of temperature. We attempted to eliminate the difference between heating and cooling scans by performing runs at slower scan rates with the 60:40 POPE/TMCL mixture (see below).

We chose a lipid:peptide ratio of 20 (Figures 2 and 3A,B), at which the lipid/peptide mixtures formed homogeneous suspensions and the ratio is in the range of values found for peptide disruption of membranes in bacteria. Peptide concentrations at the membrane in bacteria can be many fold higher than the bulk concentration, and disruption of membranes has been shown to occur in a range of low lipid to peptide ratios (*31*). The range of temperatures used for DSC avoids the denaturation of the peptides, while at the same time, the system is in the liquid crystalline state, corresponding to the phase in which the bacterial membranes themselves are found.

The property of clustering anionic lipids in mixtures with zwitterionic ones is also exhibited by cationic polymers, like poly-L-lysine (PLL), poly-L-arginine (PLA), and poly-L-ornithine (PLO), with the following order of efficiency: PLA > PLL > PLO (Figure 3). Because of their high density of charge, the lipid:peptide ratios at which this phenomenon occurs are much higher than those with small peptides. PLL and PLA have been shown to have antimicrobial activity (*32*). K and R are common charged residues found in antimicrobial peptides, unlike O or Dab. However, MSI-843 is one of the few antimicrobial compounds carrying O as the main cationic residue, as is the linear MSI-1254 containing Dab. Other antimicrobial peptides containing Dab are generally cyclic peptides, and well-known examples of these are polymyxin and its derivatives.

DSC with 60:40 POPE/TMCL Mixtures. Studies were also performed with the 60:40 POPE/TMCL lipid mixture at different scan rates in the temperature range of 24−38 °C (Figure 3). In this lipid system, the pure anionic component, CL, has a transition temperature above that of the mixture itself (at 41 °C) in contrast with the 75:25 POPE/TOCL mixture where the pure zwitterionic lipid has a transition at 25 °C that is above that of the mixture itself. The PE/CL ratio is different than for the POPE/TOCL system so that a separation between the transition temperature of the higher melting TMCL and that of the POPE/TMCL mixture can be maintained. When the peptides bind to this mixture, they also cause phase separation, binding preferentially to the anionic component and causing a region of the membrane to be depleted of TMCL, thus shifting the transition temperature of this region lower, closer to that of the pure POPE component. This shows that the peptides bind to the anionic component in the mixture, regardless of whether it is the higher or lower melting component of the mixture. Changing the scan rate from 0.5 to 0.125 °C/min did not eliminate the small differences between heating and cooling.

CD. In buffer, all the peptides are in a disordered conformation (Figure 4A), which has been reported previously for peptides MSI-78 (*33*), MSI-103 (*34*), and MSI-843 (*26*). When cationic peptides bind to an anionic membrane, they often acquire more structure, also demonstrating that the peptides are binding to vesicles, as shown already by DSC. The large absorbance of the lipid/peptide mixtures in the far UV prevents us from obtaining more accurate quantitative data, as the presence of lipid contributes to scattering, which reduces somewhat the magnitude of the ellipticity. It has previously been reported that MSI-78 was helical in POPC SUVs at a lipid:peptide ratio of 100 (*33*), that MSI-103 was helical in POPG LUVs at a lipid:peptide ratio of 20 (*34*), and that MSI-843 exhibited an increased level of structure but with a large contribution from disordered regions in POPC SUVs at a lipid:peptide ratio of 100 (*26*). No report exists in the literature about the secondary or tertiary structure of MSI-469 or of MSI-1254. Therefore, we performed comparative CD studies in SUVs of the 75:25 POPE/TOCL mixture, at a lipid:peptide ratio of 80 (Figure 4B). The short decapeptides become only partially structured, as expected from their length and as has already been shown for MSI-843, but MSI-103 and MSI-469 appear to be distinctly α-helical. For the sake of comparison, we also present the CD spectra of magainin 2 in 75:25 POPE/TOCL SUVs. The peptides MSI-78, MSI-843, and MSI-1254 under these experimental conditions appear to be somewhat aggregated in SUVs; this was confirmed by the fact that upon performance of temperature scans up to 95 °C no denaturation transition was observed (data not shown). In contrast, MSI-103 and MSI-469

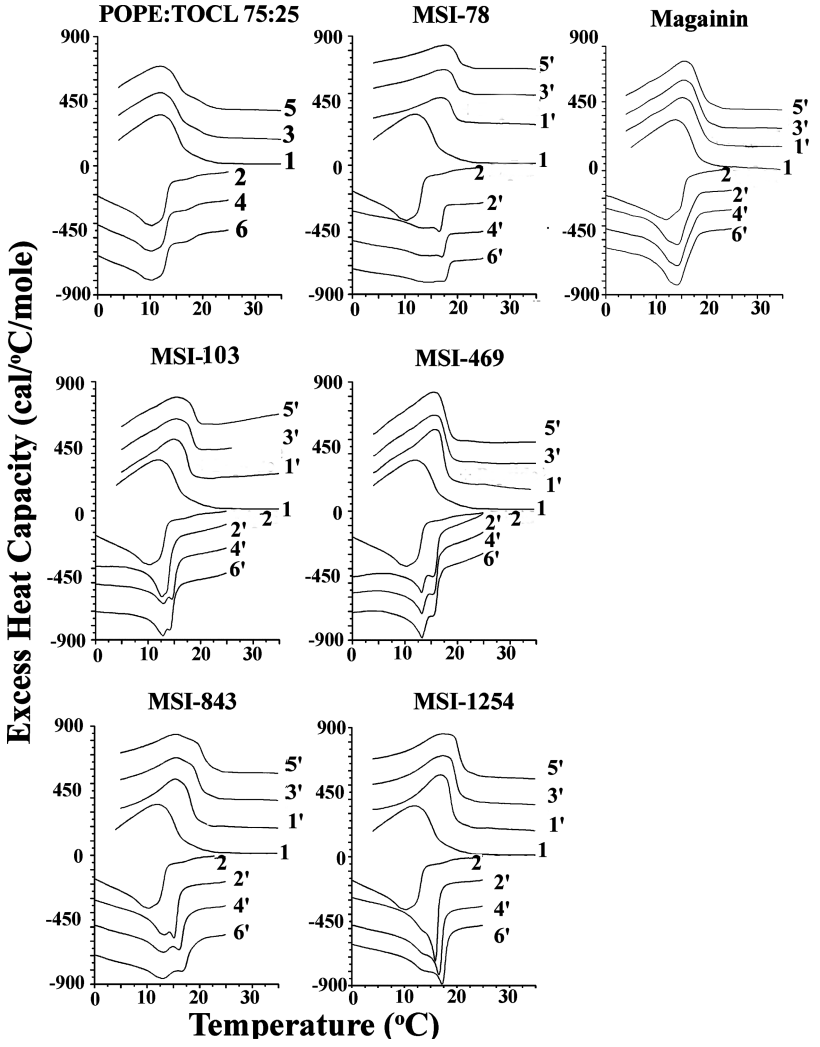

FIGURE 2: DSC conducted with 2.5 mg/mL 75:25 POPE/TOCL mixture at a scan rate of 1 °C/min in the absence and presence of one of the peptides, in 20 mM Pipes (pH 7.4) (0.14 M NaCl and 1 mM EDTA). A peptide solution was added at room temperature to the lipid film to give the final lipid:peptide ratio of 20. Odd-numbered scans represent heating scans and even-numbered ones cooling scans. Heating and cooling scans 1 and 2 correspond to the lipid alone. Primed numbers refer to the lipid with peptide.

have irreversible denaturation thermal transitions, accompanied by aggregation and coagulation (data not shown). We observed that MSI-469 has a higher melting temperature (~60 °C) than MSI-103. MSI-103 denatures in a POPE/TOCL mixture gradually, starting at lower temperatures (~25 °C). This indicates that the structure of MSI-103 is undergoing some structural disorganization and exhibits greater conformational flexibility at temperatures at which most of the assays are performed. It also indicates that MSI-469 is stabilized by the insertion of the octyl chain in the membrane.

These CD spectra were acquired in a membrane whose composition mimics more closely that of the headgroups in the cytoplasmic membrane of Gram-negative bacteria like *E. coli*. For CD studies, one is not limited to lipid mixtures whose compositions exhibit suitable phase transition temperatures, as is required in DSC. The same results were obtained by repeating the CD scans in 85:15:5 POPE/DOPG/TOCL SUVs (data not shown).

ITC. ITC studies were conducted to assess the role of the outer wall and the differences these peptides exhibit in their interaction with LPS from Gram-negative bacteria as opposed to LTA from Gram-positive bacteria, both negatively charged complex macromolecules, which the peptides have to traverse on their way to the cytoplasmic membrane.

Recently, the structure of a cationic antimicrobial peptide, MSI-594, was reported in LPS micelles (*35*). MSI-594 is a hybrid of the cationic N-terminus of MSI-78 and the hydrophobic N-terminus of melittin placed at the C-terminus of MSI-594. It was found that although unstructured in solution, it has a helical hairpin structure (helix−loop−helix) in LPS micelles and interacts with the phosphate groups of the Lipid A moiety. The amphiphilic nature of the LPS-bound structure allowed it to have optimal interaction with the macromolecule.

Because of the heterogeneity of LPS (from *E. coli* O111:B4) and LTA (from *S. aureus*) preparations extracted from bacteria, it is not possible to obtain accurate binding constants, although one could approximate values by using an average molecular weight. Some of the peptides, in addition, cause aggregation that results in very complex titration curves. We chose not to report binding constants but rather to compare features of the interaction of the peptides with these two macromolecules. Several factors can contribute to the enthalpy of binding of these cationic peptides to LPS or LTA. These include the exothermic processes of peptide helix formation, membrane insertion, intermolecular salt bridge formation, and peptide aggregation. There can also be endothermic contributions from disaggregation of the macromolecule, the break up of hydrogen bonds, and, in LPS, also displacement

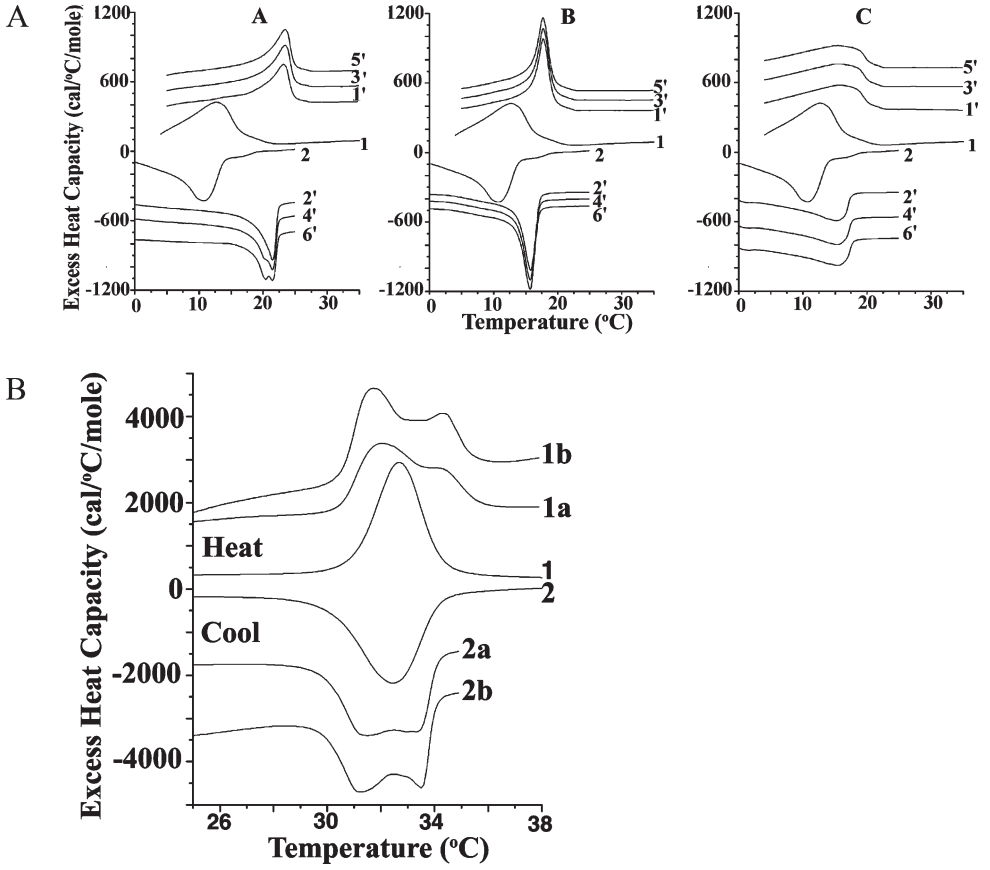

FIGURE 3: (A) DSC conducted with 2.5 mg/mL 75:25 POPE/TOCL mixture at a scan rate of 1 °C/min in the absence and presence of one of the polymers, in 20 mM Pipes (pH 7.4) (0.14 M NaCl and 1 mM EDTA). A polymer solution was added to the lipid film at room temperature to give the final lipid:polymer ratio of 100. Odd-numbered scans represent heating scans and even-numbered ones cooling scans. The weight-average molecular weight of these polymers is in the range of $30-60 \times 10^3$: (A) PLA, (B) PLL, and (C) PLO. Heating and cooling scans 1 and 2 correspond to the lipid alone. Primed numbers refer to the lipid with peptide. (B) DSC conducted with 2.5 mg/mL 60:40 POPE/TMCL mixture in 20 mM Pipes (pH 7.4) (0.14 M NaCl and 1 mM EDTA). A solution of MSI-78 was added to the lipid film at room temperature to give the final lipid:peptide ratio of 20, and DSC was conducted at 0.5 °C/min (scans 1a and 2a) and 0.125 °C/min (scans 1b and 2b). Odd-numbered scans represent heating scans and even-numbered ones cooling scans. Heating and cooling scans 1 and 2 correspond to the lipid alone.

of Mg^{2+} ions or removal of water from phosphate groups in Lipid A. The overall enthalpy change is the result of contributions from all of these processes.

Titrations in LPS show very complex behavior with all the peptides (Figure 5). MSI-78 and MSI-1254 exhibit similar titration patterns, characterized by a strong aggregation with the macromolecule at ~12 μM peptide. After this point, a very large exothermic heat is produced with every single additional injection of peptide.

In LTA, titrations are simpler and there are two distinct modes of interaction. Peptides MSI-78, MSI-843, and MSI-1254 exhibit initial electrostatic neutralization followed by endothermic reactions, while MS-103 and MSI-469 exhibit very little exothermic heat evolved, indicating poor binding (Figure 6).

These studies confirm that, even though both LPS and LTA are complex negatively charged macromolecules, the outer wall in Gram-negative bacteria constitutes a greater barrier for peptides to gain access to the cytoplasmic membrane.

DISCUSSION

The aim of this study was to probe whether AHCAPs with different sequences but with a high density of charge were capable of clustering anionic lipids away from zwitterionic ones and if this property was related to the species specificity of their toxicity. For this purpose, we looked at their ability to cluster CL in POPE/TOCL mixtures, a mimetic system that has been previously shown by many other techniques to provide a good test for clustering. In addition, information about whether these peptides were able to cause leakage of nitrocefin and ONPG through the outer and cytoplasmic membranes, respectively, of Gram-negative bacteria was obtained. ITC was used to determine the degree of interaction with negatively charged macromolecules that regulate their access to the membrane. With CD we wished to assess their degree of secondary structure change in going from buffer to a lipid mixture containing a zwitterionc and an anionic lipid as a criterion of their conformational flexibility.

Antimicrobial peptides can exhibit several different mechanisms of toxicity against bacteria. A contributing mechanism that has been shown to be important for certain oligo-acyl-lysines (*10*), fragments of the cathelicidin, LL-37 (*11*), Arg-rich nonapeptides (*12*), and other peptides, is their ability to cluster anionic lipids in zwitterionic−ainionic mixtures (*21*), i.e., the "charge cluster mechanism". As indicated above, DSC results suggest that all of the peptides studied except magainin 2 can cluster anionic lipids in the presence of zwitterionic ones. By this mechanism, an agent would be predicted not to be as toxic to *S. aureus* that is largely devoid of zwitterionic lipids as it is to *E. coli*. To varying degrees, this is the case for all of the five peptides studied (Table 2).

The series of agents used in this work have different sequences, but they all could form amphipathic helices. MSI-78 has been

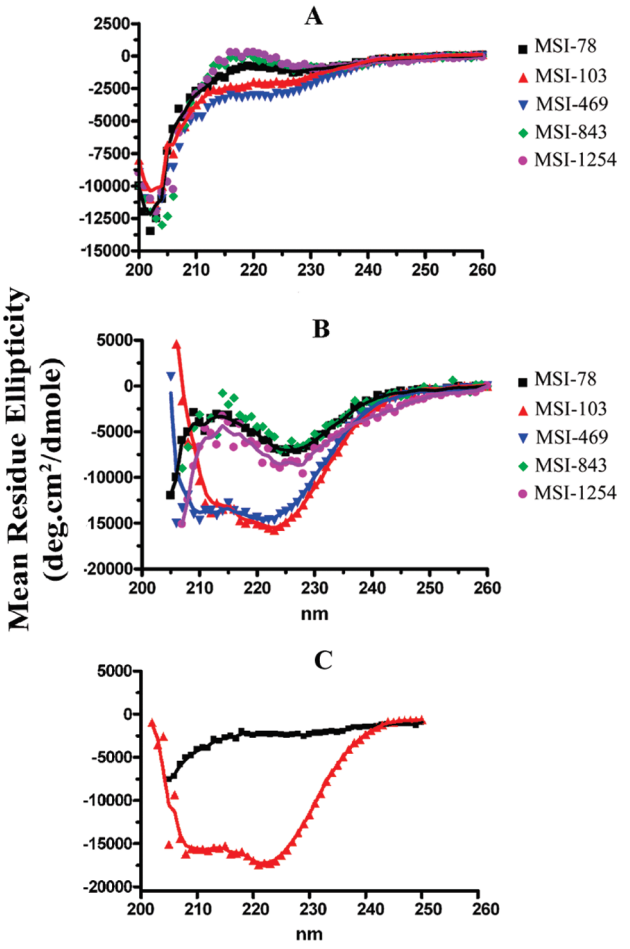

FIGURE 4: (A) CD spectra comparing the five peptides in HEPES buffer (pH 7.4) (0.14 M NaCl and 1 mM EDTA). (B) CD spectra in a 75:25 POPE/TOCL mixture at a lipid:peptide ratio of 80. (C) Spectra of magainin 2 in buffer (black) and in 75:25 POPE/TOCL SUVs at a lipid:peptide ratio of 35 (red). The peptide concentration was 125 μM. All the experiments were conducted at 25 °C.

studied by NMR and shown to be unstructured in aqueous solution but to acquire helical structure in the presence of detergents (*36*). In detergents, this peptide forms a helical antiparallel dimer that is similar to the arrangement of magainin 2 in detergents (*37*). It was also shown by REDOR that MSI-78 remained helical when incorporated into 75:25 POPC/POPG bilayers (*36*). In the 75:25 POPE/TOCL binary mimetic mixture, we found an increased level of structure, although the peptide aggregated (Figure 4B). MSI-103 is also known to dimerize and was found to be helical in the presence of POPG SUVs (*34*) and in oriented membranes (*8*, *38*, *39*). This peptide and its N-octyl analogue, MSI-469, were also found to have a distinct α-helical CD spectrum in POPE/TOCL SUVs in this study (Figure 4B). However, there is not a simple relationship between the helical content of these peptides and their antimicrobial activity (Table 2).

Lipopeptides MSI-843 and MSI-1254 have a similar number of residues, charges, and chemical structures, and they both carry an octyl chain at the amino terminus. The only difference is in the length of the side chain of their cationic residue. The ornithine residues in MSI-843 have one additional CH_2 group compared with the 2,4-diaminobutyric acid residues in MSI-1254. Interestingly, the peptide with the shorter charged side chain, MSI-1254, interacts more strongly with LPS (Figure 5), perhaps because of its capacity to form stronger aggregates.

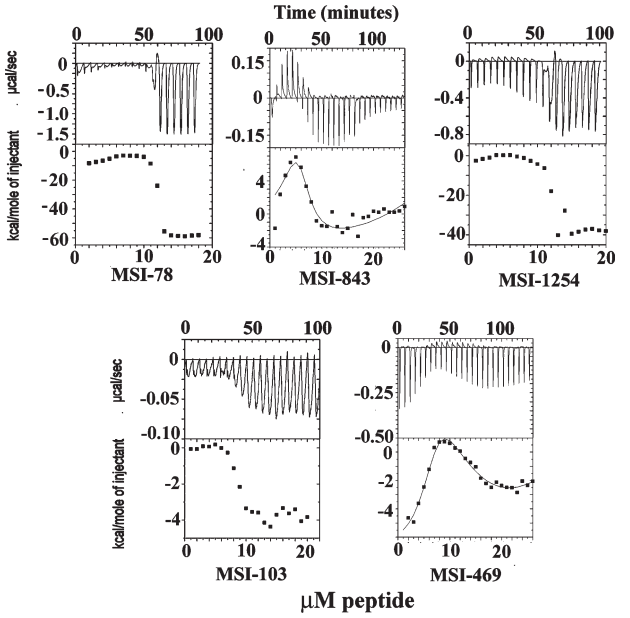

FIGURE 5: ITC of LPS with 10 μL injections of a solution of 200 μM peptide in the syringe. The buffer consisted of 10 mM HEPES and 0.14 M NaCl (pH 7.4). Titration was conducted at 30 °C. The cell contained 125 μg/mL LPS from *E. coli* 0111:B4. The top panel in each of the plots shows heat flow (microcalories per second) as a function of time (minutes). The bottom panel shows the kilocalories per mole of injectant, obtained from the integration of each of the titration peaks, as a function of the micromolar peptide concentration in the cell.

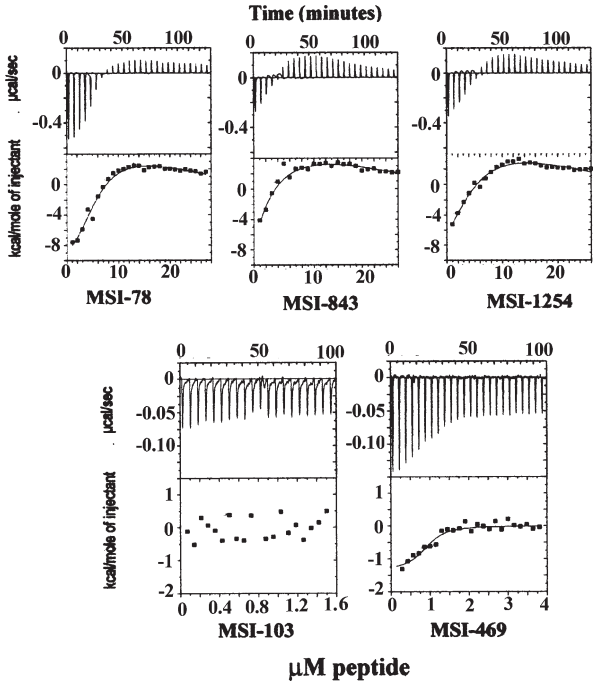

FIGURE 6: ITC of LTA with 10 μL injections of a solution of 200 μM peptide in the syringe. The buffer consisted of 10 mM HEPES and 0.14 M NaCl (pH 7.4). Titration was conducted at 30 °C. The cell contained 125 μg/mL LTA from *S. aureus*. The plots show heat flow (microcalories per second) as a function of time in minutes (top) and kilocalories per mole of peptide as a function of the micromolar peptide concentration in the cell (bottom), obtained from the integration of each of the titration peaks.

AHCAPs make up an important class of antimicrobial peptides. All of the peptides in this study, except for magainin 2, were capable of clustering anionic lipids in a mixture with zwitterionic

Table 3: Specificity of Antimicrobial Agents and Charges

agent	charge[a]	charge per residue	MIC *S. aureus* / MIC *E. coli*	ref
MSI-78	10	0.45	2	this work
MSI-103	7	0.33	15.6	this work
MSI-469	6	0.29	4	this work
MSI-843	6	0.6	4	this work
MSI-1254	6	0.6	1	this work
KR-12	6	0.5	>4	*11*
GF-17	6	0.35	0.5	*11*
GF-17 D3	6	0.35	>8	*11*
PR-9	5	0.56	*32*[c]	*12*
RR-9	5	0.56	*64*[c]	*12*
PI-9	5	0.56	*8*[c]	*12*
cateslytin	5	0.33	>3	*41*
$C_{12}K\text{-}7\alpha_8$	8	8[b]	16	*42*
$C_{12(\omega 7)}K\text{-}\beta_{12}$	3	3[b]	<0.1	*43*

[a]Estimated for pH 7 using $+^1/_2$ for His. [b]Not a peptide, but an oligoacyl-lysine (OAK). [c]Ratio of Gram-positive bacteria without PE (*S. aureus*) to Gram-positive bacteria with PE (*B. megaterium*).

ones (Figure 2). A critical test of the importance of this mechanism to the antimicrobial action of a peptide is its relative activity against *E. coli* compared with *S. aureus* (Table 3), because the lipid compositions of these two bacterial species are very different. The major lipid of the cytoplasmic membrane of *E. coli* is the zwitterionic lipid, PE, but this membrane also has significant amounts of anionic lipids. These anionic lipids are thus able to phase separate in this membrane. In contrast, the *S. aureus* membrane is comprised largely of anionic lipids. It should be noted that some strains of *S. aureus* contain the cationic lipid, lysyl phosphatidylglycerol. This lipid is more abundant in drug-resistant strains of *S. aureus* but is normally found on the inner leaflet of the cytoplasmic membrane and therefore is not exposed to the cell surface (*40*). Hence, the mechanism of zwitterionic−anionic lipid clustering would be effective against only *E. coli*. In accord with a contribution from this mechanism, all of the MSI peptides have a ratio of the MIC against *S. aureus* to the MIC against *E. coli* that is greater than 1 (Table 3), even though *E. coli* has the additional barrier of the outer membrane and is usually more resistant to antimicrobial agents than *S. aureus*. This higher ratio of their MICs in *S. aureus* to their MICs in *E. coli* is expected if the charge cluster mechanism is present. MSI-1254 is an exception, with a ratio of 1; however, we have shown that this agent is unable to permeabilize *E. coli* membranes, and it interacts very strongly with LPS (Figures 1 and 5).

One peptide that stands out as having the greatest specificity for *E. coli* relative to *S. aureus* is MSI-103. MSI-103 and MSI-469 have the same sequence, and they exhibit some of the same properties, i.e., acting as strong permeabilizers of the cytoplasmic membrane of *E. coli* (Figure 1), being more helical (Figure 4), and showing very little affinity for LTA (Figure 6). MSI-469 differs from MSI-103 only by the addition of an octyl group at the N-terminus, which also results in the loss of one positive charge. This causes a marked reduction in the ratio of MIC for *S. aureus* to that for *E. coli*. In fact, none of the three N-octyl peptides, i.e., MSI-469, MSI-843, and MSI-1254, exhibits a high specificity for *E. coli*. For the lipopeptides MSI-843 and MSI-1254, we show that access of these lipopeptides to the cytoplasmic membrane is facilitated by the presence of EDTA, indicating that LPS is the barrier for their entry.

It is also interesting to compare MSI-78 with MSI-103. Both are AHCAPs with similar, although not identical, numbers of amino acid residues and charge, yet MSI-103 exhibits a much higher specificity for *E. coli* and a greater toxicity against this species. There are also large differences in the interaction of these peptides with both LPS (Figure 5) and LTA (Figure 6). With MSI-78 at lower concentrations, the access of this peptide to the cytoplasmic membrane of Gram-negative bacteria appears to be somewhat retarded by the outer membrane, although it can eventually permeate it (Figure 1). In both LPS and LTA, MSI-78 exhibits much stronger and complex interactions than MSI-103, and this is reflected in their bacteriostatic effects.

Conformational flexibility has also been suggested to play a role in enhancing the ability of peptides to cause clustering of anionic lipids (*21*). Conformational flexibility in this group of AHCAPs is more evident in the small decapeptides MSI-843 and MSI-1254 (Figure 4) and in MSI-78. With MSI-103, its secondary structure begins to unravel at low temperatures, as observed by the performance of CD studies at 222 nm as a function of temperature in POPE/TOCL mixtures.

In the case of the two most helical peptides, MSI-103 and MSI-469, a larger contribution to the energy of domain formation and stabilization will come from helix formation upon membrane binding, which is on the order of -0.4 kcal mol^{-1} residue^{-1}. For MSI-78, besides a very high charge of 10 and helix formation, there are hydrophobic Phe residues that can insert in the membrane and contribute to the energy of cluster formation and stabilization. For the lipopetides, besides helix formation, insertion of the octyl chain will provide energy for domain stabilization.

To summarize, many cationic amphipathic helical antimicrobial peptides can cluster anionic lipids from zwitterionic ones, making them more toxic to *E. coli* than to *S. aureus*, provided they have a sufficient density of positive charges in their sequence and are able to pass through the barrier of the cell wall. It is now recognized that antimicrobial cationic peptides can act by a variety of different mechanisms. Some of these peptides have been described previously as forming toroidal pores. Anionic lipid charge clustering should be considered a contributing mechanism, which does not exclude other mechanisms of action previously proposed for antimicrobial peptides, including the carpet mechanism and pore formation. The charge cluster mechanism of toxicity results from the first encounter of the peptide with the outer leaflet of the cytoplasmic membrane, causing a large reorganization of the membrane, increasing the concentrations of anionic lipid and cationic peptide within a membrane domain. This could result in small defects or in some cases even proceed to large membrane permeabilization and/or in functional impairment of proteins adjacent to the anionic lipids that become recruited by these antimicrobial agents.

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