Published in final edited form as:

Biochemistry. 2010 May 18; 49(19): 4076–4084. doi:10.1021/bi100378m.

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 are those 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 which efficiently cluster anionic lipids generally have a ratio of MIC for *S. aureus* to that for *E. coli* >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). 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 the current study, MSI-78, an analog of magainin 2, has also been suggested to act by forming a toroidal type pore (7) as has MSI-103, based on

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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 the present 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 the contribution of anionic-zwitterionic lipid clustering is 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 the current 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 designing novel antimicrobial agents, those favouring charge clustering over other mechanisms, like pore formation, may provide some advantage. 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), ³¹P MAS/NMR (10;11), FTIR (14), ²H 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, flexible to be applied to a wide variety of systems and which 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 behaviour 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 vs. 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 the 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 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 aminoacids that only differ by one methylene group in their side-chain (MSI-843 and MSI-1254), were examined both in bacterial membranes and in mimetic systems for their properties as well as their contribution to the charge cluster mechanism.

Experimental Procedures

Materials

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

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 were 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 CFU/mL. Plates were incubated at 37°C for 18 hours and then 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-fold 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 3 washings in phosphate buffer at pH 7.4 (10 mM phosphate buffer, 0.1 M NaCl), the bacterial culture was diluted to 10^6 CFU/mL in incubation buffer (phosphate buffer pH 7.4 containing 300 μ g/mL TSB) and added to all wells in a non-treated polystyrene microplate, together with increasing concentrations of peptide (0–100 μ g/mL) in duplicates, which were later averaged. The solutions of peptide were also made in phosphate buffer. Each well also contained 30 μ M nitrocefin in phosphate buffer and 2.5 mM ONPG in phosphate buffer. Absorbance was followed simultaneously at 486 nm and at 420 nm for 60 minutes, taking readings every two minutes at 37°C, in a SpectraMax Pro microplate reader equipped with MaxSoft Plus software (Molecular Devices, Sunnyvale, CA), with shaking. Seven different concentrations of each peptide were followed for 30 minutes, taking absorbance points every two minutes, in order to construct permeabilization curves.

Preparation of phospholipid vesicles

Lipid films were made by dissolving appropriate amounts of lipids in chloroform/methanol 2/1 (v/v) 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 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 obtained 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 measured in the absence or presence of small unilamellar vesicles (SUVs) of POPE:TOCL 75:25 or of POPE:POPG:TOCL 80:15:5, at a lipid to peptide ratio of 80. Solvent baselines with or without SUVs were subtracted from the spectra of samples with peptide, as appropriate.

ITC

Titration 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, 0.14 M NaCl, pH 7.4. LPS or LTA at a concentration of 400 $\mu\text{g}/\text{mL}$ in 10 mM HEPES, 0.14 M NaCl, pH 7.4, were titrated with 5 μL injections of peptide for LPS, or 10 μL injections of peptide for LTA, at 30°C . A quantitative thermodynamic analysis of the LPS and LTA titrations was not carried out because of the high degree of heterogeneity of the sugar chains in LPS and LTA. Data was analyzed with the program Origin v.7.0.

DSC

Measurements were made in a Nano II Differential Scanning Calorimeter (Calorimetry Sciences Corp, Linden, UT). The method was adapted from that reported by Epand *et al.* (30). MLVs of POPE:TOCL 75:25 were prepared by extensive vortexing to hydrate the films with 20 mM PIPES buffer pH 7.4, 1 mM EDTA, 140 mM NaCl. Peptides were incorporated into the lipid by hydrating 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 $1^{\circ}\text{C}/\text{min}$. When mixtures of POPE:TMCL 60:40 were used, heating was done in the range of 24 – 38°C , at different scan rates from $0.5^{\circ}\text{C}/\text{min}$ to $0.125^{\circ}\text{C}/\text{min}$. Curves were plotted with the program Origin v.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 comparing the relative activities of this group of peptides the values determined in the present study have the advantage that they are all done 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.

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 (Fig. 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 (Fig. 1).

DSC with POPE:TOCL 75:25 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 POPE:TOCL 75:25, 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, as 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 behaviour 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 towards 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 POPE:TOCL (75:25) (Fig. 2) and is the only peptide of this group that does not induce rearrangement of these lipid species into domains. Interestingly, MSI-78 that 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 similarly to 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 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 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 mixture POPE:TMCL 60:40 (see below).

We chose a lipid to peptide ratio of 20 (Figs. 2, 3A and 3B), 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. Concentrations of peptide 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 the bacterial membranes themselves are found to be.

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 an efficiency following the order of PLA>PLL>PLO (Fig. 3A). Because of their high density of charge, the lipid to 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 POPE:TMCL 60:40 mixtures

Studies were also performed with the lipid mixture POPE:TMCL 60:40 at different scan rates and in the temperature range 24–38°C (Fig. 3B). 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 POPE:TOCL 75:25 mixture where the pure zwitterionic lipid has a transition at 25°C that is above that of the mixture itself. The ratio of PE to CL is different than for the POPE:TOCL system so as to maintain a separation between the transition temperature of the higher melting TMCL and that of the POPE:TMCL mixture. 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°C/min 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 (Fig. 4, panel A), as has been reported before 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 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 to peptide ratio of 100 (33); that MSI-103 was helical in POPG LUVs at a lipid to peptide ratio of 20 (34) and that MSI-843 showed increased structure but with a large contribution from disordered regions in POPC SUVs at a lipid to 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 POPE:TOCL 75:25, at a lipid to peptide ratio of 80 (Fig. 4, panel B). 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 distinctly α -helical. For comparison we present also the CD spectra of magainin 2 in SUVs of POPE:TOCL 75:25. The peptides MSI-78, MSI-843 and MSI-1254 under these experimental conditions appear somewhat aggregated in SUVs; this was confirmed by the fact that performing temperature scans up to 95°C no denaturation transition was observed (data not shown). In contrast, MSI-103 and MSI-469 have irreversible denaturation

thermal transitions, accompanied by aggregation and coagulation (data not shown). We observed that MSI-469 has a higher melting temperature ($\sim 60^{\circ}\text{C}$) than MSI-103. MSI-103 denatures in POPE:TOCL gradually, starting at lower temperatures ($\sim 25^{\circ}\text{C}$). 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 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 SUVs of POPE:DOPG:TOCL 85:15:5 (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 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.

Titration in LPS show very complex behaviour with all the peptides (Fig. 5). MSI-78 and MSI-1254 show similar titration patterns to each other, characterized by a strong aggregation with the macromolecule at $\sim 12\ \mu\text{M}$ 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 show initial electrostatic neutralization followed by endothermic reactions, while MSI-103 and MSI-469 show very little exothermic heat evolved indicating poor binding (Fig. 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. To this purpose, we looked at their ability to cluster CL in POPE:TOCL mixtures, a mimetic system which has been previously shown by many other techniques to provide a good test for clustering. In addition, information was obtained as to whether or not these peptides were able to cause leakage of nitrocefin and ONPG through the outer and cytoplasmic membranes, respectively, of Gram negative bacteria. 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 zwitterionic 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-anionic 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, compared with *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 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 bilayers of POPC:POPG 3:1 (36). In the binary mimetic mixture POPE:TOCL 75:25 we found increased structure although the peptide aggregated (Fig. 4, panel B). MSI-103 is also known to dimerize and was found to be helical in the presence of SUVs of POPG (34) and in oriented membranes (8;38;39). This peptide and its N-octyl analog, MSI-469 were also found to have a distinct α -helical CD spectrum in POPE:TOCL SUVs in the present study (Fig. 4, panel B). However, there is not a simple relationship between the helical content of these peptides and their antimicrobial activity (Table 2).

The lipopeptides MSI-843 and MSI-1254 have a similar number of residues, charges, 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 CH₂ 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 (Fig. 5), perhaps due to its capacity to form stronger aggregates.

AHCAPs are 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 ones (Fig. 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 normally it is 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 only be effective against *E. coli*. In accord with a contribution from this mechanism, all of the MSI peptides have a ratio of MIC against *S. aureus*/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 compared with *S. aureus*. This higher ratio of their MIC 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 (Figs. 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* (Fig. 1), being more helical (Fig 4), showing very little affinity for LTA (Fig 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, exhibit 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 (Fig. 5) and LTA (Fig. 6). With MSI-78 at lower concentrations, the access of this peptide to the cytoplasmic membrane of Gram negative bacteria appears somewhat retarded by the outer membrane, although it can eventually permeate it (Fig. 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 (Fig. 4) and in MSI-78. With MSI-103, its secondary structure begins to unravel at low temperatures, as observed by performing 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 of the order of -0.4 kcal/mole/residue. 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 lipopeptides, 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 either 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 then become recruited by these antimicrobial agents.

Acknowledgments

This work was supported in part by grant MOP 86608 from the Canadian Institutes of Health Research (RME) and funds from NIH (AI054515 to A.R.).

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-oleoyl phosphatidylethanolamine
POPC	1-palmitoyl-2-oleoyl phosphatidylcholine
POPG	1-palmitoyl-2-oleoyl phosphatidylglycerol
DOPG	1,2-dioleoyl phosphatidylglycerol
DMPG	1,2-dimyristoyl phosphatidylglycerol
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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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.

Figure 2.

DSC carried out with 2.5 mg/mL POPE:TOCL (75:25) 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, 1 mM EDTA). 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 numbers cooling scans. Heating and cooling scans 1 and 2 correspond to the lipid alone. Primed numbers refer to lipid with peptide.

Figure 3A.

DSC carried out with 2.5 mg/mL POPE:TOCL (75:25) 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, 1 mM EDTA). 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 numbers cooling scans. The weight average molecular weight of these polymers is in the range of $30\text{--}60 \times 10^3$. Panel A, PLA. Panel B, PLL. Panel C, PLO. Heating and cooling scans 1 and 2 correspond to the lipid alone. Primed numbers refer to lipid with peptide.

Figure 3B.

DSC carried out with 2.5 mg/mL POPE:TMCL (60:40) in 20 mM Pipes pH 7.4 (0.14 M NaCl, 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 the DSC was carried out 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 numbers cooling scans. Heating and cooling scans 1 and 2 correspond to the lipid alone.

Figure 4.

Panel A. CD spectra comparing the five peptides in HEPES buffer pH 7.4 (0.14 M NaCl, 1 mM EDTA).

Panel B. CD spectra in POPE:TOCL 75:25 at a lipid to peptide ratio of 80.

Panel C. Spectra of magainin 2 in buffer (black) and in SUVs of POPE:TOCL 75:25. L/P=35 (red).

The peptide concentration was 125 μ M. All the experiments were carried out at 25°C.

Figure 5.

ITC of LPS with 10 μ L injections of a solution of 200 μ M peptide in the syringe. Buffer was 10 mM HEPES, 0.14 M NaCl, pH 7.4. Titration was carried out at 30°C. The cell contained 125 μ g/mL LPS from *E. coli* 0111:B4. The upper panel in each of the plots show heat flow (μ cal/sec) as a function of time (minutes). The lower panel shows kcal/mole of injectant, obtained from the integration of each of the titration peaks, as a function of μ M peptide in the cell.

Figure 6.

ITC of LTA with 10 μL injections of a solution of 200 μM peptide in the syringe. Buffer was 10 mM HEPES, 0.14 M NaCl, pH 7.4. Titration was carried out at 30°C. The cell contained 125 $\mu\text{g/mL}$ LTA from *S. aureus*. The plots show heat flow ($\mu\text{cal/sec}$) as a function of time in minutes (upper panel) and kcal/mole of peptide as a function of μM peptide in the cell (lower panel). The upper panel in each of the plots show heat flow ($\mu\text{cal/sec}$) as a function of time (minutes). The lower panel shows kcal/mole of injectant, obtained from the integration of each of the titration peaks, as a function of μM peptide in the cell.

Table 1

Sequence of antimicrobial peptides studied and comparison with magainin 2

Peptide Name	Sequence	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

O=Ornithine; X=2,4 diaminobutyric acid (Dab)

Table 2

Antimicrobial action of peptides

Peptide	<i>S. aureus</i> ATCC 29213		<i>E. coli</i> K12	
	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

Table 3

Specificity of Antimicrobial Agents and Charge

Agent	Charge ¹	Charge/residue	MIC <i>S. aureus</i> /MIC <i>E. coli</i>	Reference
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	<u>32</u> ³	(12)
RR-9	5	0.56	<u>64</u> ³	(12)
PI-9	5	0.56	<u>8</u> ³	(12)
Cateslytin	5	0.33	>3	(41)
C ₁₂ K-7a ₈	8	8 ²	16	(42)
C ₁₂₍₀₇₎ K-β ₁₂	3	3 ²	<0.1	(43)

¹ Estimated for pH 7 using +1/2 for His.

² Not a peptide, but an oligo-acyl-lysine (OAK).

³ Ratio of Gram positive bacteria without PE (*S. aureus*) to Gram positive bacteria with PE (*B. megaterium*).