Roles of Hydrophobicity and Charge Distribution of Cationic Antimicrobial Peptides in Peptide-Membrane Interactions*

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Background: Cationic antimicrobial peptides offer an alternative to conventional antibiotics, as they physically disrupt bacterial membranes, causing cell death.

Results: Peptides designed with high hydrophobicity display strong self-association that is minimized by distribution of positive charges at both peptide termini.

Conclusion: Balancing peptide hydrophobicity and charge distribution promotes efficient antimicrobial activity.

Significance: Routes to optimization of peptide sequences are valuable for devising therapeutic strategies.

Cationic antimicrobial peptides (CAPs) occur as important innate immunity agents in many organisms, including humans, and offer a viable alternative to conventional antibiotics, as they physically disrupt the bacterial membranes, leading to membrane lysis and eventually cell death. In this work, we studied the biophysical and microbiological characteristics of designed CAPs varying in hydrophobicity levels and charge distributions by a variety of biophysical and biochemical approaches, including in-tandem atomic force microscopy, attenuated total reflection-FTIR, CD spectroscopy, and SDS-PAGE. Peptide structural properties were correlated with their membrane-disruptive abilities and antimicrobial activities. In bacterial lipid model membranes, a time-dependent increase in aggregated β-strand-type structure in CAPs with relatively high hydrophobicity (such as KKKKKKALFALW-LAFLA-NH₂) was essentially absent in CAPs with lower hydrophobicity (such as KKKKKKAFAAWASHAFAA-NH₂). Redistribution of positive charges by placing three Lys residues at both termini while maintaining identical sequences minimized self-aggregation above the dimer level. Peptides containing four Leu residues were destructive to mammalian model membranes, whereas those with corresponding Ala residues were not. This finding was mirrored in hemolysis studies in human erythrocytes, where Ala-only peptides displayed virtually no hemolysis up to 320 μM, but the four-Leu peptides induced 40–80% hemolysis at the same concentration range. All peptides studied displayed strong antimicrobial activity against Pseudomonas aeruginosa (minimum inhibitory concentrations of 4–32 μM). The overall findings suggest optimum routes to balancing peptide hydrophobicity and charge distribution that allow efficient penetration and disruption of the bacterial membranes without damage to mammalian (host) membranes.

Antimicrobial peptides occur naturally as important innate immunity agents in a wide range of living organisms ranging from plants to insects to mammals, including humans (1); as such, they have become increasingly recognized in current research as templates for prospective antibiotic agents. Also termed cationic antimicrobial peptides (CAPs),2 they are characterized by their positive net charge, modest length (12–50 residues), and good solubility in water (2–4). CAPs offer a viable alternative to conventional antibiotics, as they bind to and penetrate bacterial cell membranes, physically disrupting them, leading to membrane lysis and eventually cell death (5). The advantages of these CAPs are that (i) they are highly selective against the negatively charged bacterial membrane versus the zwitterionic mammalian membranes of a human host and that (ii) there is no specificity in targeting, as the peptides function by physically disrupting the bacterial membrane and therefore are unlikely to evoke bacterial resistance (6).

Our laboratory previously designed a novel category of synthetic hydrophobic membrane-active CAPs that display antimicrobial activity against multiple bacteria and yeast strains without toxicity to mammalian cells (7, 8), of which a lead candidate is 6K-F17 (sequence KKKKKKAFAAWASHAFAA-NH₂). In designing this peptide, Ala was chosen as the background residue due to its midrange hydrophobicity and frequent occurrence in membrane domains. As well, the AxxxA (“small-xxx-small”) sequence motif promotes peptide dimerization in membranes (9, 10), which has been suggested to enhance antimicrobial activity (11). The Trp residue in the hydrophobic core serves as the fluorescent probe, whereas the positively charged Lys residues were included at the terminus to maintain cationic properties and enhance peptide solubility. On the basis of the highly active CAP 6K-F17, we went on to design a series of CAPs with a range of hydrophobicity values and observed a “threshold hydrophobicity” for selective bacterial membrane

2 The abbreviations used are: CAP, cationic antimicrobial peptide; Fmoc, N-[9-fluorenylmethoxycarbonyl]; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-sn-glycero-3-phospho(1'-rac-glycerol); DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; BisTris, 2-[bis(2-hydroxyethyl)amin]-2-(hydroxymethyl)propane-1,3-diol; AFM, atomic force microscopy; ATR, attenuated total reflection; MIC, minimum inhibitory concentration; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine.
insertion (11–13). However, once the core segment hydrophobicity of the CAPs is beyond an upper threshold, as in the case in which the sequence contains two or more Ala-to-Leu substitutions, the CAPs generally have reduced antimicrobial activity and display increased toxicity to mammalian membranes (14). In agreement with these findings, other workers confirmed the importance of peptide hydrophobicity in membrane selectivity and insertion and for antimicrobial activity (15, 16). In addition to hydrophobicity, the net positive charge of a given CAP also plays an important role in peptide-membrane interactions, particularly in attracting the CAP efficiently to the anionic surface of bacterial membranes (17–19). However, the interplay of CAPs with varying hydrophobicity levels versus varying distributions of positive residues along the CAP sequence as a determinant of bioactivity remains to be clarified.

In this work, we report the biological activity (and the biochemical characteristics of peptide-membrane interactions) of selected designed CAPs varying systematically in hydrophobicity levels and charge distributions to answer the following questions. 1) Why does increasing hydrophobicity lead to poorer antimicrobial activity and greater hemolytic toxicity? 2) Will altered charge distribution improve the activity of CAPs with the same hydrophobicity level? 3) Which factor, hydrophobicity or charge distribution, is ultimately the more important contributor to effective CAP design and bioactivity?

**EXPERIMENTAL PROCEDURES**

**Reagents**—The reagents for peptide synthesis, cleavage, and purification included (Fmoc-aminomethyl-3,5-dimethoxyphenoxy)valeric acid-polyethylene glycol-polystyrene resin and piperidine (Applied Biosystems); Fmoc-protected amino acids and 2-(7-aza-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluxonium hexafluorophosphate (GL Biochem (Shanghai) Ltd.); N,N-diisopropylethylamine and triisopropylsilane (Aldrich); Ultrapure™ buffer-saturated phenol (Invitrogen); TFA (DURAN Group); and peptide-grade N,N-dimethylformamide, methanol, diethyl ether, and acetonitrile (Caledon Laboratories Ltd.). Reagent kits for micro-BCA protein assays were purchased from Pierce. Cation-adjusted Mueller-Hinton broth was from Sigma. Lipids, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), and 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG) were from Avanti Polar Lipids. Buffers were prepared in double-distilled water with pH adjustment when necessary.

**Peptide Synthesis and Purification**—Peptides were synthesized by the continuous flow Fmoc solid-phase method on a Protein Technologies PS3 peptide synthesizer using the standard cycle (20). (Fmoc-aminomethyl-3,5-dimethoxyphenoxy)valeric acid-polyethylene glycol-polystyrene resin was used to produce an amidated C-terminus. 2-(7-Aza-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluxonium hexafluorophosphate and N,N-diisopropylethylamine were used as the activation pair. Deprotection and cleavage of peptides were performed in a mixture of 88% TFA, 5% phenol, 5% water, and 2% triisopropylsilane for 2 h in the dark at room temperature. The crude peptides were purified on a reverse-phase C4 preparative HPLC column using a linear gradient of acetonitrile in 0.1% TFA. The purities of the peptides were confirmed by their molecular weights using MALDI-MS. The concentrations of the peptides were determined using the micro-BCA protein assay.

**Circular Dichroism**—CD spectra were recorded on a Jasco J-810 spectropolarimeter using a 1-mm path length quartz cell at 25 °C. Peptide (20 μM each) in 20 mM Tris buffer and 10 mM NaCl with and without 10 mM SDS at pH 7.0 was measured in an average of three scans with the buffer background subtracted.

**SDS-PAGE**—50-ng portions of each peptide were prepared in Novex sample buffer and incubated at room temperature for >30 min prior to being loaded onto NuPAGE Novex 12% BisTris precast gels (1.0-mm thickness, 10 wells) in MES buffer according to the manufacturer’s protocols. Silver staining was performed using the SilverXpress staining kit (Invitrogen) to visualize peptides on gels. Apparent molecular weights were estimated from the migration of Novex sharp unstained protein standard (Invitrogen). The gel was analyzed using the NIH ImageJ program, and $MW_{theor}$/ $MW_{exp}$ values were calculated from the ratios of the experimental to theoretical molecular weights of the CAPs.

**Liposome Preparation**—To mimic bacterial and mammalian membranes, desired ratios of lipid solutions in chloroform were mixed and dried by rotary evaporation for a minimum of 1 h. HEPES buffer (10 mM HEPES, 150 mM NaCl (pH 7.4), and 1 mM CaCl$_2$) was added to rehydrate the lipid mixture to a final concentration of 1 mM, followed by sonication at a temperature higher than the melting temperatures of all of the lipids for 40 min prior to atomic force microscopy (AFM) measurements. For the FTIR experiments, the lipids were rehydrated in CaCl$_2$-free deuterated HEPES buffer to a final concentration of 2 μM and used immediately.

**AFM**—AFM images were acquired in fluid tapping mode using a Digital Instruments MultiMode scanning probe microscope comprising a Nanoscope IIIA controller equipped with either a J scanner (maximum scan area of 116 × 16 μm) or an E scanner (maximum lateral scan area of 14.6 × 14.6 μm). All images were acquired using an SNL-10 short thin tip (Veeco Probes, Camarillo, CA) fitted to a combined tapping mode/contact mode glass fluid cell fitted with inlet and outlet ports. Images were collected at a resolution of 512 × 512 pixels using a scan angle of 0° and a scan rate of 1 Hz. The tip drive frequency was generally set between 7 and 10 kHz with a drive amplitude set point of 0.2–0.6 V. The flow-through glass fluid cell was sealed against freshly cleaved mica with a silicone O-ring to create a 200-μl sample compartment. To facilitate liposome fusion and bilayer formation, the freshly cleaved mica sealed in the fluid cell was incubated for ~10 min with HEPES buffer prior to the introduction of ~500 μl of hydrated liposomes heated to ~70 °C. After ~30 min of incubation at room temperature to allow for bilayer formation, the fluid cell was flushed with liposome-free buffer. Reference AFM images of the bilayers were then acquired prior to the addition of an ~300-μl aliquot of the CAP of interest at 8 μM in HEPES buffer to the fluid cell. All AFM images were acquired at room temperature. Image analysis was performed using the Nanoscope software (version 5.30r1, Digital Instruments) on images that had been subjected to zero-order flatten and second-order plane fit filters. Relative height differences were determined using a horizontal section line along the slow scan axis.
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**Attenuated Total Reflection (ATR)-FTIR**—The FTIR spectra were collected using a Nexus 670 FTIR spectrometer equipped with an EverGlo mid-IR source, a liquid N$_2$-cooled MCT/A detector, a KB beam splitter, a SmartOrbit single-bounce ATR accessory fitted with a diamond internal reflectance element, and a custom flow-through sealed fluid cell at a resolution of 2 cm$^{-1}$ using an average of 128 scans over a scan range of 4000–700 cm$^{-1}$, referenced against the spectra of a clean diamond internal reflectance element. 500 µl of freshly sonicated liposomes in 10 mM deuterated HEPES and 150 mM NaCl (pH 7.4) were injected into the custom fluid flow cell (as described above) covering the diamond internal reflectance element and flushed with buffer after the lipids fused onto the surface, as confirmed with IR scans. Each tested peptide (8 µM) in HEPES buffer was added to the fused lipids, and scans were taken over time. All spectra were analyzed using Omnic software (version 5.2a, Nicolet Instrument Inc.) with lipid spectra subtracted and H$_2$O- and base line-corrected. Fourier self-deconvolution was performed on the amide peak from 1700 to 1600 cm$^{-1}$ using a full-width at half-height of 13 cm$^{-1}$ (typically 12–20 cm$^{-1}$ (21)) and an enhancement factor (K) of 2.4 (typically 2–3 (22)).

**Antibacterial Activity**—The antimicrobial activity of each peptide was tested in sterile 96-well plates by standard microtiter dilution protocols in cation-adjusted Mueller-Hinton broth (23). *Pseudomonas aeruginosa* strain PAO1 (a kind gift from Dr. Lynne Howell, Hospital for Sick Children) was grown to a final concentration of 5$^{10^8}$ forming units/ml. 11 l of diluted bacterial suspension. Plates were incubated at 37 °C overnight for 20 h, and the minimum inhibitory concentration (MIC) was taken as the concentration at which the bacterial growth was fully inhibited, as detected at A$_{540}$ using a Genesys 5 microplate autoreader spectrophotometer.

**Hemolytic Activity**—The hemolytic toxicity levels of the designed CAPs were measured in human RBCs as described previously (14). Freshly collected venous human blood with heparin was centrifuged at 1000 × g for 5 min at 11 °C to remove the buffy coat, and the RBCs obtained were washed three times with PBS. Peptides were diluted in PBS to 100 µl and mixed with 100 µl of 4% (v/v) RBC suspension to final concentrations ranging from 320 to 5$^{10^3}$ colony-forming units/ml. 11 µl of peptides at 2-fold serial dilutions were added to 100 µl of diluted bacterial suspension. Plates were incubated at 37 °C overnight for 20 h, and the minimum inhibitory concentration (MIC) was taken as the concentration at which the bacterial growth was fully inhibited, as detected at A$_{540}$ using a Genesys 5 microplate autoreader spectrophotometer.

**RESULTS**

**Helical Induction of CAPs in SDS Micelles**—The TM-Finder (25) predicts that the hydrophobic core segments of the peptides should adopt an α-helical secondary structure in membrane-mimetic environments. Both the 6K peptides and their derived 3K-3K partners (Table 1) displayed “random coil” structures in aqueous buffer (Fig. 1, dashed lines). In the membrane-mimetic environment of SDS micelles, the CAPs all

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**TABLE 1**

Sequences, molecular weights, and hydrophobicity values of designed CAPs

| Peptide | Amino acid sequence | Molecular weight | Hydrophobicity$^a$ |
|---------|---------------------|------------------|-------------------|
| 6K-F17  | KKKKKKAFAAWAFAANH$_2$ | 1836             | 1.48              |
| 6K-F17-4L | KKKKKKALFAWLAFAANH$_2$ | 2005             | 1.14              |
| 3K-F17-3K | KKKAAFAWAAFAANH$_2$ | 1836             | 1.48              |
| 3K-4L-3K | KKKALFAWLAFLAKK-NH$_2$ | 2005             | 3.14              |

$^a$ Hydrophobicity is the mean residue hydrophobicity of the peptide core segment, calculated from the Liu-Deber scale (13). Lys residues are not included.

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**FIGURE 1. CD spectra of CAPs in aqueous solution and SDS micelles.** The spectra were measured at 20 µM each CAP in 10 mM Tris-HCl and 10 mM NaCl (pH 7.0) in the presence (solid lines) and absence (dashed lines) of 10 mM SDS. The peptide sequences are given in Table 1. The curves are based on triplicate measurements with the buffer background subtracted. deg, degrees.
underwent an α-helical conformational transition, signified by the minima of their CD spectra at 208 and 222 nm (Fig. 1, solid lines). There was no significant difference between the induced helicity of the 6K peptides versus their 3K-3K analogs (Fig. 1, A and B).

Designed CAPs Are Dimeric on SDS-PAGE—The sequences of the designed peptides contain at least one pair of “small” residues separated by three residues (AxxxA motifs) that are known to promote helix-helix dimerization in transmembrane segments (9, 10). The F17 sequence contains three AxxxA motifs (two AAFAA sequences and a central AAWAA sequence); the F17-4L sequence retains the central motif (ALWLA). Consistent with our previous findings (11, 14), the $MW_{exp}/MW_{theor}$ values for both 6K-F17 and 6K-F17-4L are 2.4 (Fig. 2), indicating that these two CAPs form SDS-resistant dimers on SDS-polyacrylamide gels, even at the low loading levels used in silver stain analysis. Peptides 3K-F17-3K and 3K-F17-4L-3K migrate with $MW_{exp}/MW_{theor}$ ratios of 2.0 and 2.1, respectively, also indicative of dimer formation, but the slower movement of the 6K versus 3K-3K peptides may signal some qualitative difference in the strength of dimer interfaces (see “Discussion”).

CAP Interactions with Model Bacterial Membranes—Because the core segment hydrophobicity of CAPs is a key factor that affects bacterial membrane insertion directly and antimicrobial activity indirectly (11, 14), it becomes crucial to understand how peptide hydrophobicity influences their membrane-disruptive ability, particularly where host membranes are at stake. To address this, we compared the mechanistic details of the interactions of two CAPs with contrasting core hydrophobicities (6K-F17 with a “low” core segment hydrophobicity (1.48) and 6K-F17-4L with a “high” core segment hydrophobicity (3.14)) (Table 1) in a bacterial membrane lipid bilayer model using a combination of “simultaneous” AFM and ATR-FTIR techniques. In a typical run, CAPs (8 μM) were added to a lipid bilayer mixture composed of 3:1 POPE/DOPG, which resembles the phosphoethanolamine/phosphoglycerol ratio found in the inner membrane of Gram-positive and Gram-negative bacteria (26, 27). The in situ AFM images revealed that direct fusion of the lipid vesicles onto mica resulted in a molecularly smooth intact surface with no or few defects (Fig. 3A, left panel). The addition of the CAPs to the membrane bilayers led to an immediate remodeling or restructuring of the surface (28). Over time, CAP-induced membrane defects (darker region) were observed in the entire bilayer (Fig. 3A, right panel). Notably, we did not observe detachment of the membrane from the supporting mica; rather, the membrane itself appeared structurally roughened, indicating the destabilization and disruption of the membrane bilayer. In tandem to the AFM measurements that revealed the structural nature of the peptide-bilayer interaction, FTIR spectra were monitored continuously to pinpoint any accompanying structural changes in the CAPs that may underlie their membrane-disruptive properties (29, 30). As shown in Fig. 4A, 6K-F17 adopted a largely α-helical structure (indicated by the amide I band at ~1650 cm$^{-1}$) over the corresponding time course, and the spectral intensity increased over time as additional amounts of CAPs penetrated the lipids. In contrast, 6K-F17-4L initially induced an α-helical conformation in the bacterial membrane lipids and eventually exhibited a time-dependent increase in aggregated β-strand species (indicated by the amide I band at ~1625 cm$^{-1}$) with a concomitant relatively decreased population of α-helical spe-
not result in an aggregated association of these two CAPs with the bacterial membrane did and the absence of the diagram (Fig. 4, amide I region of their FTIR spectra in bacterial membranes intensity increased over time as additional amounts of CAPs penetrated the lipids, and no further changes were detected beyond the final time point in each diagram (black lines). The 6K-F17 peptide tested in E is the β-isomer; the l-enantiomer gives comparable results (data not shown).

Antimicrobial activities of designed CAPs

| Peptide | MIC μM |
|---------|--------|
| 3K-F17-3K | 32     |
| 6K-F17   | 4      |
| 3K-F17-4L-3K | 8    |
| 6K-F17-4L | 16     |

MIC is the minimum inhibitory concentration, defined as the lowest peptide concentration required to fully inhibit bacterial growth, as tested against P. aeruginosa strain PAO1. See “Experimental Procedures” for further details of these experiments.

Positive Charges on Both CAP Termini Minimize Aggregation in Bacterial Membranes—Peptide 6K-F17-4L discussed above likely self-oligomerizes and aggregates in an antiparallel manner because the grouping of the six Lys positive charges at the N terminus mitigates against parallel association. Therefore, placement of three positive charges on each of the N and C termini while an identical peptide core sequence is maintained might be expected to produce a mixture of parallel and antiparallel dimers, but likely with reduced higher level aggregation in membranes due to charge repulsion. To test this hypothesis, we designed the CAPs 3K-F17-3K and 3K-F17-4L-3K as charge distribution analogs of the parent CAPs 6K-F17 and 6K-F17-4L, respectively (Table 1). In contrast to 6K-F17-4L, the interactions of 3K-F17-3K and 3K-F17-4L-3K with the bacterial membrane mimic were less well defined, appearing to result in a reduction in the lateral stability of the membrane itself rather than outright restructuring. This was seen in the emergence of sunken regions and the ability of the AFM tip to induce local-
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**FIGURE 5. Hemolytic activities of the designed CAPs.** The percent hemolysis (±error) was tested at peptide concentrations ranging from 5 to 320 μM with 4% (v/v) RBCs. Values calculated from at least triplicate measurements are listed below the bar graph. See “Experimental Procedures” for details of these experiments.

Hemolytic Activity—The hemolytic activities of the designed CAPs were measured against human RBCs at concentrations ranging from 320 to 10 μM (Fig. 5). Peptides with low hydrophobicity, namely 3K-F17-3K and 6K-F17, displayed no hemolysis even at high concentrations (320 and 160 μM, respectively). In striking contrast, peptides with high hydrophobicity (3K-F17-4L) showed significant membrane destabilization (supplemental Fig. S2B) and very minimum structures in the zwitterionic mammalian membrane model (Fig. 4G), whereas 3K-F17-4L-3K adopted significant helical structure detected by FTIR (Fig. 4H) and induced visible small “holes” in the bilayer (supplemental Fig. S2C).

**DISCUSSION**

Our laboratory has developed a novel category of synthetic hydrophobic membrane-active peptides that display high antimicrobial activity (7, 8, 12, 14). Relating their biology to their biochemical mechanism(s) of action can aid in optimization of the properties of sequence that underlie their potency. In this study, we examined four such bioactive peptides, selected to highlight the extremes of two such properties: core sequence hydrophobicity (four Ala residues substituted with four Leu residues) and positive charge distribution (six Lys residues on the N terminus versus three Lys residues on each of the N and C termini). The peptides were subjected to microbiological assays and a panel of biophysical experiments, including CD, SDS-PAGE, AFM, and FTIR. Among key results, we found that CAPs can inflict serious membrane damage on model bacterial membranes, yet CAPs with low hydrophobicity (6K-F17 and 3K-F17-3K) are inert to mammalian membranes. It was also observed that a CAP with relatively higher hydrophobicity (i.e. 6K-F17-4L) undergoes a structural transition in contact with bacterial-type membranes from α-helical to β-strand-type structure, whereas the corresponding CAP with lower hydrophobicity (i.e. 6K-F17) largely retains its α-helical conformation upon entering the membrane. This phenomenon is likely attributable to a charge neutralization effect as the peptides bind to the surface of the anionic bacterial membranes, and the resulting dehydrated environment facilitates the formation of β-strand-type aggregates of the CAP with a sequence of higher hydrophobicity (34, 35). This result indicates that (overly) high core segmental hydrophobicity can lead to an increased potential to peptide self-association at the membrane surface (and possibly to precipitation), thus limiting the concentration of peptide actually impacting on the bacterial membrane and consequently reducing antimicrobial activity; this latter notion is supported by the relatively lower MIC for the 6K-F17 peptide (4 μM) versus its Leu-containing counterpart (16 μM).

Because 6K-F17-4L can self-aggregate at the bacterial membrane surface and undergo oligomerization via propagation of antiparallel aggregation (and hence staggered 6K regions), we sought to inquire whether separated charge distribution would prevent such self-association without altering the helix-forming and dimerization ability of the peptide in the membrane environment, which may be the crucial properties for bioactivity in the 6K-F17 series of novel CAPs (11, 14). We found that charge distribution itself does not dominate the impact on activity because it improves the antimicrobial activity of only the CAP with high hydrophobicity (3K-F17-4L-3K) but reduces the activity of the CAP with low hydrophobicity (3K-F17-3K) (Table 2). As described above, this situation can be explained by the fact that CAPs still need to self-dimerize to function upon binding to the bacterial membrane (see below), yet separated charges are seen to mitigate against the parallel or antiparallel aggregation of the 3K-3K peptides, ostensibly via repulsions of the positive changes at the peptide termini.

In addition to their ability to induce defects in bacterial membrane mimics, the four peptides examined also display a facile propensity to insert into micelles of the anionic detergent SDS and adopt helical conformations (Fig. 1). As well, all four peptides migrate as dimers on SDS-polyacrylamide gels (Fig. 2). However, when gel migration behavior is examined in detail, several further observations that relate CAP structure to function emerge. First, it was noted that each four-Leu-containing peptide migrated perceptibly slower than its four-Ala counterpart. This observation can be attributed to a charge neutralization effect as the peptides anomalously on SDS-PAGE due to varying levels of detergent.

**FIGURE 6. Hemolytic activities of the designed CAPs.** The percent hemolysis (±error) was tested at peptide concentrations ranging from 5 to 320 μM with 4% (v/v) RBCs. Values calculated from at least triplicate measurements are listed below the bar graph. See “Experimental Procedures” for details of these experiments.
binding as a function of sequence hydrophobicity (36); thus, regardless of the four-Leu positions versus their four-Ala counterparts, the 6K-F17-4L peptide should be migrating significantly more slowly than the 6K-F17 peptide, yet its position is virtually identical. Given that all four peptides are dimeric (Fig. 2), this latter finding may be explicable by the fact that the Leu residues in 6K-F17-4L largely line the peptide-peptide dimer interface, thus exposing mainly Ala rather than Leu residues to detergent, as would be the case for the 6K-F17 peptide as well.

The same analysis holds for 3K-F17-3K versus 3K-F17-4L-3K, which also display comparable migration positions. These findings allow us to choose representative CAP dimer models from CHI clusters, as shown in Fig. 6. In CAPs where Ala is not insertions allow us to choose representative CAP dimer models from which also display comparable migration positions. These findings allow us to choose representative CAP dimer models from CHI clusters, as shown in Fig. 6. In CAPs where Ala is not insertions allow us to choose representative CAP dimer models from which also display comparable migration positions. These findings allow us to choose representative CAP dimer models from CHI clusters, as shown in Fig. 6. In CAPs where Ala is not insertions allow us to choose representative CAP dimer models from which also display comparable migration positions. These findings allow us to choose representative CAP dimer models from CHI clusters, as shown in Fig. 6. In CAPs where Ala is not insertions allow us to choose representative CAP dimer models from which also display comparable migration positions. These findings allow us to choose representative CAP dimer models from CHI clusters, as shown in Fig. 6. In CAPs where Ala is not insertions allow us to choose representative CAP dimer models from which also display comparable migration positions. These findings allow us to choose representative CAP dimer models from CHI clusters, as shown in Fig. 6. In CAPs where Ala is not insertions allow us to choose representative CAP dimer models from which also display comparable migration positions. These findings allow us to choose representative CAP dimer models from CHI clusters, as shown in Fig. 6. In CAPs where Ala is not insertions allow us to choose representative CAP dimer models from which also display comparable migration positions. These findings allow us to choose representative CAP dimer models from CHI clusters, as shown in Fig. 6. In CAPs where Ala is not insertions allow us to choose representative CAP dimer models from which also display comparable migration positions. These findings allow us to choose representative CAP dimer models from CHI clusters, as shown in Fig. 6. In CAPs where Ala is not ins...
9. Lear, J. D., Stouffer, A. L., Gratkowski, H., Nanda, V., and Degrado, W. F. (2004) Association of a model transmembrane peptide containing Gly in a heptad sequence motif. Biophys. J. 87, 3421–3429

10. Schneider, D., and Engelman, D. M. (2004) Motifs of two small residues can assist but are not sufficient to mediate transmembrane helix interactions. J. Mol. Biol. 343, 794–804

11. Chang, Y., Guarnieri, M. T., Vasil, A. I., Vasil, M. L., Mant, C. T., and Glukhov, E., Burrows, L. L., and Deber, C. M. (2008) Membrane interactions of designed cationic antimicrobial peptides: the two thresholds. Biopolymers 47, 41–62

12. Liu, L. P., Li, S. C., Goto, N. K., and Deber, C. M. (1996) Threshold hydrophobicity dictates helical conformations of peptides in membrane environments. Biopolymers 39, 465–470

13. Liu, L. P., and Deber, C. M. (1997) Anionic phospholipids modulate peptide insertions into membranes. Biochemistry 36, 6122–6132

14. Goormaghtigh, E., Raussens, V., and Ruysschaert, J. M. (1999) Attenuated total reflection infrared spectroscopy of proteins and lipids in biological membranes. Biochim. Biophys. Acta 1422, 105–185

15. Tam, L. K., and Tatulian, S. A. (1997) Infrared spectroscopy of proteins and peptides in lipid bilayers. Q. Rev. Biophys. 30, 365–429

16. Wu, M., and Hancock, R. E. (1999) Interaction of the cyclic antimicrobial cationic peptide bactenecin with the outer and cytoplasmic membrane. Biochemistry 38, 7745–7752

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17. De Kroon, A. I., Soekarjo, M. W., De Gier, J., and De Kruijff, B. (1990) The diastereomeric assembly of polylysine is the low volume pathway for helical peptides. J. Mol. Biol. 212, 212–219

18. Dathe, M., Nikolenko, H., Meyer, J., Beyermann, M., and Biener, M. (2001) Optimization of the antimicrobial activity of magainin 2 amide in interaction with membranes. Biochemistry 30, 8229–8240

19. Dathe, M., Milon, H., Becker, M., Beyermann, M., and Biener, M. (2001) Optimization of the antimicrobial activity of magainin peptides by modification of charge. FEBS Lett. 501, 146–150

20. Lepthin, S., Har, J. Y., Wohland, T., and Ding, J. L. (2010) Correlation of charge, hydrophobicity, and structure with antimicrobial activity of S1 and MIRIAM peptides. Biochemistry 49, 9161–9170

21. Liu, L. P., and Deber, C. M. (1997) Anionic phospholipids modulate peptide insertion into membranes. Biochemistry 36, 5476–5482

22. Goormaghtigh, E., Raussens, V., and Ruysschaert, J. M. (1999) Attenuated total reflection infrared spectroscopy of proteins and lipids in biological membranes. Biochim. Biophys. Acta 1422, 105–185

23. Tam, L. K., and Tatulian, S. A. (1997) Infrared spectroscopy of proteins and peptides in lipid bilayers. Q. Rev. Biophys. 30, 365–429

24. Wu, M., and Hancock, R. E. (1999) Interaction of the cyclic antimicrobial cationic peptide bactenecin with the outer and cytoplasmic membrane. J. Biol. Chem. 274, 29–35

25. Adams, P. D., Engelman, D. M., and Brünger, A. T. (1996) Improved prediction for the structure of the dimeric transmembrane domain of glycophorin A obtained through global searching. Protein 26, 257–261

26. Debarbouillre, L., Wang, C., Liu, L. P., Prior, A. S., Agrawal, S., Muskat, B. L., and Cuticchia, A. J. (2001) TM Finder: a prediction program for transmembrane protein segments using a combination of hydrophobicity and nonpolar phase helicity scales. Protein Sci. 10, 212–219

27. van der Does, C., Swaving, J., van Klompenburg, W., and Driessen, A. J. (2000) Non-bilayer lipids stimulate the activity of the reconstituted bacterial protein translocase. J. Biol. Chem. 275, 2472–2478

28. Oreopoulos, J., and Yip, C. M. (2009) Combinatorial microscopy for the study of protein-membrane interactions in supported lipid bilayers: order parameter measurements by combined polarized TIRFM/AFM. J. Struct. Biol. 168, 21–36

29. Yin, L. M., Lee, S., Edwards, M. A., Yip, C. M., and Deber, C. M. (2011) Peptide-lipid interactions: a comparative study based on tryptophan fluorescence measurements combined with the use of aqueous and hydrophobic quenchers. Biochemistry 50, 846–856

30. Govan, J. R., and Deretic, V. (1996) Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. Microbiol. Rev. 60, 539–574

31. Florin-Christensen, J., Suarez, C. E., Florin-Christensen, M., Wainselbaum, M., Brown, W. C., McElwain, T. F., and Palmer, G. H. (2001) A unique phospholipid organization in bovine erythrocyte membranes. Proc. Natl. Acad. Sci. U.S.A. 98, 7736–7741

32. Mukherjee, S., Chowdhury, P., and Gai, F. (2007) Infrared study of the role of charge and hydrophobicity in peptide-lipid interaction: a comparative study based on tryptophan fluorescence measurements combined with the use of aqueous and hydrophobic quenchers. Biochemistry 46, 7206–7215

33. Florin-Christensen, J., Suarez, C. E., Florin-Christensen, M., Wainselbaum, M., Brown, W. C., McElwain, T. F., and Palmer, G. H. (2001) A unique phospholipid organization in bovine erythrocyte membranes. Proc. Natl. Acad. Sci. U.S.A. 98, 7736–7741

34. Mukherjee, S., Chowdhury, P., and Gai, F. (2007) Infrared study of the role of charge and hydrophobicity in peptide-lipid interaction: a comparative study based on tryptophan fluorescence measurements combined with the use of aqueous and hydrophobic quenchers. Biochemistry 46, 7206–7215

35. Dzwolak, W., Ravindra, R., Nicolini, C., Jansen, R., and Winter, R. (2004) Lipid-induced organization of a primary amphipathic peptide: a coupled AFM-monolayer study. J. Membr. Biol. 167, 241–249

36. Verity, J. E., Chhabria, N., Sinnathamby, K., and Yip, C. M. (2009) Tracking molecular interactions in membranes by simultaneous ATR-FTIR-AFM. Biophys. J. 97, 1225–1231

37. Vie, V., Van Mau, N., Chaloin, L., Lesniewska, E., Heitz, F., and Le Grimmellec, C. (1999) Lipid-induced organization of a primary amphipathic peptide: a coupled AFM-monolayer study. J. Membr. Biol. 167, 241–249

38. Verity, J. E., Chhabria, N., Sinnathamby, K., and Yip, C. M. (2009) Tracking molecular interactions in membranes by simultaneous ATR-FTIR-AFM. Biophys. J. 97, 1225–1231

39. Mukherjee, S., Chowdhury, P., and Gai, F. (2007) Infrared study of the role of charge and hydrophobicity in peptide-lipid interaction: a comparative study based on tryptophan fluorescence measurements combined with the use of aqueous and hydrophobic quenchers. Biochemistry 46, 7206–7215

40. Mukherjee, S., Chowdhury, P., and Gai, F. (2007) Infrared study of the role of charge and hydrophobicity in peptide-lipid interaction: a comparative study based on tryptophan fluorescence measurements combined with the use of aqueous and hydrophobic quenchers. Biochemistry 46, 7206–7215

41. Mukherjee, S., Chowdhury, P., and Gai, F. (2007) Infrared study of the role of charge and hydrophobicity in peptide-lipid interaction: a comparative study based on tryptophan fluorescence measurements combined with the use of aqueous and hydrophobic quenchers. Biochemistry 46, 7206–7215