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Toxins and antimicrobial peptides: Interactions with membranes

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Abstract

The innate immunity to pathogenic invasion of organisms in the plant and animal kingdoms relies upon cationic antimicrobial peptides (AMPs) as the first line of defense. In addition to these natural peptide antibiotics, similar cationic peptides, such as the bee venom toxin melittin, act as nonspecific toxins. Molecular details of AMP and peptide toxin action are not known, but the universal function of these peptides to disrupt cell membranes of pathogenic bacteria (AMPs) or a diverse set of eukaryotes and prokaryotes (melittin) is widely accepted. Here, we have utilized spectroscopic techniques to elucidate peptide-membrane interactions of alpha-helical human and mouse AMPs of the cathelicidin family as well as the peptide toxin melittin. The activity of these natural peptides and their engineered analogs was studied on eukaryotic and prokaryotic membrane mimics consisting of <200-nm bilayer vesicles composed of anionic and neutral lipids as well as cholesterol. Vesicle disruption, or peptide potency, was monitored with a sensitive fluorescence leakage assay. Detailed molecular information on peptide-membrane interactions and peptide structure was further gained through vibrational spectroscopy combined with circular dichroism. Finally, steady-state fluorescence experiments yielded insight into the local environment of native or engineered tryptophan residues in melittin and human cathelicidin embedded in bilayer vesicles. Collectively, our results provide clues to the functional structures of the engineered and toxic peptides and may impact the design of synthetic antibiotic peptides that can be used against the growing number of antibiotic-resistant pathogens.

Keywords

Fluorescence; melittin; cathelicidin; Raman; spectroscopy; membranes; LL-37; protein folding

INTRODUCTION

The interaction between lytic peptides and membranes is a unique class of protein-membrane interactions. Antimicrobial peptides (AMPs) and hemolytic peptides (toxins) are membrane-associated biomolecules with typically less than 50 residues that have amphipathic structures and are soluble in the aqueous phase. Despite being water soluble, these peptides can spontaneously insert into the hydrophobic core of membranes. AMPs and toxins are similar to membrane proteins in that the insertion of the peptide into a bilayer is

closely associated with formation of its functional secondary structure. However, in contrast to membrane proteins, AMPs and toxins function primarily as membrane disruptors. The paucity of data describing the molecular interactions between membranes and these unique amphipathic, cell-killing peptides combined with the observation that these peptides display characteristics of both soluble and membrane proteins motivate the current study.

Melittin, a 26 amino-acid peptide that is the main component in European honeybee venom, is one of the most well studied hemolytic, toxic peptides.¹ Melittin is cationic (+6 net charge) and exhibits amphipathic α -helical secondary structure upon spontaneous insertion into lipid membranes. Under some aqueous conditions, such as high pH, high salt environment, and high peptide concentration, melittin self-associates to form α -helical tetramers (Figure 1).^{2, 3} In this tetrameric conformation, the charged and polar residues are located towards the ends and periphery of the structure, and a hydrophobic pocket remains in the center (Figure 1). The peptide is highly soluble in water (>250 mg/mL) despite the fact that residues 1–20 are predominantly hydrophobic.³ Melittin perturbs the lipid structure of model membranes and increases the permeability of vesicles, inducing leakage of encapsulated ions and markers.^{4–6} It has been proposed that this peptide perforates the membrane by forming pores.^{4, 5} Melittin is believed to reach the membrane through the aqueous phase as a random coil, so elucidating structure of melittin in the aqueous phase is of biological relevance.⁷ Because the function of melittin is directly related to its structure in the lipid membrane, the structural analysis of this peptide upon interaction with lipid membranes is critical to understanding the behavior of melittin.

In contrast to toxins such as melittin, AMPs that comprise the innate immunity of species across the animal and plant kingdoms have evolved to destroy pathogens during infection. The basis for the selectivity for pathogenic bacteria is partly electrostatic: AMPs have a high affinity for the highly anionic membranes of bacteria. In the human innate immune system, two major classes of AMPs have been identified, the cathelicidins and defensins.⁸ LL-37 (Figure 2) is derived from the only human cathelicidin gene identified to date, hCAP18, and is expressed primarily in neutrophils and epithelial cells.⁹ Like the majority of antimicrobial peptides found in nature, LL-37 is cationic with an overall charge of +6; its functional structure is an α -helix.¹⁰ LL-37 exhibits broad-spectrum antimicrobial activity, binds and neutralizes bacterial lipopolysaccharide, plays a role in wound healing, and is directly and indirectly chemotactic.⁸ The expression and activity of LL-37 have been implicated in a variety of medical conditions, including rosacea, atopic dermatitis, psoriasis, and cystic fibrosis.⁸ Cathelicidins from different species exhibit variable potencies; for example, it has previously been reported that the mouse cathelicidin mCRAMP is less potent than LL-37 against some bacteria.^{11, 12} The importance of these abundant AMPs in immunity and disease combined with the lack of detailed structural information on LL-37-membrane interactions further motivate the current report.

In this work, we report structural differences among the broad-spectrum toxin, melittin, and the mammalian AMPs LL-37 and mCRAMP in a variety of conformations, including random coil in aqueous solution, α -helical oligomers in aqueous solution, and membrane-associated, functional forms (see Table 1). Lipids were selected to model the environment that induces the peptide's antimicrobial activity (vesicles composed of anionic and neutral

lipid molecules) and hemolytic activity (vesicles composed of neutral lipid and cholesterol molecules). We have applied a combination of electronic and vibrational spectroscopy to explore the local environment of site-specific residues and secondary structure of these peptides in these different conditions. Intrinsic fluorescence was utilized to elucidate the local environment of native and engineered tryptophan residues of melittin and LL-37 while a sensitive leakage assay based on fluorescence of extrinsic dye molecules helps reveal the relative potency of these peptides. UV Resonance Raman spectroscopy (UVR) is a powerful technique used to examine the vibrational structure of the backbone and the local environment of side chains;¹³ here we utilize this structure-sensitive technique to elucidate the overall backbone secondary structure as well as hydrogen bonding state of the carbonyl group near the single proline residue in melittin and LL-37. Finally, circular dichroism spectroscopy was also utilized to probe the secondary structure of these peptides, and these results are compared to UVR data.

MATERIALS AND METHODS

Chemicals

Melittin purchased from Axxora and wild-type LL-37 and mouse cathelicidin (mCRAMP) purchased from Anaspec were used as received. LL-37 mutants F6W and F17W were purchased with >95% purity from Genscript and used without further purification. Anionic lipid 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt, POPG) and neutral lipid 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids as chloroform solutions. Cholesterol (Alfa Aesar) was used as received. Triton X-100 (TritonX) detergent was purchased from MP Biomedicals, Inc. All other chemicals and reagents were purchased from Fisher Scientific. Peptide concentration for all experiments was 10–50 μ M. The buffer used for melittin experiments was 20 mM potassium phosphate buffer at pH = 7.3, and the buffer used for LL-37 and mCRAMP experiments was 50 mM sodium bicarbonate, 1.1 mM sodium monobasic phosphate at pH=7.4. Dye leakage assays were performed using fluorophore 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) and quencher *p*-xylenebis(pyridinium)bromide (DPX) from Invitrogen.

Vesicle preparation

To make 2:1 molar ratio POPC:POPG (anionic) vesicles, a chloroform solution containing 5 mg of POPG was added to a chloroform solution containing 10 mg of POPC. The resulting solution was dried under a stream of argon. Dried lipids were re-suspended in buffer using a bath sonicator. For dye-containing vesicles, the buffer also contained 50 mM ANTS and 50 mM DPX. 2:1 molar ratio POPC:cholesterol vesicles (cholesterol-containing neutral vesicles) were made by replacing the POPG solution with 2.5 mg cholesterol after drying the POPC solution. All vesicles were prepared by extruding the lipid suspension eleven times through a polycarbonate filter with pore size 200 nm using a liposome extruder or via probe sonication to create <100 nm vesicles as determined by dynamic light scattering experiments. These vesicle solutions were filtered through a 0.45 μ m filter and passed through a gravity driven desalting column. The elution containing vesicle was collected and allowed to equilibrate for >2 hrs at 37 °C. The final lipid concentration used in experiments was 1 mg/mL.

Circular dichroism and fluorescence

Peptides were incubated in 1 mg/mL vesicle solution for 1–4 hours at 37°C. Circular dichroism (CD) spectra were obtained on an Aviv202 spectropolarimeter. Buffer- or vesicle-only spectra were subtracted from all peptide spectra. Fluorescence spectra were acquired on a Jobin Yvon Horiba Fluorolog-3 spectrofluorometer. The excitation wavelength for tryptophan was 290 nm, and fluorescence measurements were recorded at a constant temperature of 30 or 37 °C. For leakage assay experiments, dye-containing vesicle solutions were allowed to incubate with peptide at 37 °C. The excitation wavelength was 386 nm. TritonX detergent was added to dye-containing vesicle solutions to determine the maximum fluorescence intensity corresponding to 100% dye leakage. Control experiments of dye-containing vesicle solutions in the absence of peptide were also performed to measure spontaneous leakage of dyes.

UV resonance Raman spectroscopy

The UVRR setup has been described elsewhere.¹⁴ Vibrational spectra of the backbone were obtained with the 210 nm fourth-harmonic output of the 840 nm fundamental beam. A typical sample volume of 2 mL was flowed through a vertically-mounted fused silica capillary at a rate sufficient to ensure fresh sample for each laser pulse. The UV power was ~1–2 mW at the sample. UVRR spectra of all appropriate blank solutions were also collected and subtracted from corresponding melittin spectra. Accuracy of $\pm 2 \text{ cm}^{-1}$ was determined using standard ethanol peaks and the spectral bandpass was $\sim 9 \text{ cm}^{-1}$.

RESULTS

Fluorescence

Steady state fluorescence spectra of 50 μM melittin in buffered solution containing anionic vesicles, 2 M NaCl, and cholesterol-containing neutral vesicles are shown in Figure 3. Spectra of melittin and the model compound L-tryptophan (L-trp) in buffer-only solutions are also displayed. Melittin in anionic vesicles exhibits the most blue-shifted λ_{max} of 336 nm while melittin in buffer solution has a λ_{max} of 355 nm. In cholesterol vesicles, the emission λ_{max} continued to evolve from 347 nm (2 hours) to 335 nm (14 hours) and stabilized at 335 nm up to the 38-hour observation period. Tryptophan fluorescence of melittin folded in 2 M NaCl and in vesicles shifts upon addition of ~3 M guanidinium hydrochloride (GdmHCl) to 355 – 357 nm. Normalized steady-state tryptophan fluorescence spectra of 40 μM LL-37 mutant F17W in the presence of anionic vesicles, buffer, and distilled (DI) water are also shown in Figure 3. Emission maxima are most blue-shifted in the presence of anionic and cholesterol vesicles at 328 nm. In carbonate-containing buffer and DI water, the fluorescence maxima are 336 and 355 nm, respectively. The F6W mutant displayed nearly identical emission spectra in all conditions with the exception of carbonate-containing buffer; in the presence of carbonate, the emission maximum of F6W is 344 nm.

Fluorescence leakage assay results are presented in Figure 4 for melittin, LL-37, and the mouse cathelicidin mCRAMP at increasing peptide concentrations. Peptide-induced leakage is reported relative to TritonX-induced leakage, which is 100%. For melittin, leakage was high at low concentrations and did not vary significantly in the concentration range tested

here. While there was some variability in leakage under different experimental conditions (e.g. different vesicle size and incubation time), the leakage graphs for melittin displayed a general plateau after $\sim 20 \mu\text{M}$. For the cathelicidins, leakage depended linearly on peptide concentration in the range 5 to $40 \mu\text{M}$. LL-37 and its F6W and F17W mutants displayed identical behavior within the experimental error. The comparison to mCRAMP was motivated by previous findings that LL-37 and mCRAMP exhibit different potencies^{11, 12} and this variation in potency is supported by the leakage assay data. Within this concentration range there is no spectroscopic evidence of peptide-peptide interactions in the presence of vesicles. Scattering from vesicles was present at all concentrations after 1 hr of incubation with peptide.

Circular dichroism

Circular dichroism (CD) spectra of melittin, LL-37, and mCRAMP are shown in Figure 5 under conditions that stabilize the random coil conformation and α -helical forms. All peptides exhibited unfolded structures in buffer (melittin) or DI water (LL-37 and mCRAMP). In the presence of 2 M NaCl (melittin) or carbonate (LL-37), these two peptides exhibited α -helical structures in solution. In contrast, mCRAMP did not exhibit α -helical structure in carbonate. The anomalous CD spectrum of melittin in 2 M NaCl has been reported previously (see discussion). In the presence of anionic and cholesterol-containing vesicles, the peptides form α -helical secondary structure consistent with the CD minima at 208–210 nm and 222 nm as well as a maximum at 193–196 nm. CD spectra of the LL-37 mutants F6W and F17W under all conditions were identical to those of native LL-37 within the S/N (data not shown).

UVR spectra of the peptide backbone

Vibrations coupled to the amide $\pi \rightarrow \pi^*$ transition of the peptide backbone are enhanced with 210 nm excitation (Figure 6). While this wavelength is still resonant with the B_b electronic transition of the tryptophan residue, Raman scattering is dominated by amide I, II, and III backbone modes. The 210 nm Raman spectrum of model compound L-trp shows negligible signal in the backbone region. Analysis of the amide III ~ 1250 to $\sim 1350 \text{ cm}^{-1}$ region via decomposition of the UVR bands using a least-squares fitting technique confirms the secondary structure assignments of random coil and α -helix.^{15, 16} For example, the amide S band at $\sim 1390 \text{ cm}^{-1}$, a C_{α} -H bending mode that disappears when an α -helix forms,¹⁷ is clearly visible in the spectrum of melittin in buffer (Figure 6D) and is missing in the spectra of melittin in other environments. However, subtle variations in the amide III region indicate that the α -helical structure of melittin in the presence of cholesterol vesicles is different from that in the presence of anionic vesicles. For example, inclusion of a fourth peak in the decomposition of the amide III region of cholesterol-containing vesicles (Figure 6C) was necessary to provide a reasonable fit to the raw data; this minor peak contributes $\sim 5\%$ to the total integrated area and may reflect different backbone structures not observable in CD spectra. These and other differences between CD and UVR interpretations are discussed below. The amide IIp band is a sensitive indicator of hydrogen bonding of the leu-pro (melittin) or val-pro (LL-37) carbonyl groups and is found between 1425 and 1475 cm^{-1} . This band was decomposed into two Gaussian bands corresponding to strong hydrogen bonded ($\sim 1460 \text{ cm}^{-1}$) and weak hydrogen bonded ($\sim 1440 \text{ cm}^{-1}$) carbonyl

groups.¹⁸ The ratios of the intensities of the $\sim 1460\text{ cm}^{-1}$ and $\sim 1440\text{ cm}^{-1}$ ($R_p = I_{1460}/I_{1440}$) are 2.3 for melittin in buffer, 0.9 for melittin in anionic vesicles, 1.3 for melittin in cholesterol vesicles, 0.6 for LL-37 in anionic vesicles, and 0.6 for LL-37 in cholesterol vesicles. The error for the intensity ratio R_p is $\sim 20\%$.

DISCUSSION

For over 50 years, melittin has been of great interest in the scientific community because of its abundance in honeybee venom and formation of functional structure upon insertion into a lipid membrane. This peptide has since become a model compound to study peptide-membrane interactions. The mechanisms of membrane disruption have been studied using a variety of experimental and computational techniques including NMR, CD, fluorescence, and molecular dynamics simulations. Melittin also forms secondary structure in environments that promote self-association (high pH, high ionic strength, and high peptide concentration).³ In these cases, melittin forms an α -helical tetramer with a hydrophobic core that originates from the sequestering of hydrophilic and hydrophobic amino acids upon helix formation.¹⁹ We presently investigated the structure of melittin in four conditions: A) melittin in the presence of 2 M NaCl (α -helical tetramer), B) melittin in the presence of anionic vesicles (bacterial membrane mimic), C) melittin in the presence of cholesterol-containing neutral vesicles (eukaryotic membrane mimic) and D) melittin in the presence of buffer only (random coil). Results from fluorescence, circular dichroism, and UV resonance Raman experiments contribute to our understanding of melittin-membrane interactions by providing insight into the functional structures as well as details about local regions in melittin under relevant conditions.

In contrast to the plethora of studies on the broad-spectrum toxin melittin, very few biophysical studies have been published for the amphipathic α -helical antimicrobials that are part of the innate immunity of plants and animals. These AMPs, including the cathelicidins, differ significantly from toxins such as melittin in that AMPs have evolved to primarily destroy pathogenic bacteria. While the primary basis for this selectivity is electrostatic in nature, specific peptide-lipid interactions are expected to play important roles in the selectivity and mechanisms of membrane disruption. The fact that these toxins and AMPs have the same net charge (Table 1) but variable distribution of the charged and polar residues (Figures 1 and 2) validates the significance of specific molecular interactions for determining selectivity and potency. Here, we probe the mechanisms by which the human AMP cathelicidin acts on synthetic vesicles by utilizing single-point mutations in which a native phenylalanine residue was converted to a tryptophan residue in position 6 or 17 to yield two mutant LL-37 peptides, F6W and F17W. In addition to gaining insight into LL-37, the current study seeks to compare peptide-membrane interactions of toxins and AMPs. Finally, results presented here illustrate the wealth of information that may be gained from the combination of the inherently structure-sensitive technique of resonance Raman spectroscopy with typical electronic spectroscopic tools such as fluorescence and CD.

Potency of peptides

At the lowest concentration of peptides studied here, melittin is found to be more effective at disrupting synthetic membranes than LL-37. This result is consistent with prior reports that melittin has much lower minimal inhibitory concentrations (MICs) than LL-37 for a number of bacteria.^{12, 20} Data in Figure 4 illustrates that the *in vitro* leakage assay utilized here and by others may be a reliable indicator of peptide potency. In addition to these measurements of relative potency, the leakage assay also provides evidence that a single point mutation in position 6 or 17 from phenylalanine to tryptophan in LL-37 did not affect the peptide's ability to cause leakage of anionic vesicles. Secondary structure differences among these variants of LL-37 were also negligible in all tested conditions (data not shown). Similar results have been reported previously; mutation from phenylalanine to tryptophan in position 27 of LL-37 was shown to have negligible effect on antimicrobial activity against *B. subtilis* and *S. aureus* as well as negligible effect on secondary structure.²¹ The lack of observable change in peptide potency upon incorporation of a tryptophan residue may be counterintuitive since thermodynamic studies have indicated that tryptophan partitions into the bilayer interface more readily than phenylalanine.²²

While tryptophan is an important residue in membrane-associated proteins, the LL-37 results described here are not without precedent. Single-tryptophan mutants of magainin-2 F5W and F16W were equipotent against *E. coli* and in causing leakage from anionic large unilamellar vesicles relative to wildtype.²³ Tryptophan to phenylalanine mutations of all five tryptophan residues (38% of the peptide by residue count) of indolicidin²⁴ and the single tryptophan residue of cecropin²⁵ had negligible effect on antimicrobial activity but lessened or eliminated hemolytic activity. While the replacement of phenylalanine with tryptophan has varying effects on peptide potency, omission of the single tryptophan residue in cecropin²⁵ and melittin²⁶ greatly reduced both hemolytic and antimicrobial activities, suggesting that aromatic residues as a general class are critical. Our observation that the phenylalanine-to-tryptophan mutation in LL-37 had negligible structural and potency effects relative to wild-type is consistent with these and other reports and supports the use of these mutations to elucidate LL-37 action.

Tryptophan local environment

The fluorescence spectra of melittin and LL-37 mutants indicate that the single tryptophan residues are in different environments under the various folding conditions. The variation in emission maxima for trp-19 in melittin follows the general trend: Trp-19 is in the most hydrophobic environment when folded in 2 M NaCl and in anionic vesicles, and in the least hydrophobic environment when unordered in buffer solution. One interesting result is that the fluorescence maximum of trp-19 in cholesterol-containing vesicles evolved over 14 hours to the most blue-shifted value of 335 nm; after 14 hours, no changes were observed up to the 38-hour observation time. This slow evolution of the fluorescence maximum is not due to a change in vesicle diameter since the cholesterol-containing vesicles maintained a constant diameter of ~200 nm throughout the 38 hours (data not shown). Instead, this evolution may be a result of the stiffness of the cholesterol membrane due to tighter packing of the lipids,²⁷ causing peptide insertion to be modulated by this stiff membrane.

The emission maxima spectra indicate that melittin in 2 M NaCl or vesicles could be completely unfolded in 3 M GdmHCl but not 8 M urea. We have performed dynamic light scattering experiments and determined that the presence of up to 3 M of GdmHCl does not alter the size (~200 nm diameter) of anionic or cholesterol vesicles (data not shown), indicating that unfolding from the vesicle is due to melittin-GdmHCl interactions. The inability of urea to unfold melittin reflects the significance of electrostatic interactions in the stability of α -helical melittin. GdmHCl is known to be a more potent denaturant than urea because in addition to breaking backbone and residue hydrogen bonds, GdmHCl easily destabilizes electrostatic interactions in proteins.^{28, 29} The need for a charged denaturant such as GdmHCl to unfold melittin supports the idea that electrostatic interactions play a significant role in melittin stability as an α -helix in tetrameric form and bound to vesicles. The fluorescence maxima of unfolded melittin are similar and range from 355–358 nm. The emission maximum of trp-19 in unordered melittin in buffer solution undergoes a slight but reproducible red-shift from 355 to 358 nm upon addition of 3 M GdmHCl, supporting the picture that unordered melittin maintains residual or collapsed structure in the absence of denaturant. In the presence of vesicles and GdmHCl, however, melittin may still interact with the vesicle, evidenced by the 355 nm emission max of the cholesterol vesicles.

The fluorescence spectra of unfolded and membrane-bound LL-37 are qualitatively similar to those of melittin: the single trp residues are in the most hydrophobic pocket when bound to anionic vesicles, and in the least hydrophobic when unordered in DI water. However in contrast to melittin, the fluorescence spectrum of LL-37 was immediately blue-shifted upon addition of peptide to cholesterol-containing vesicles, and the emission maximum did not evolve over the >7 hour observation window. This result indicates that alterations in lipid packing due to the presence of cholesterol had no observable effect on the binding of LL-37 to membranes. The introduction of carbonate-containing buffer leads to the formation of α -helical secondary structure of LL-37 as determined by circular dichroism. Some, albeit less, α -helical structure was observed in 5 mM HEPES, 0.1 mM EDTA, pH 7.0 buffer for the F27W mutant of LL-37.²¹ The formation of secondary structure in carbonate-containing buffer is consistent with previous results for native LL-37 which suggested the formation of oligomers in the presence of some salts, including sodium bicarbonate.³⁰ In the current experiments, sodium bicarbonate buffer was chosen because of its biological relevance in the context of AMP-expressing tissues as well as studies that suggested that carbonate enhances susceptibility of bacteria to LL-37.³¹ This increase in susceptibility has largely been attributed to carbonate's direct effect on the bacterial cell wall and membrane composition as well as alterations in gene expression, as opposed to a direct effect on LL-37.³¹ However, since the disruptive activity of LL-37 on membranes is concomitant with the formation of α -helical structure, we were motivated to probe the α -helical structure induced via salt effects and/or interpeptide interactions as well as the membrane-bound, functional structure.

Peptide structure

The UVRR and CD spectra of melittin, LL-37, and mCRAMP in the presence of salts, anionic vesicles, or neutral cholesterol vesicles support the predominant presence of α -helical structures under these conditions. However, differences between UVRR and CD

interpretations exist. For example, the presence of non-native species in the two-hour UVRR and fluorescence spectra of melittin in cholesterol-containing neutral vesicles contrasts with results from the CD spectrum, which indicate pure α -helical structure. In addition, the anomalous CD spectrum of melittin in 2 M NaCl has been interpreted to reflect an equilibrium between predominantly unordered monomer and α -helical tetramer.³² In contrast, the absence of an amide S peak and the decomposed Gaussian frequencies in the 210 nm UVRR spectrum suggest the presence of α -helical species only, and no detectable amount of unordered species. These differences between CD and UVRR interpretations illustrate the disparate sensitivities of these two techniques. Because normal mode frequencies largely reflect variations in backbone dihedral angles, UVRR can report on a single turn of an α -helix.³³ On the other hand, exciton coupling dominates the features in a typical CD spectrum of an α -helix and as a result, CD reports on the global average of the secondary structure of the peptide.³⁴ A consequence of this difference is that UVRR intensities scale linearly with the fraction of α -helix³³ whereas in CD spectroscopy, the molar ellipticity per residue decreases as α -helical content decreases, and a threshold length of α -helix must persist in order to give results that correspond to an α -helical secondary structure.³⁵ Exciton coupling is especially important in CD spectra of short α -helical peptides; short peptides result in CD spectra with variations in peak positions and relative intensities.³⁶ An additional challenge inherent to CD spectra of membrane-bound peptides is that differential light scattering and absorption flattening from membrane vesicles may alter the observed shapes and relative intensities in CD spectra.³⁷ In UVRR spectroscopy, enhanced scattering from vesicles may directly impact the signal-to-noise ratio, but peak positions and relative intensities are unaffected under the appropriate experimental conditions. In the current set of experiments, the addition of vesicles to peptide samples had minimal effect on the background scattering level.

There are subtle but reproducible differences in frequencies of decomposed Gaussian peaks and relative intensities in the UVRR spectra that may reflect differential coupling of the amide III modes to side chains or to the C_{α} -H bending mode, variation in the Ramachandran dihedral angles, or alterations in hydration level under these different folding conditions.^{38–40} The decompositions indicate that one of the bands in the amide III region at $\sim 1341\text{ cm}^{-1}$ remains unchanged in all three folding environments. This constancy supports previous reports that this mode of the α -helix is not sensitive to conformation or hydration.^{39, 40} Other peaks in the amide III region, however, depend on both conformation and hydration. Specifically, it was suggested that dehydration of the backbone causes a slight downshift in the lowest energy α -helical amide III peak near $\sim 1265\text{ cm}^{-1}$.⁴⁰ Our Gaussian decompositions suggest that this amide III mode appears at $\sim 1265\text{ cm}^{-1}$ for melittin in 2 M NaCl, but then downshifts $\sim 15\text{--}25\text{ cm}^{-1}$ in the neutral cholesterol-containing and anionic vesicles. This relatively large shift may reflect dehydration upon folding in vesicles relative to the 2 M NaCl tetrameric structure as well as indicate differences in Ramachandran angles for these different forms of melittin. The large intensity of this low-frequency amide III peak in the presence of cholesterol-containing vesicles may indicate a minor population of partially folded species.

Based on the observed differences between UVRR and CD spectroscopy and interpretation of the UVRR backbone spectra, we hypothesize that the partially folded 2-hour species in

cholesterol-containing neutral vesicles has α -helical secondary structure that may not be the native conformation. In addition, the population of nominally monomeric melittin species in 2 M NaCl also appears to maintain a significant degree of α -helicity according to the 210 nm UVR spectra. The CD spectrum of melittin under high ionic strength conditions in Figure 5 is consistent with a prior report;³² in addition to strong α -helical signal due to the presence of tetrameric melittin, the CD spectrum may also exhibit features characteristic of short α -helical peptides as opposed to purely unordered monomer species. The effect of short peptides on the CD spectrum of melittin is a reasonable concern given that the presence of pro-14 may create two short peptides on the N- and C-terminus ends of the peptide. An earlier proposal that monomeric peptide maintains highly structured regions in fragments 5–9 and 14–20 supports our finding that residual monomeric melittin in 2 M NaCl may not be a purely unordered species.⁴¹ Overall, we speculate that the α -helical secondary structures of tetrameric melittin in 2 M NaCl, anionic vesicles, and cholesterol-containing neutral vesicles vary; for example, the range of Φ and Ψ angles as well as extent of hydration differ in these unique folding environments. A previous study concluded similar results in that the α -helical tetrameric and micelle-bound forms possess variations in structure based on differences in NMR spectra.⁴²

The amide IIP band found near 1440 and 1460 cm^{-1} was analyzed for differences in hydrogen-bonding structure of the backbone near the single proline residue in melittin and LL-37. This band is mostly composed of C-N stretching of the amide backbone, and is shifted from the amide II band ($\sim 1520\text{--}1560\text{ cm}^{-1}$) because the hydrogen atom usually found on the amide nitrogen between leu-13 and pro-14 in melittin or val-32 and pro-33 in LL-37 is replaced by the proline side chain. The amide IIP band is sensitive to the hydrogen bonding state of the carbonyl group between the leu-13 and pro-14 or val-32 and pro-33 because the presence of a strong hydrogen bond decreases the bond order of carbonyl, and accordingly increases the C-N bond order.¹⁸ This coupling results in a band at $\sim 1460\text{ cm}^{-1}$ that corresponds to strongly hydrogen-bonded carbonyl, and a band at $\sim 1440\text{ cm}^{-1}$ that corresponds to the non-hydrogen bonded population.^{18, 43}

The conserved single proline residue plays an important structural role in melittin.⁴⁴ Pro-14 is responsible for the bend in the helix found in the structure of melittin and is not hydrogen bonded to thr-10. This region is flexible, and is therefore known as the “hinge” region of the peptide.⁴⁵ A P14A mutant eliminated the bend and this mutant exhibited decreased membrane-lytic potency relative to wild-type.⁴⁴ It was also reported that the presence of the proline residue allows melittin to form more stable ion channels.⁴⁵ The amide IIP region shown in Figure 6, illustrates that the leu-pro carbonyl of melittin in buffer (Figure 6D) has the strongest hydrogen bond to presumably water molecules in the hinge region ($R_p = 2.3$). In contrast, the amide IIP peak of melittin in NaCl ($R_p = 0.8$) and bound to anionic vesicles ($R_p = 0.9$) is broad with a low frequency component, indicating that the leu-pro carbonyl is not strongly hydrogen bonded under these conditions. This result indicates that the carbonyl oxygen is strongly hydrogen-bonded to water when melittin is unfolded in buffer, and that replacement of water for an amide proton in the α -helical forms of melittin weakens the hydrogen-bond strength of the backbone carbonyl oxygen. This finding is consistent with the correlation of amide IIP frequency with solvent hydrogen-bond donating capability;¹⁸

water has a larger acceptor number than formamide⁴⁶ and therefore, the amide IIp band is expected to have a higher frequency for carbonyl oxygen that is hydrogen-bonded to water than to amide backbone. It is not clear whether the change in R_p value reflects a shift in population or homogeneous change in hydrogen-bond strength. Melittin in cholesterol-containing vesicles exhibits an intermediate R_p value of 1.3, suggesting moderate hydrogen-bond strength and/or similar populations of hydrogen bonded and non-hydrogen bonded leu-pro carbonyl groups. In addition to change in hydrogen-bond strength due to different hydrogen-bond donors, variations in R_p may also reflect modulations of the hydrogen-bond strength by the local environment.

Unfortunately, the presence of relatively strong signal from the phe residues and low S/N in the amide III region of the UVRR spectra of LL-37 and mCRAMP prevented us from performing a detailed backbone analysis of these AMPs. While the CD spectra indicate that LL-37 and mCRAMP are α -helical in anionic and cholesterol vesicles, it is not clear if these structures are identical. Additional UVRR experiments are ongoing. The amide IIp region of the LL-37 spectra, however, is relatively isolated and yielded insight into the local structure. In contrast to melittin, the single proline residue of LL-37 does not appear to play an important role in LL-37 function. For example, a study of the C-terminally truncated peptide in which six residues, including pro-33, were removed indicated that this truncated peptide, LL-31, is as potent as native LL-37.⁴⁷ In addition, the localization of LL-31 to the cell perimeter of *C. albicans* was identical to that exhibited by LL-37.⁴⁷ While the specific role of pro-33 in LL-37 has not yet been reported, it has been proposed that the presence of proline residues in AMPs serves to help prevent proteolytic degradation.⁴⁸ Our results on LL-37 indicate that the microenvironment and hydrogen-bonding environment of the val-pro carbonyl oxygen are similar in the presence of cholesterol and anionic vesicles. Specifically, the low R_p values suggest that this carbonyl oxygen does not participate in hydrogen bonding and instead, is buried in the lipid membrane and excluded from solvent. This interpretation is consistent with our studies of tryptophan fluorescence experiments that localize the peptide in the interior of the bilayer (data not shown) as well as with the NMR structure that shows no secondary structure in the C-terminus tail.

CONCLUSION

As one of the most potent antimicrobial and hemolytic peptides in nature, melittin has become a benchmark system for the study of peptide-membrane interactions. In contrast, far less biophysical and biochemical data are available for ubiquitous AMPs that are part of the innate immune system. In the absence of high-resolution structures of toxins and AMPs in membrane-bound conformations, our current work provides one of the first in-depth structural studies of these ubiquitous peptides using UV resonance Raman spectroscopy. The 210 nm UVRR spectra indicate variations in the secondary structure as well as the hydrogen bonding state of the important hinge region of melittin. Specifically, while melittin is found to be primarily α -helical in NaCl, anionic vesicles, and cholesterol vesicles, there is evidence for differences among these α -helices, such as variations in Φ and Ψ angles and alterations in the level of hydration, which may be related to the functions in these environments. Comparison of UVRR and CD spectra reveals that these two techniques provide similar results; however, the effect of peptide length may be an important

consideration for interpretation of CD spectra. In addition, while CD provides information on global structure, UVRR is capable of providing site-specific molecular information. Analysis of the amide I_{ip} region reveals variability in the hydrogen-bonding state of the leu-pro carbonyl in the hinge region. Melittin in NaCl and in vesicles exhibits a population of weak hydrogen-bonded species presumably to backbone amide protons, consistent with the α -helical form. The val-pro carbonyl on the C-terminus of LL-37 exhibits little to no hydrogen-bond character when folded in vesicles; this finding is consistent with localization of LL-37 in the lipid bilayer and with the NMR structure that indicates lack of secondary structure near the pro-33 region. In summary, the abundance of aromatic amino acids makes UVRR a promising technique for gaining molecular-level understanding of AMP activity; the experimental findings reported here provide important foundations for ongoing studies on the mechanisms of membrane disruption by peptides while illustrating the potential of this technique to yield insight of the hundreds of tryptophan- or phenylalanine-containing antimicrobial peptides.

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Figure 1. Melittin from PDB 2MLT. Dark green regions represent charged residues, light green corresponds to polar residues, and gray denotes hydrophobic residues. The tetramer is rotated 90° to show the hydrophobic pocket with the pro-14 and trp-19 regions expanded. The leu-pro carbonyl oxygen is in red and the indole nitrogen of tryptophan is in blue. The absorption spectra of tetrameric melittin (2 M NaCl, green) and unordered melittin (buffer, black) are also presented.

Figure 2.

LL-37 (PDB 2K6O). Dark green regions represent charged residues, light green corresponds to polar residues, and gray denotes hydrophobic residues. The four native phe residues are indicated; F6W and F17W mutants are probed in the current report. The single pro residue is shown.

Figure 3.

Tryptophan fluorescence spectra. Left: Melittin in anionic vesicles (A), NaCl (B), cholesterol vesicles after 2 hours (C), and buffer only (D). L-trp in buffer is spectrum (E). Inset: Trp-19 fluorescence of melittin incubated with cholesterol vesicles for 2 hours (dash) and 14 hours (dotted). Melittin in the presence of 3 M GdmHCl and cholesterol vesicles is shown as a solid line (355 nm). Right: LL-37 mutant F17W in anionic vesicles (A), cholesterol vesicles (B), carbonate-containing buffer (C) and DI water (D).

Figure 4.

Fluorescence leakage assay results. The ordinate indicates fluorescence signal of peptide relative to 100% leakage due to complete disruption of vesicles by TritonX detergent. Left: melittin in anionic (circles) and in cholesterol (squares) vesicles. Right: Native LL-37 (squares), F17W (triangles), and mCRAMP (circles) in anionic vesicles.

Figure 5. Circular dichroism spectra of melittin (left), native LL-37 (middle), and mCRAMP (right). Solid curves: melittin in phosphate buffer, LL-37 and mCRAMP in DI water. Dash dotted curves: Melittin in 2 M NaCl, LL-37 and mCRAMP in carbonate-containing buffer. Dotted curves: peptides in the presence of anionic vesicles. Dashed curves: peptides in the presence of cholesterol-containing vesicles.

Figure 6. 210 nm UVRR spectra of melittin and L-trp. Melittin in 2 M NaCl (A), anionic vesicles (B), cholesterol vesicles (2 hours) (C), and buffer (D), with contributions from model compound L-trp (E) subtracted. Gaussian decompositions and resulting sums are also displayed.

Table 1
Summary of peptides and conditions probed in current report. Key residues are highlighted in bold.

Peptide	Function	Primary Sequence	Net charge	Random coil	Aqueous α -helix	Membrane-bound α -helix (prokaryotic)	Membrane-bound α -helix (eukaryotic)
Melittin	Toxin	GIGAVLKVLTTGLPALISWIKRQRQ	+6	Buffer	NaCl	anionic vesicles	cholesterol vesicles
LL-37	AMP	LLGDFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	+6	DI water	HCO ₃ ⁻	anionic vesicles	cholesterol vesicles
mCRAMP	AMP	GLLRKGGEGKEKLLKKIGQKIKNFFQKLVPQPEQ	+6	DI water, HCO ₃ ⁻	----	anionic vesicles	cholesterol vesicles