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Towards Mapping Electrostatic Interactions between Kdo₂-Lipid A and Cationic Antimicrobial Peptides via Ultraviolet Photodissociation Mass Spectrometry

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Abstract

Cationic antimicrobial peptides (CAMPs) have been known to act as multi-modal weapons against Gram-negative bacteria. As a new approach to investigate the nature of the interactions between CAMPs and the surfaces of bacteria, native mass spectrometry and two MS/MS strategies (ultraviolet photodissociation (UVPD) and higher energy collisional activation (HCD)) are used to examine formation and disassembly of saccharolipid•peptide complexes. Kdo₂-lipid A (KLA) is used as a model saccharolipid to evaluate complexation with a series of cationic peptides (melittin and three analogs). Collisional activation of the KLA•peptide complexes results in the disruption of electrostatic interactions, resulting in apo-sequence ions with shifts in the distribution of ions compared to the fragmentation patterns of the apo-peptides. UVPD of the KLA•peptide complexes results in both apo- and holo-sequence ions of the peptides, the latter in which the KLA remains bound to the truncated peptide fragment despite cleavage of a covalent bond of the peptide backbone. Mapping both the N- and C-terminal holo-product ions gives insight into the peptide motifs (specifically an electropositive KRKR segment and a proline residue) that are responsible for mediating the electrostatic interactions between the cationic peptides and saccharolipid.

Introduction

Gram-negative bacteria are responsible for some of the most persistent infections and deadliest pandemics in the world. The degree of pathogenicity of Gram-negative bacteria is attributed to the highly variable lipopolysaccharide (LPS) coating that extends outwards from the bacterial outer membrane.^{1–3} LPS is comprised of three key regions: the O-antigen region, the core sugars, and lipid A.² The polysaccharide O-antigen region is connected to a less variable saccharide core, which in turn is linked to a hydrophobic domain (known as lipid A) responsible for anchoring the entire LPS to the bacterial membrane.^{4,5} Variability in the hydrophobic domain such as acyl chain length and connectivity as well as phosphate content has been shown to modulate the pathogenicity and resistance to antibiotics of Gram negative bacteria.^{6–9}

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Antimicrobial peptides (AMPs) hold significant promise in terms of their potential as an underexploited class of antimicrobials – an issue that is critically important given the constant evolution of antibiotic resistant strains of bacteria. Cationic antimicrobial peptides (CAMPs) are of particular interest owing to their ability to interact with LPS of Gram negative bacteria, depolarizing the membrane and increasing permeability.^{10,11} Both naturally occurring and synthetic CAMPs display extensive variability in both their amino acid sequences and conformations. Typically smaller than 10 kDa in molecular weight, CAMPs frequently have an unusually high number of basic residues, resulting in their characteristic positive charge. The mechanism of action of AMPs may involve both disintegration of the lipid bilayer and interaction with intracellular targets after cell penetration. The amphipathicity of AMPs allows their hydrophobic and hydrophilic regions to bind both the lipid and phospholipid groups of LPS, respectively.^{12–14} Some AMPs can diffuse through the bacterial membrane and inhibit cellular pathways such as DNA synthesis or other enzymatic processes.^{15–17} CAMPs have shown activities against both Gram-negative and Gram-positive bacteria, fungi, and cancer cells.^{18–21}

New insight into the mechanisms, structures, and activities of biological molecules has been obtained through the development and application of an arsenal of new biophysical tools, including native mass spectrometry.^{22–24} Native spray mass spectrometry has emerged as a powerful technique to characterize the structures of biological molecules and complexes, including the elucidation of primary through quaternary organization and interactions between molecules.^{25–27} Protein-ligand and protein-protein interactions have been successfully conserved during the transfer of macromolecules from solution to the gas phase via electrospray ionization of aqueous solutions containing high salt concentrations.^{28–32} Stoichiometries of non-covalent assemblies have been derived from the m/z values of ions observed in the resulting native mass spectra,^{33–37} and MS/MS methods have been used to infer structural arrangements based on the resulting fragmentation patterns of mass-selected ions.^{38–43} Collisional activation of non-covalent complexes often results in disruption of weak non-covalent interactions and disassembly of the complexes, manifested as the ejection of a subunit in a protein-protein complex^{44–47} or the release of a small molecule ligand, such as the separation of heme from holo-myoglobin.⁴⁸ Electron and photon-based activation techniques, such as electron transfer and electron capture dissociation (ETD, ECD),^{49–51} ultraviolet photodissociation (UVPD),⁴⁸ and surface induced dissociation (SID),⁵² have been shown to more effectively preserve non-covalent interactions between proteins and small molecule ligands, thus providing insight into the binding sites of ligands and nature of secondary structure based on the fragmentation patterns.^{27,35,53–56}

In the present study, native mass spectrometry coupled with 193 nm UVPD and higher energy collisional dissociation (HCD) is used to investigate the sequence motifs of cationic peptides that contribute to formation of complexes with a truncated LPS analog (Kdo₂-lipid A) and to localize the key amino acids involved in electrostatic interactions with Kdo₂-lipid A. Kdo₂-lipid A (KLA), a saccharolipid comprised of a bis-phosphorylated hexaacylated lipid A with two appended keto-deoxyoctulosonate sugars, serves as the simplest LPS analog. UVPD of the saccharolipid•peptide complexes yields both informative apo fragment ions (peptide sequence ions that do not retain the saccharolipid) and holo fragment ions (i.e. ones comprised of a peptide sequence ion plus the appended saccharolipid) that allow

localization of the electrostatic binding sites between the lipid and the peptide. HCD of the saccharolipid•peptide complexes yields distributions of apo sequence ions that differ from the sequence ions produced by the peptide alone.

Experimental

Mass Spectrometry

Kdo₂-lipid A (KLA) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Melittin (from honey bee venom (85%)) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Other melittin analogs were purchased from United Biosystems Inc. (Herndon, VA, USA). All materials were used without additional purification. The saccharolipid KLA was mixed with each peptide in a five-to-one molar ratio and diluted to net 20 μM KLA and 4 μM peptide with 5 mM aqueous ammonium acetate. High resolution measurements for the native KLA:peptides complexes were acquired using a Thermo Scientific Orbitrap™ Fusion Lumos mass spectrometer (San Jose, CA, USA) coupled with a Coherent 193 nm excimer laser (Santa Clara, CA, USA) to perform photodissociation in the low pressure linear ion trap at 120 K resolution, as described previously.⁵⁷ Uncoated static emitters with an applied voltage of 1300 V (via an inserted platinum wire) were used to transport the analytes into the gas phase via electrospray ionization. The complexes were analyzed in the positive ion mode and subjected to CID, HCD, ETD, and UVPD. Spectra were deconvolved with a signal-to-noise threshold of two using the Xtract algorithm and analyzed via UV-POSIT (<http://uv-posit.cm.utexas.edu>)⁵⁸ to calculate the fragmentation yields along the peptide backbone with a mass accuracy of ±5 ppm. Briefly, apo- (no saccharolipid) and holo- (retaining saccharolipid) sequence ions were mapped for all peptide backbone cleavage positions in which the exact mass of the saccharolipid was searched as a variable modification. Ten product ion types were searched when considering N- and C-terminal fragments, including *a*, *a*+1, *x*, *x*+1, *b*, *y*, *y*-1, *y*-2, *c*, and *z* ions, all of which are product ions consistently identified via UVPD analysis of peptides or proteins.⁵⁹ All N-terminal product ions (*a_n*, *b_n*, and *c_n* ions) arising from backbone cleavages that occur C-terminal to a specific amino acid were summed and plotted alongside all the summed C-terminal product ions (*x_{R-n+1}*, *y_{R-n+1}*, and *z_{R-n+1}* ions) arising from cleavages that occur N-terminal to the same amino acid, where *n* is the residue number and *R* is the total number of amino acids in the peptide.

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Impact of Peptide on Cell Viability

Pseudomonas aeruginosa Pa01 pMRP9-1 (constitutively expressing green fluorescent protein) from freezer stock was grown overnight at 37°C on a heated shaker in tryptic soy broth (TSB). TSB, from Remel Thermo Scientific (Waltham, MA, USA), was treated with 10 mg L⁻¹ carbenicillin (carb10) from Fisher Bioreagents (Fairlawn, NJ, USA) to maintain the plasmid pMRP9-1. 200 μL of concentrated cells in stationary phase (OD₆₀₀ 2-3) were then taken from the overnight tube and diluted into 4 mL of fresh TSB. Following 1.25 hours of growth on the heated shaker, 200 μL of cells in exponential phase (OD₆₀₀ 0.2-0.3) were transferred into each well of an 8-well chambered coverslip from Lab-Tek Thermo Scientific (Omaha, NE, USA) and allowed to grow for 3 hours at 37 °C with no shaking. Each well was then rinsed with fresh, 1/3 strength TSB in dH₂O to remove cells not attached to the

surface of the coverslip. For each of the four peptides, three test concentrations of 12.5 µg/mL, 25 µg/mL, and 50 µg/mL were used for dosing. Two wells were treated with melittin, two with melittin-RS, two with melittin-S, and two with melittin-noKRKR. All experimental wells were dosed with 200 µL of peptide. Each test concentration was also performed with a control to which no peptide was administered. All were incubated at room temperature (~22°C) for 2 hours. The TSB was then removed from each well and 200 µL of 30 µM propidium iodide (Acros Organics; Geel, Belgium), a cell viability stain, was added. The chamber was encased in foil and incubated at 22°C for 15 minutes prior to imaging. Imaging was performed using a Zeiss 710 Laser Scanning Confocal Microscope (Pleasanton, CA) and accompanying Zen 2012 Black Edition software (Roschdale, UK). Scans were optimized for GFP and propidium iodide data collection. Live/dead ratios were calculated in ImageJ (NIH; Washington, DC, USA) as determined by overall pixel intensity in each respective channel.

Circular Dichroism

Circular dichroism (CD) spectra were collected at room temperature on a JASCO J-815 spectrometer with a 1 mm path length quartz cell (Starna Cells). Peptide samples were prepared at 20 µM in 10 mM aqueous potassium phosphate buffer at pH=7.0 or 5 mM ammonium acetate and were background subtracted against either buffer alone or buffer with the addition of 100 µM KLA. Scans were taken from 280 nm to 190 nm, with a data pitch of 0.5 nm, a bandwidth of 1 nm, response time of 4.0 s, and at a rate of 50 nm/min. Results are truncated to a range of 190 nm - 250 nm and are reported as the average of at least 5 scans. CD spectra were deconvoluted into contributions from different secondary structure motifs using the online server BeStSel.⁶⁰

Results and Discussion

While CAMPs normally encounter a layer of LPS on the outer membrane of Gram negative bacteria, the heterogeneity and complexity of this lipid layer makes it impractical for fundamental investigations of peptide/lipid interactions and motivated our use of KLA as a surrogate for LPS in the present study. It is known that KLA but not lipid A activates toll-like receptor 4 (TLR-4) with an activity similar to LPS, suggesting that KLA more closely mirrors LPS than does the more truncated lipid A (which lacks the two keto-deoxyoctulosonate sugars).^{6,61} The sequences of the four peptides (melittin plus three analogs, melittin-RS, melittin-S, and melittin-noKRKR) are given in Table 1. The analogs were designed to create similar peptide constructs with subtle changes in prominent sequence motifs to better understand the role of electrostatic interactions between the peptide and KLA.

ESI of melittin under native MS conditions produces 3+ and 4+ charge states. ESI of a solution containing only KLA resulted in the production of mostly doubly charged deprotonated KLA in the negative mode, whereas in the positive ion mode showed only a low abundance of doubly charged molecular ion (Figure S1). ESI of solutions containing different stoichiometries and concentrations of the peptide and KLA, as well as different solvent compositions, were evaluated in order to optimize the formation of non-covalent

KLA•peptide complexes. Concentrations ranging from 1 μ M to 20 μ M of each constituent and covering molar ratios of KLA to peptide from 10:1 to 1:10 were examined. Aqueous solutions containing 20 μ M KLA and 4 μ M peptide (5:1) with 5 mM ammonium acetate (pH 6.5) resulted in optimal formation of 1:1 KLA•peptide complexes in the 4+ charge state as well as predominantly 3+ and 4+ charge states of the saccharolipid-free apo-peptide (Figure S2). The abundances of the KLA•peptide complexes relative to the abundances of the unbound peptide ions were used to estimate the relative binding affinities of the complexes. Based on the MS1 spectra shown in Figure S2, the binding affinity trend was estimated as melittin > melittin-RS > melittin-S. Melittin-noKRKR produced no detectable complexes. ESI of solutions containing the simpler lipid A (without the two appended Kdo sugars) rather than KLA did not yield any detectable complexes with any of the peptides. The absence of lipid A•peptide complexes was attributed to inherent solubility issues associated with the more hydrophobic lipid A species in aqueous solution as well as the critical omission of the di-keto-deoxyoctulosonate group (found on KLA) which may participate in multivalent stabilizing hydrogen-bonding interactions with the peptide.⁶¹

The various KLA•melittin complexes (4+) were characterized by ETD, HCD and UVPD as illustrated in Figure 1. Melittin (4+), melittin-RS (4+), and melittin-S (4+) were characterized by HCD and UVPD in Figures S3 – S5, and HCD and UVPD of the KLA•peptide complexes (4+) for melittin-RS and melittin-S are shown in Figures S6 – S7. The primary fragmentation pathways of the complexes are summarized in Figure 2. The fragmentation varied greatly based upon the activation mode. ETD of the KLA•peptide complexes primarily resulted in charge reduction with formation of few or no informative sequence ions (Figure 1a) and was not pursued further. It should be noted that while ETD did not provide diagnostic fragmentation of the noncovalent complex, ECD has been shown to be sensitive to protein and peptide conformations in native-like mass spectrometry experiments, and should be considered for a similar experiment.^{62–65} According to findings from several groups, helical regions are less susceptible to fragmentation via ECD or are more prone to CID-like fragmentation,^{62–65} giving insight into secondary structural features. ETD of the 4+ charge state of melittin alone yielded several *c*- and *z*-type fragment ions, with the most abundant ones arising from backbone cleavages close to the termini of the peptide (*c*₂₅ and *z*₂₄ ions), as shown in Figure S8. HCD resulted in disruption of electrostatic interactions between the saccharolipid and peptide, ultimately generating only apo-peptide sequence ions that did not retain the saccharolipid (Figure 1b). The sequence ions derived from HCD of the apo-peptide and the KLA•peptide complexes show differences, as described in the subsequent sections. 193 nm UVPD of the KLA•peptide complexes generated rich spectra comprised of both apo- and holo- (saccharolipid-retaining) product ions (Figure 1c), in which the apo- ions are conventional peptide sequence ions (analogous to the general type produced by HCD) and the holo-ions are those that contain a diagnostic truncated peptide sequence ion (*a*, *b*, *c*, *x*, *y*, or *z*) with retention of the entire saccharolipid. The holo-ions produced through UVPD are the key ones for deciphering the putative locations of electrostatic interactions between the peptide and the saccharolipid. It is expected that the phosphate groups of the saccharolipid engage in interactions with positively polarized side-chains (Arg, Lys) of the peptide,⁶⁶ and strong interactions may suppress fragmentation of the peptide backbone during UVPD. Through the UVPD spectra, specific motifs and residues

related to the peptide sequence are identified as prominent candidates for mediating and conserving electrostatic interactions with saccharolipids.

The relative abundances of apo- and holo- N-terminal *a*, *b*, *c* and C-terminal *x*, *y*, *z* fragment ions produced by UVPD and HCD of melittin (4+) and the KLA•melittin complex (4+) are shown in Figure 3 in a histogram format as a function of the backbone cleavage position of the peptide sequence. Both UVPD and HCD of melittin alone (Figure S3) or the KLA•melittin complexes (Figure 1b,c) afforded extensive sequence coverage of the peptide based on formation of primarily *a*, *b*, and *y* product ions, as well as some additional *c*, *x*, and *z* ions for UVPD. Preferential fragmentation N-terminal to proline at position 14 resulted in the prominent $y_{13}2+$ ion in both the UVPD and HCD spectra of melittin. With the exception of the preferential proline cleavage, there was generally evenly dispersed fragmentation throughout the backbone of melittin as well as broader coverage for UVPD (Figure 3c) compared to HCD (Figure 3d) with fragmentation that extended to the C-terminus. HCD of the KLA•melittin complex (Figure 3d) produced mostly apo *y* product ions via backbone cleavages adjacent to residues V5 to R24 with a dominant $y_{13}2+$ ion from backbone cleavage at the P14 position as noted also for apo-melittin (Figure 3b). Additionally, a few N-terminal *b*-type product ions were formed from backbone cleavages adjacent to residues G3 to V8. Holo-product ions were not observed upon HCD of the KLA•melittin complexes (Figure 3f). In general, HCD of apo-melittin and the KLA•melittin complex demonstrate qualitatively similar fragmentation trends, thus suggesting that KLA is promptly released during activation and has little impact on the subsequent backbone cleavages that lead to the characteristic *b/y* sequence ions. EThcD (50 ms ETD period with supplemental HCD (30 NCE) activation) was also evaluated for the KLA•melittin complexes, and one set of results is shown in Figure S9. EThcD produced abundant charge-reduced complexes and an array of apo-peptide sequence ions, but no holo ions were produced (similar to the outcome of ETD alone). The lack of holo product ions limited the ability to probe the lipid/peptide interactions using EThcD.

UVPD of the KLA•melittin complexes resulted in formation of a large array of apo (without bound saccharolipid) and holo (with bound saccharolipid) product ions (Figure 3c,e). Interestingly, virtually no apo C-terminal *x*, *y*, *z* fragment ions were produced upon UVPD (Figure 3c, lack of red bars), yet many holo- *x*, *y*, *z* ions were formed (Figure 3e, red bars) that mirrored the extensive series of large C-terminal ions observed upon UVPD of apo-melittin (Figure 3a, red bars). A number of apo N-terminal *a*, *b*, *c* ions were observed upon UVPD of KLA•melittin (Figure 3c, blue bars), just as was observed for apo melittin (e.g., a_7 , a_8 , a_9 , a_{10}) (Figure 3a, blue bars). Some longer holo *a*, *b*, *c* fragment ions were produced upon UVPD of KLA•melittin (Figure 3e, blue bars), but the smaller and medium-sized fragments (e.g., b_{11} , b_{12} , a_{13} , b_{13}) were exclusively observed as saccharolipid-free apo products (Figure 3c, blue bars). The fact that the mid-sized N-terminal ions are primarily observed as apo ions and the larger N-terminal ions are predominantly found as holo ions suggests that KLA is associated with the KRKR motif located near the C-terminal end of melittin, presumably via a network of electrostatic interactions. The differences in the HCD and UVPD spectra for holo-melittin and the KLA•melittin complex are striking, thus motivating the evaluation of analogs of melittin to more fully decipher the role of charge sites on the fragmentation patterns of the complexes.

Three analogs with similar constructs to melittin but with variation in the location or presence of the KRKR motif were examined (Table 1). Melittin-RS (2875.53 Da) was designed to have a sequence similar to the reverse of melittin, but with preservation of the lysine- and arginine-rich motif located near the C-terminus. Melittin-S (2859.57 Da) was constructed to have the same general sequence as naturally occurring melittin, but with the KRKR motif shifted closer to the mid-section. Melittin-noKRKR (2746.37) was designed to evaluate a system in which the lysine- and arginine-rich motif was absent. Two of the peptides, melittin-RS and melittin-S, formed complexes with KLA as shown in Figure S2b,c. The third analog, melittin-noKRKR, did not form complexes with KLA, presumably owing to the lack of a region of concentrated basic amino acids and charge sites, thereby eliminating electrostatic interaction with the negatively charged phosphate residues of the saccharolipid.

UVPD and HCD of the melittin-RS peptide (4+) yielded MS/MS spectra (Figure S4) similar to the ones observed for melittin (Figure S3). UVPD resulted in a diverse series of *a*, *b*, *c* and *x*, *y*, *z* fragment ions arising from backbone cleavages localized between residues Q2 through G12, and a second lower abundance series covering K17 to A24. HCD yielded a somewhat more truncated series of diagnostic ions stretching from residues S6 to G12. A significant preferential cleavage N-terminal to Pro occurred for both UVPD and HCD, producing abundant *y*₁₇ ions. The histograms showing the distribution of fragment ions for UVPD and HCD of melittin-RS (Figure 4a,b) parallel the ones shown in Figure 3a,b, albeit shifted according to the reversal of sequence.

The mass spectra obtained upon UVPD and HCD of the corresponding KLA•MEL-RS complexes are illustrated in Figure S6 and summarized in histogram format in Figure 4. Activation of the KLA•MEL-RS complex resulted in similar trends in the formation of apo-product ions as noted for the KLA•MEL complex. Rather evenly distributed N-terminal *a*, *b*, *c* apo-product ions were generated via UVPD, with a few C-terminal fragments arising from cleavages adjacent to Q2, P10, and R20 residues (Figure 4c). One notable difference in the apo-ions produced by HCD and UVPD for the KLA•MEL-RS complexes compared to apo MEL-RS was the decrease in abundance of the previously dominant C-terminal Pro-cleavage product ions as well as the emergence of new C-terminal ions from cleavages adjacent to K19 and R20. The pattern of holo-product ions generated upon UVPD of the KLA•MEL-RS complex (Figure 4e) shared some similarities to the pattern observed for the KLA•MEL complex: formation of C-terminal *x*, *y*, *z* ions up to the proline residue (P10) and N-terminal fragment ions covering the KRKR motif (residues 17–20). However, the lower relative abundances of these ions and absence of holo N-terminal ions along the C-terminal region suggests that the saccharolipid-peptide interactions of KLA•MEL-RS may be less specific than those of the KLA•MEL complex. HCD again generated no holo-product ions for the KLA•MEL-RS complex (Figure 4f).

UVPD and HCD of apo MEL-S (4+) resulted in a significant shift in the fragmentation patterns compared to the other two peptides (see MS/MS spectra in Figure S5 and backbone cleavage histograms in Figure 5a,b). A series of longer N-terminal product ions were favored (*b*₂₂, *b*₂₃, *b*₂₄, *b*₂₅) for UVPD along with evenly distributed, low abundance C-terminal *x*, *y*, *z* ions via backbone cleavages throughout nearly the entire MEL-S backbone.

HCD fragmentation of MEL-S also produced longer *b* ions covering the PALIS motif near the C-terminus, whereas a series of low abundance *b* and *y* ions covered the N-terminus. The notable absence of the dominant proline cleavage that was observed for MEL and MEL-RS suggests that the peptide may adopt a different conformation related to the isomerization state of the proline residue. It has been demonstrated that cis/trans conformational changes of proline isomerization state result in notable changes in backbone fragmentation adjacent to the proline residues, as shown for bradykinin and synthetic analogs.^{67,68} The significant dominance of the longer N-terminal ions upon both UVPD and HCD is consistent with the position of the highly basic KRKR motif in the mid-section of the peptide, thus facilitating charge retention by the larger N-terminal products. UVPD of MEL-S again yielded more extensive backbone cleavage throughout the peptide, resulting in higher net sequence coverage than HCD (Figure 5a versus 5b).

The mass spectra obtained upon UVPD and HCD of the KLA•MEL-S complexes are displayed in Figure S7 and summarized in histogram format in Figure 5c – 5f. The distribution of backbone cleavages observed upon HCD (Figure 5d) is very similar to the distribution exhibited by the KLA•MEL complexes (Figure 3d), with a large array of longer C-terminal *y* ions and no holo product ions (Figure 5f). The dramatic shift in fragmentation (mostly large N-terminal fragments for the peptide alone to mostly large C-terminal fragments for the saccharolipid•peptide complex) upon HCD illustrates the alteration of the protonation scheme and/or peptide conformation that occurs upon interaction between the two biomolecules.

UVPD of the KLA•MEL-S complex (Figure 5c) generated mostly N-terminal apo-product ions (blue bars) in addition to two prominent C-terminal apo-product ions (*y*₇ and *z*₂₂, large red bars). With respect to holo product ions, UVPD of the KLA•MEL-S complex yielded primarily N-terminal *a*, *b*, *c* ions owing to cleavages adjacent to or beyond the lysine- and arginine-rich motif (residues 14 – 17) (Figure 5e). A few low abundance C-terminal holo-product ions were also detected that encompassed the KRKR motif as a potential anchor site for the KLA ligand. The notable suppression of C-terminal holo ions for the MEL-S complex in comparison to the MEL and MEL-RS complexes also points to the potential impact of the location of the proline residue with respect to the KRKR motif. For the MEL and MEL-RS complexes, C-terminal holo ions were identified up to the position of the proline residue, which was positioned N-terminal to the KRKR motif. For UVPD of the KLA•MEL-S complex, the near absence of C-terminal holo-ions suggests a link to the location of the proline residue which is C-terminal to the KRKR motif, significantly decreasing the likelihood of an available mobile proton to form the longer C-terminal holo-ions covering the N-terminus of the peptide.

The distributions of holo and apo fragment ions produced by UVPD are displayed in a ratio format for each of the KLA•peptide complexes, in which the abundance of holo ions was divided by the summed abundances of holo and apo ions relative to each peptide backbone position (see Figure S10). This ratio format highlights the distribution of holo versus apo product ions generated upon disassembly of the KLA•peptide complexes. Based on this alternative data format, the convergence of N- versus C-terminal ions near the putative KRKR binding motif is underscored for the KLA•peptide complexes.

As an orthogonal means of exploring peptide/lipid interactions, the effects of melittin and its three analogs on cell viability were examined based on the mortality rates of *Pseudomonas aeruginosa*, a bacterium commonly used in determining the experimental minimum inhibitory concentration (MIC) of various AMPs.^{69–72} Three dosing concentrations of 12.5, 25, and 50 µg/mL were created from stock solutions of each of the four peptides. These concentrations were chosen for consistency with typical cell viability studies.^{70,71} The mortality rates at each peptide concentration, as well as representative confocal images for 25 µg/mL dosing, are shown in Figure 6. Additional confocal images obtained using dosing concentrations of 12.5 and 50 µg/mL, respectively, are shown in Figures S11 and S12. The live/dead ratios obtained for each peptide concentration confirmed that while melittin resulted in the highest mortality rate of the four peptides tested, two of the other three analogs also showed noticeable toxicity towards *P. aeruginosa*. At higher concentrations, MEL-S was the most comparable to MEL in terms of cell toxicity at 25 µg/mL, with mortality rates of 85% for MEL-S and 94% for MEL, respectively. At all concentrations MEL-RS was significantly less effective, exhibiting negligible impact on cell survival. It should be noted that at the highest concentration tested (50 µg/mL), MEL-NoKRKR showed a substantial increase in mortality rate, nearly approaching the effectiveness of MEL-S. Additionally, cells showed significant degradation when dosed with 50 µg/mL of melittin, MEL-S, and MEL-NoKRKR. These effects are evident in the bright field images shown in Figure S13 for which lysed cells are clustered together, and debris from lysed cells is observed on the coverslip. These results are consistent with previous reports demonstrating how melittin interacts with cellular membranes, causing destabilization, permeabilization, and potential lysis of cells.⁷¹

Circular dichroism (CD) experiments were performed in order to establish a link between the bacterial studies with *P. aeruginosa* and the holo-ion products observed through the photodissociation experiments, as demonstrated in Figure 7. These experiments were undertaken first using potassium phosphate, a conventional salt frequently used for CD measurements, rather than ammonium acetate, which was used for the native MS experiments. The carbonyl group of acetate exhibits strong absorbance in the far UV range, thus obscuring signals arising from the peptides of interest in solution and making the use of ammonium acetate less desirable for CD experiments. For the solutions containing potassium phosphate, two main spectral features were used to assess the secondary structures of the peptides: 1) right-handed α -helix, which yields a positive peak at 190 nm and negative peaks at 205 and 222 nm; and 2) β -sheet which, though typically showing more spectral diversity because of twisting and parallel/antiparallel orientations, contributes to a positive peak near 195 nm and single negative peak around 215 nm.^{73,74} Proteins and peptides often contain one or both of these motifs as well as random coils and bends, yielding CD spectra that are a linear combination of the secondary structures present in solution. Multiple methods exist to de-convolute CD spectra to account for the contributions of secondary structure and calculate the secondary structure content empirically.⁷⁵ In this work, the BeStSel online server was used because it is an easily accessible program and provided the best fits to the experimental data.⁶⁰ Based on the fits of the CD spectra of each peptide compared to the fits of the spectra of each peptide in the presence of KLA, the interaction of each peptide with the lipid is correlated with the change in secondary structure

of the peptide(Table S1). Both MEL and MEL-S (Figure 7a and 7b) exhibit similar α -helical features in the unbound state and adopt a higher degree of β -sheet properties upon addition of KLA. Interestingly, MEL-RS (Figure 7c) displays a diverse range of structures and converts to a more helical conformation in the presence of KLA. Finally, MEL-NoKRKR (Figure 7d) assumes even more α -helical (both regular and distorted) character upon addition of KLA relative to its initial helical properties. The highly basic KRKR motif is critical for observation of the gas-phase complexes and may mediate the initial interactions with KLA, but it is clear from the efficacy and structural studies of MEL-RS that this motif is not the singular cause of AMP activity.

The same CD experiments were also performed using the solvent composition used for the native ESI experiments (5 mM ammonium acetate) in order to confirm that the peptide structure is not significantly different in the MS buffer compared to the original CD buffer (10 mM potassium phosphate), and the results are summarized in Figure S14 and Table S2. Additionally, background CD spectra (no peptide) are shown for solutions containing 10 mM potassium phosphate and 5 mM ammonium acetate both with and without KLA in Figure S15. Several differences were noted for the spectra acquired in ammonium acetate (Table S2) compared to the potassium phosphate buffer. In general, the signal-to-noise ratio was notably lower in ammonium acetate, especially below 200 nm, owing to the absorption of the ammonium acetate background in the far-UV. This region of interference made it more difficult to estimate the ratios of helical to beta sheet character for each construct and impeded the quantitative predictions of secondary structure content. It has been reported previously that melittin exists as a helical dimeric/tetrameric structure in low pH and high salt concentration solutions based on NMR spectroscopy and x-ray diffraction experiments.^{76–78} For the CD measurements undertaken in the present study, melittin adopts greater helical character in 10 mM potassium phosphate relative to 5 mM ammonium acetate, an outcome consistent with previous work and the impact of increased ionic strength. However, the interaction of the melittin constructs with KLA seems to convolute this pattern for the other CD experiments, perhaps because dimeric and trimeric structures are less accessible in the presence of the lipid. The CD profiles of melittin-RS and melittin-NoKRKR in the presence of KLA in ammonium acetate diverge most significantly compared to the corresponding structures in potassium phosphate. Despite these differences, the addition of KLA to each peptide solution appears to affect the resulting structure of the peptide to a greater degree than does the change in the buffer based on comparison of the corresponding CD spectra.

As an additional reference point of interest, Florance *et al.* suggested that melittin retained some helical content in the gas phase based on hydrogen-deuterium exchange and ion mobility experiments, concluding that it is appropriate to study the structure of membrane-inserting peptides in the gas phase.⁷⁹ While the traditional solution phase conditions most widely used to favor helical melittin structures were not met in the present ESI-MS experiments, the use of ammonium acetate as an additive to aqueous solutions to study protein and peptide conformations in the gas phase has become increasingly popular as an MS-friendly alternative compared to potassium phosphate.

Two possible explanations for the lower UVPD fragmentation efficiency and patchy production of holo KLA-containing fragment ions of the KLA•melittin-RS complexes relative to the KLA•melittin and KLA•melittin-S complexes are offered based on the supplemental information from the CD experiments. 1) The interaction between the MEL - RS peptide and KLA may be less specific as a consequence of the diversity of initial peptide structures. 2) Owing to the adoption of a helical conformation upon lipid binding, the UVPD fragmentation patterns are significantly altered. The anti-bacterial efficacy of the MEL-NoKRKR at higher concentrations also raises interesting questions regarding the mechanism of activity; it is possible that without the highly basic motif to initiate binding, efficacy at low concentrations is reduced, as was observed. However, higher concentrations of peptide may shift the equilibrium leading to eventual binding between the peptide and saccharolipid, ultimately resulting in peptide restructuring (to the observed β -sheet conformation) and subsequent cell death.

Conclusion

UVPD of the KLA•melittin complexes affords significant sequence coverage of the peptides and provides insight into the electrostatic interactions between the two partners as evidenced by the holo-product ion analysis. In essence, the lipid may be retained by *a*, *b*, *c* and *x*, *y*, *z* fragment ions when the lysine/arginine-rich motif is conserved during truncation of the peptide by UVPD. Re-location of the highly basic KRKR binding motif illustrates its role in the transfer and survival of KLA•melittin complexes in the gas phase and the fragmentation pathways during disassembly of the complexes. In contrast, collisional activation of the complexes yields no holo lipid-containing sequence ions and results in qualitatively similar fragmentation trends relative to the apo-peptides, suggesting that the electrostatic interactions between the peptide and saccharolipid are promptly disrupted upon collisional activation. Cell viability and CD experiments provide orthogonal strategies for evaluating complementary aspects of peptide function and structure. Extending the native MS and MS/MS characterization strategy to larger complexes may reveal additional insights into mechanisms of CAMPs in the destruction of Gram negative bacteria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

MS/MS spectra of the Kdo₂-lipid A•melittin complex (4+). (a) ETD (50 ms) produces a charge-reduced radical species. (b) HCD (NCE 35) generates an array of apo-peptide ions. (c) UVPD (1 pulse at 3.5 mJ) produces both apo- and holo-peptide ions (holo-ions are denoted with a superscript purple dot).

Figure 2.
Fragmentation pathways for the Kdo₂-lipid A•CAMP complexes using ETD, HCD, and UVPD.

Figure 3. Normalized fragmentation yields via UVPD and HCD of melittin (4+) and Kdo₂-lipid A•melittin complexes (4+). N-terminal ions (all *a*, *b*, *c* ions) are denoted in blue and C-terminal ions (all *x*, *y*, *z* ions) are denoted in red. For the complexes, apo-peptide sequence ions and holo-peptide sequence ions (containing Kdo₂-lipid A) are shown in separate histograms.

Figure 4.

Normalized fragmentation yields via UVPD and HCD of melittin-RS (4+) and Kdo₂-lipid A•melittin-RS complexes (4+). N-terminal ions (all *a*, *b*, *c* ions) are denoted in blue and C-terminal ions (all *x*, *y*, *z* ions) are denoted in red. For the complexes, apo-peptide sequence ions and holo-peptide sequence ions (containing Kdo₂-lipid A) are shown in separate histograms.

Figure 5. Normalized fragmentation yields via UVPD and HCD of melittin-S (4+) and Kdo₂-lipid A•melittin-S complexes (4+). N-terminal ions (all *a*, *b*, *c* ions) are denoted in blue and C-terminal ions (all *x*, *y*, *z* ions) are denoted in red. For the complexes, apo-peptide sequence ions and holo-peptide sequence ions (containing Kdo₂-lipid A) are shown in separate histograms.

Figure 6.

Confocal microscopy images demonstrating the mortality rates of *Pseudomonas aeruginosa* based on 25.0 μg/mL dosing of (a) MEL, (b) MEL-RS, (c) MEL-S, and (d) MEL-NoKRKR. Scale bars in the bottom right of each image are 5 μm. (e) Percentage cell death at varying concentrations of the representative melittin variants (confocal images for 12.5 μg/mL and 50.0 μg/mL peptide concentrations are shown in Figures S8 and S9, respectively). Green (GFP fluorescence) indicates metabolically active (i.e., live) cells; red reveals where cellular membranes have been damaged, allowing propidium iodide to enter the (dead) cells.

Figure 7.

CD spectra of 20 μ M (a) melittin, (b) melittin-S, (c) melittin-RS, and (d) melittin-NoKRKR alone (solid lines) and with 100 μ M KLA (dashed lines) in 10 mM potassium phosphate at pH 7.0.

Table 1

Peptide sequences used to probe the electrostatic interactions between Kdo₂-lipid A and CAMPs. Monoisotopic mass given in parenthesis. The basic residues are highlighted in red font.

Name (M)	Sequence
MEL (2845.76 Da)	GIGAVLKVLTITGLPALISWIKRKRQQ
MEL-RS (2874.75 Da)	QQRIWSILAPLGTTLVKRKRLVAGIG
MEL-S (2858.78 Da)	GIGAVLKVLTIGLKRKRWIPALISQQ
MEL-noKRKR (2744.66 Da)	GRIGAVLKVLTIGLPRALISWIAAQQ