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Helical Hairpin Structure of a Potent Antimicrobial Peptide MSI-594 in Lipopolysaccharide Micelles by NMR Spectroscopy

Anirban Bhunia,[a] Ayyalusamy Ramamoorthy,*[b] and Surajit Bhattacharjya*[a]

Antimicrobial peptides (AMPs) are naturally occurring host defense molecules that exist in all life forms.[1] In current years, structure–activity studies of AMPs, naturally occurring or designed, have been widely carried out in order to develop new antibiotics against drug-resistance bacterial pathogens.[2,3] As a mode of their action, many AMPs target the cytoplasmic membranes of microorganisms. Amphipathic property of cationic AMPs allows them to interact aptly with inner cell membranes leading to cell lysis.[4] However, in addition to cytoplasmic membranes, Gram-negative bacteria contain an outer membrane predominantly composed of a glycolipid called lipopolysaccharide (LPS).[5] LPS serves as a permeability barrier and plays a key role in bacterial resistance hydrophobic antibiotics and antimicrobial agents.[5] Recent studies suggested that LPS (Scheme S1, Supporting Information) is actively involved in modulating binding and insertion of AMPs into Gram-negative organisms.[6] Structure–activity relationship (SAR) studies of AMPs often show a lack of correlations with the structures derived from model membranes composed of synthetic lipids.[7] However, structures and interactions of AMPs with LPS are poorly understood.[8] Here, we present 3D structure of a highly active antimicrobial peptide MSI-594 in LPS micelles using transferred nuclear Overhauser effect (Tr-NOE) or exchanged transferred nuclear Overhauser effect (Et-NOE) spectroscopy.[9] This unique structure obtained provides insights into the mechanisms by which MSI-594 traverse the LPS barrier of the outer membrane and neutralizes LPS endotoxic activities.[5]

A series of peptides based on naturally occurring magainin (magainin 2 and PGLa) have been designed for potential medical applications by Genaera and Adis.[10] Among these peptides, MSI-78 and MSI-594 demonstrated more potent antimicrobial activities than magainin 2.[10,11] MSI-594 is a hybrid of MSI-78, an analogue of magainin 2, and melittin, a hemolytic peptide from bee venom (Figure 1a). The hybrid MSI-594 was derived based on the premise that the cationic N-terminus of MSI-78 will provide binding to negatively charged membrane head groups, whereas the hydrophobic N-terminus of melittin, placed at the C-terminus of MSI-594, would interact with alkyl chains of lipids (Figure 1a). The MSI-594 peptide was found to be non-hemolytic.[10,11] In order to gain structural insights into the outer-membrane disruption by MSI peptides, we determined the 3D structure of MSI-594 in complex with LPS (Figure 1b–e). Free MSI-594 is largely unstructured in solution (Figure S1). Two-dimensional Tr-NOESY spectra[13] obtained in presence of LPS of *E. coli* or *S. typhimurium* showed a large number of NOE connectivities, indicating a folded structure of MSI-594 in complex with these LPS (Figure 1b). The dissociation constant K_d and the k_{off} of the MSI-594/LPS complex were estimated to be 70 μM and ~7142 s^{-1}, respectively (Table S1). The Tr-NOESY spectra acquired for *E. coli* or *S. typhimurium* LPS show almost identical pattern, which demonstrates stabilization of similar structures (Figure 1b). This observation also indicates that variable sugar moieties in different LPS do not influence structure stabilizations. Analyses of Tr-NOESY spectra reveal short- and medium-range NOEs (i to i+3, i+4) which are indicative of a helical structure[13] for residues I2–K10 and I13–L24 (Figure 1b and Figure S2). Medium-range NOEs were also identified between amide protons of the carboxyamide group of residue L24 with T21C$^{\gamma}H_3$ (Figure 1b, left panel). In addition, there were a number of long-range unambiguous NOE contacts involving aromatic ring protons of residue F5 with C$^{\beta}$Hs of L17, L20 and also with side chain C$^{\gamma}H_2$ of residue I2 and I13 (Figure 1b, left panel). The downfield-shifted C$^{\alpha}$ proton

[a] Dr. A. Bhunia, Prof. Dr. S. Bhattacharjya
School of Biological Sciences, Nanyang Technological University
60 Nanyang Drive, Singapore 637551
Fax: (+65) 6791-3856
E-mail: surajit@ntu.edu.sg

[b] Prof. Dr. A. Ramamoorthy
Department of Chemistry and Biophysics, University of Michigan
Ann Arbor, MI 48109-1055 (USA)
Fax: (+1) 734-763-2307
E-mail: ramamoor@umich.edu

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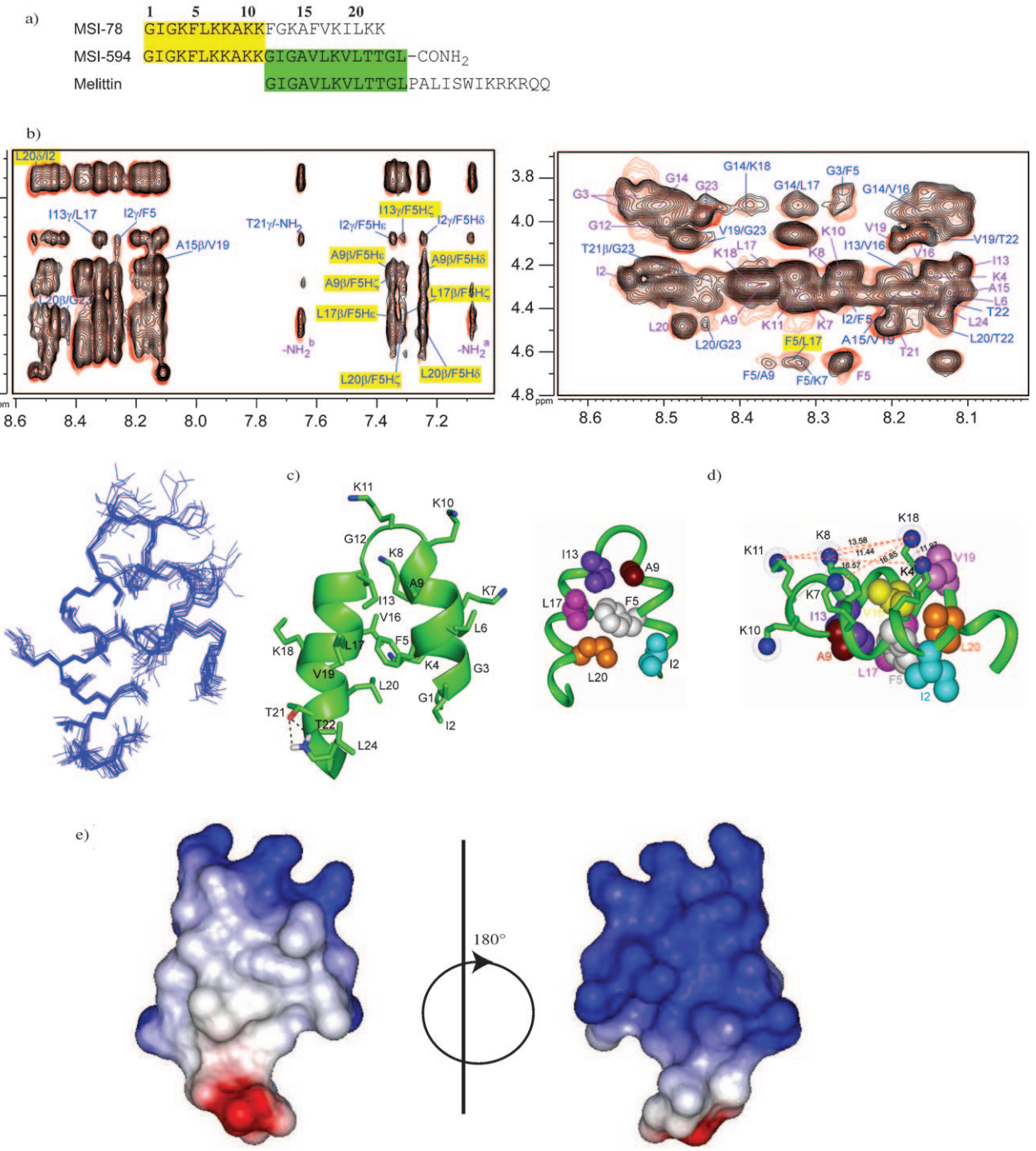

Figure 1. a) Primary amino acid sequences of MSI-78, MSI-594 and melittin. b) Superposition of two-dimensional ^{1}H–^{1}H Tr-NOESY spectra of MSI-594 obtained in LPS of *E. coli* (red) and *S. typhimurium* (black) showing long-range (marked in yellow) and medium-range NOEs between (left) aromatic ring and backbone amide protons with aliphatic sidechain protons and (right) NOEs involving amide protons with $C^{\alpha}H$ protons. Tr-NOESY spectra were acquired at a peptide:LPS molar ratio of ~20:1 in 10 mM sodium phosphate buffer at pH 4.8, 298 K. c) Left: Superposition of backbone atoms of twenty lowest energy structures of LPS-bound MSI-594. Right: A representative conformation of LPS-bound MSI-594 showing side chain disposition and backbone topology. A potential hydrogen bond is shown between the carboxyl terminal amide group with the side chain oxygen of residue Thr 21. d) Left: A space-filling representation of the side chain–side chain contacts of LPS-bound structure MSI-594 involving residues I2, F5, A9, I13, L17 and L20. Right: An amphipathic disposition of positively charged and non-polar residues of MSI-594 helical hairpin structure in LPS. Distances between ammonium groups of Lys residues between two helices are shown. e) Electrostatic potential surface of MSI-594.

(Table S2) of F5 also shows a NOE with backbone amide proton of L17 (Figure 1b, right panel). An ensemble of LPS-bound structures of MSI-594 (Figure 1c) was derived based on 203 Tr-NOE driven distance constraints including 15 long-range, 55 medium-range and 87 sequential NOEs (Table S3). Strikingly, MSI-594 adopts a helical hairpin-type

structure in the context of LPS (Figure 1c). The LPS-induced structure of MSI-594 is defined by a short helix (residues I2–K10) and a comparatively longer C-terminus helix (residues I13–L24), connected by a short loop; residues K11 and G12 (Figure 1c, left panel, Figure S3). The 15 long-range NOE contacts were critical for the determination of the tertiary fold of the MSI-594 structure in LPS. On the other hand, the αN (i to i+3/i+4) NOEs determine the helical structure. The intervening loop segment does not show αN (i to i+3/i+4) type NOEs indicating a non-helical conformation. The helical hairpin structure of MSI-594 is realized by intimate packing interactions between two helices that involves non-polar residues I2, F5, A9, I13, L17 and L20 (Figure 1d). The side chains of all Lys residues are exposed and resided at one face of the hairpin structure, whereas the hydrophobic residues are segregated onto other face forming an amphipathic structure (Figure 1e). In complex with LPS, aromatic residue F5 shows as many as ≥40 NOE contacts followed by hydrophobic residues I2, I13, L17 and L20 (Figure 2a). LPS-bound structural ensemble of MSI-594 is of high quality as revealed by low RMSD values for most of the residues (Figure S3, Table S3).

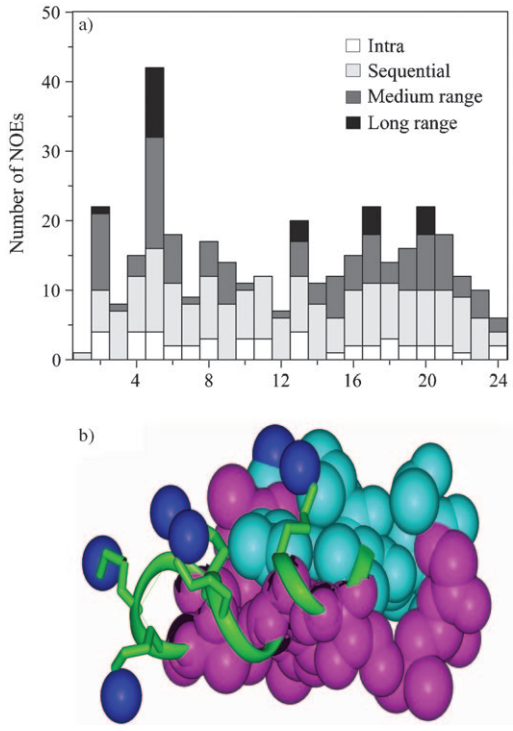

Figure 2. a) Bar diagram showing number of TR-NOEs observed per residue of MSI-594 in complex with LPS. b) Mapping of paramagnetic lipid induced resonance perturbation of MSI-594 in LPS micelles. The pink and cyan spheres represent the resonances perturbed by 16-DSA and 5-DSA, respectively.

The localization of MSI-594 in LPS micelles is investigated by two spin-labeled lipids, 5-doxyl stearic acid (5-DSA) and 16-doxyl stearic acid (16-DSA), induced relaxation enhancements as described in signal peptide and lipid vesicles interaction studies.[14] In a similar manner to Tr-NOE, NMR resonance broadening induced by the spin labels in the LPS-bound peptide is transferred to the free peptide by chemical exchange.[14] The 5-DSA and 16-DSA contain a paramagnetic nitroxide spin label at the 5th and 16th carbon position, respectively. The NMR-resonance broadening, either caused by 16-DSA or 5-DSA, will indicate a deep or sallow insertion of the amino acid residue, respectively. Analyses of paramagnetic lipids induced resonance perturbation data demonstrate that MSI-594 presumably has a parallel orientation in LPS micelles (Figure 2b). The non-polar residues I2, F5, A9, I13, A15, L17 and L24 are more perturbed in the presence of 16-DSA (Table S4), hence likely to be positioned into the hydrophobic core region of LPS micelles (Figure 2b). By contrast, positively charged Lys residues along with residues T21, T22, V16, V19 and L20 affected by 5-DSA (Table S4) lay close to the head group of the LPS micelles (Figure 2b).

In the 3D structure of MSI-594 in LPS, many of these hydrophobic residues, I2, F5, A9, I13, A15, L17 and L24, are placed in one side of the molecule whereas the cationic residues are occupying the other surface, plausibly indicating a parallel orientation. A close proximity of the MSI-594 peptide with LPS micelles is also revealed from saturation transferred difference (STD) NMR studies.[15] In the STD spectrum of MSI-594 all the proton resonances were observed, indicating that the entire peptide is closely associated with LPS micelles (Figure 3a). However, the strongest STD effects are observed for the ring protons of F5 followed by side chain methyl resonances of Leu and Ile residues (Figure 3a), implying their closest proximity to the LPS micelles. Interactions of MSI-594 with LPS phosphate head groups were further examined by ^{31}P NMR experiments. LPS micelles show two well resolved ^{31}P NMR resonances, both of them undergoing changes when titrated with MSI-594 (Figure 3b). The most upfield-shifted, ~−2.0 ppm, ^{31}P resonance shows a progressive broadening with the increasing concentrations of the peptide, whereas the ^{31}P resonance centered at 0.72 ppm becomes sharper and moves towards upfield (Figure 3b). These results indicate that MSI-594 has a direct interaction with the phosphate groups located at the lipid A moiety of LPS. The observed changes of ^{31}P resonances also indicated that LPS micelles presumably undergo structural rearrangements upon binding to MSI-594 peptide.

To the best of our knowledge, this is the first report on the atomic-resolution structure and localization of a highly active full-length antimicrobial peptide in complex with LPS. Our results demonstrate that MSI-594 peptide adopts helix–loop–helix structure in complex with LPS. Two helices are packed against each other by the aromatic residue F5 and a number of hydrophobic residues stabilizing a compact structure. NMR structures of antimicrobial peptides determined in micelles often show straight or bent helical conformations without long-range intra-peptide packing.[16] NMR structure of MSI-594 determined in DPC micelles showed a helical structure containing a bend at residues G12 and G14.[11a] Therefore, packing interactions observed between

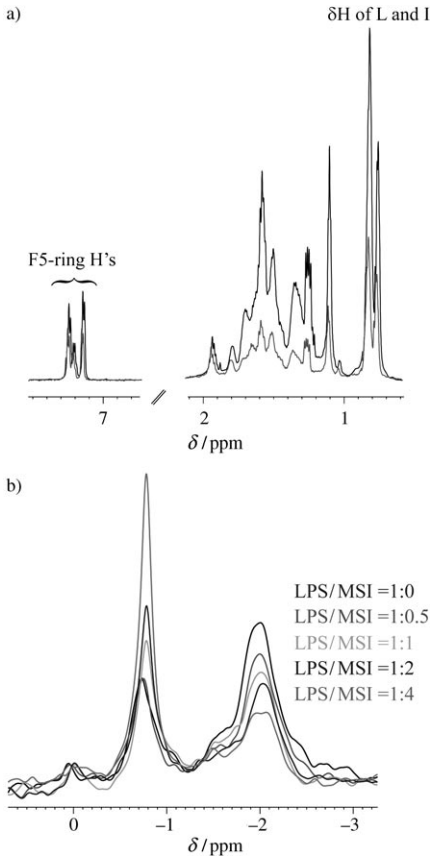

Figure 3. a) Superposition of the STD NMR spectrum (gray) and the reference NMR spectrum (in black) of MSI-594 in LPS showing a large STD effects for F5 ring protons and aliphatic sidechain protons. b) 1D ^{31}P NMR spectra of *E. coli* LPS, 0.2 mM, at a various concentrations of MSI-594 showing changes in chemical shifts and line-widths of ^{31}P resonances.

the helices in MSI-594 are specific to its interactions with LPS micelles. The structure and interactions of MSI-594 peptide with LPS provides novel insights into how a potent antimicrobial peptide overcome the LPS barrier and will stimulate further work in SAR studies of AMPs. The amphipathic nature of the LPS-bound structure allows the peptide to have optimal interactions with amphiphilic LPS molecules. However, it is noteworthy that the positively charged ammonium ($H_3\overset{+}{N}-$) groups of Lys residues across the two helices maintain a typical distance range of 12 to 15 Å (Figure 1d, right), akin to the inter-phosphate distance of the lipid A moiety of LPS, indicating a probable geometrical compatibility between peptide conformation and LPS. In addition, the compact structure of the MSI-594 in LPS may potentially aid the peptide to translocate across the LPS layer. We believe that our results could be used to explain broad-spectrum activities of AMPs and in the design of potent peptide antibiotic compounds.

Experimental Section

NMR experiments: The NMR spectra were recorded on a Bruker DRX 600 spectrometer, equipped with actively shielded cryo-probe and pulse field gradients. Data acquisition and processing were performed with the Topspin software (Bruker) running on a Linux workstation. The interaction of MSI-594 peptide with LPS was examined by recording series of one dimensional proton NMR spectra whereby 0.5 mM of MSI-594 in 10 mM sodium phosphate buffer, pH 4.8, was titrated with various concentrations, 0.05, 0.10 and 0.13 mg mL^{-1}, of either *E. coli* LPS (Sigma, St. Louis, MO) or *S. typhimurium* LPS (Sigma, St. Louis, MO) (Figure S5). The 2D tr-NOESY experiments were performed at the *E. coli* LPS concentration of 0.13 or 0.10 mg mL^{-1} of *S. typhimurium*, respectively, at 298 K, which generated a large number of TR-NOE cross-peaks. The peptide/LPS complex was found to be stable over the period of data collection at these concentrations of LPS. The 2D TR-NOESY spectra were recorded at three different (100, 150 and 200 ms) mixing times with 512 increments in t_1 and 2 K data points in t_2. The spectral width was normally 12 ppm in both dimensions. For STD-NMR studies MSI-594 (0.5 mg) was dissolved in 550 μL of 10 mM sodium phosphate D_2O buffer and pH was adjusted to 4.8. LPS was prepared by lyophilization from 99.9% D_2O. Selective saturation of LPS resonances was achieved at −2.5 ppm (40 ppm for reference spectra) using a series of 40 Gaussian-shaped pulses (49 ms, 1 ms delay between pulses), for a total saturation time of 2 s. Subtraction of the two spectra (on resonance - off resonance) by phase cycling[15] leads to the difference spectrum that contains signals arising from the saturation transfer. ^{31}P NMR spectra were acquired by adding aliquots of MSI-594, prepared in unbuffered water (pH 4.8), into a 0.2 mM of LPS in water at pH 4.8, 298 K. Paramagnetic relaxation experiments were performed by adding aliquots of paramagnetic lipids solutions, either 5-doxyl-stearic acid (5-DSA) or 16-doxyl-stearic acid (16-DSA), prepared in deuterated methanol, into the samples containing peptide-LPS complex that is, 0.5 mM MSI-594 and 0.13 mg mL^{-1} *E. coli* LPS. The samples were allowed to equilibrate for 20 min before performing 2D TOCSY experiments. The intensities of cross-peaks of MSI-594 in the presence of LPS were measured before and after the addition of the paramagnetic probes and remaining amplitudes were calculated. One-dimensional ^{1}H NMR spectra were acquired for samples containing MSI-594 alone in the presence of 5-DSA or 16-DSA (Figure S4).

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Keywords: antibiotics · lipopolysaccharides · NMR spectroscopy · peptides

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