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MINIREVIEW

Multifunctional host defense peptides: functional and mechanistic insights from NMR structures of potent antimicrobial peptides

Surajit Bhattacharjya[1] and Ayyalusamy Ramamoorthy[2]

1 Biomolecular NMR and Drug Discovery Laboratory, School of Biological Sciences, Division of Structural and Computational Biology, Nanyang Technological University, Singapore
2 Biophysics and Department of Chemistry, University of Michigan, Ann Arbor, MI, USA

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Correspondence
A. Ramamoorthy, Biophysics and Department of Chemistry, University of Michigan, Ann Arbor, MI 48109-1055, USA
Fax: +1 734 647 4865
Tel: +1 734 647 6572
E-mail: ramamoor@umich.edu
S. Bhattacharjya, Biomolecular NMR and Drug Discovery Laboratory, School of Biological Sciences, Division of Structural and Computational Biology, Nanyang Technological University, Singapore
Fax: +65 6791 3856
Tel: +65 6316 7997
E-mail: surajit@ntu.edu.sg

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The ever-increasing number of drug-resistant bacteria is a major challenge in healthcare and creates an urgent need for novel compounds for treatment. Host defense antimicrobial peptides have high potential to become the new generation of antibiotic compounds. Antimicrobial peptides constitute a major part of the innate defense system in all life forms. Most of these cationic amphipathic peptides are often unstructured in isolation but readily adopt amphipathic helical structures in complex with lipid membranes. Such structural stabilization is primarily responsible for the membrane permeation and cell lysis activities of these molecules. Understanding structure–function correlations of antimicrobial peptides is critical for the development of nontoxic therapeutics. In this minireview, we discuss atomic-resolution NMR structures of two highly potent helical antimicrobial peptides, MSI-78 and MSI-594, providing novel insights into their mechanisms of action.

Introduction

Bacterial resistance against commonly used antibiotics such as penicillin, streptomycin, vancomycin and fluoroquinolones has been increasing at an alarming rate in recent years [1,2]. The Infectious Diseases Society of America reported that in the USA, about two million people are acquiring bacterial infections every year, and that 90 000 cases have fatal outcomes [3]. Currently, bacterial strains isolated from hospital set up with resistance against multiple antibiotics, termed as multidrug-resistant (MDR) species, are the major cause of fatality. Notably, multidrug-resistant strains have been reported for a number of bacterial species, including *Mycobacterium tuberculosis*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* from different parts of the world [5,6]. Efforts to obtain a new generation of

Abbreviations
LPS, lipopolysaccharide; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; Tr-NOE, transferred-NOE.

drugs using existing antibiotic scaffolds are often challenging, as a result of their inability to penetrate the bacterial cell wall adequately. Therefore, there is a desperate need to identify new antimicrobial compounds. In this scenario, host defense antimicrobial peptides offer an attractive solution to the increasing bacterial resistance problem, and have spurred considerable scientific interest. Antimicrobial peptides, distributed in all life forms, show broad-spectrum activities against bacteria, fungi, and viruses [7–10]. Antimicrobial peptides in multicellular organisms constitute an integral part of the innate immune system, forming the first line of defense against invading microbes. They are also implicated in the stimulation and modulation of adaptive immunities [11,12]. On the basis of their structures, antimicrobial peptides can be divided into three groups, α-helical (e.g. cecropin and magainin), β-sheet or β-hairpin, stabilized by disulfide bonds (e.g. defensins, tachyplesins, and protegrins), and extended (e.g. indolicidin and PR-39) [13–15]. Although they are highly diverse in amino acid sequences and structures, a common feature shared among the antimicrobial peptides is the preponderance of positive charges (average +4 to +6) and a high (40–60%) content of hydrophobic residues [16,17]. Unlike conventional antibiotics, which act on specific intracellular targets, most of the antimicrobial peptides disrupt the structural integrity of cellular membranes. The negatively charged phospholipids of the membrane provide the initial binding sites for the cationic antimicrobial peptides through electrostatic interactions. Once anchored at the membrane surfaces, antimicrobial peptides fold into amphipathic structures, with one face of the peptide being hydrophobic and the other face containing the cluster of positively charged residues. Although acquisition of an amphipathic structure is a prerequisite for cell lysis, the exact mechanism(s) are still debated. It has been thought that such amphipathic structures might strongly interact with lipids and self-associate to form pores into the membranes or may disintegrate the membranes in a detergent-like manner [13–15]. Determination of the structure of antimicrobial peptides in appropriate lipid environments at atomic-scale resolution is an essential step in understanding the mechanism of actions of the antimicrobial peptides and development of nontoxic novel antibiotics. Global structural information can be easily obtained by use of CD, FTIR and fluorescence methods. However, an atomic-resolution structure will generate useful insights into antimicrobial peptide oligomerization states, higher-order folding, and side chain–side chain packing in complex with phospholipids. NMR, both solution-state and solid-state, has been the key method for obtaining structural determinants of a large number of antimicrobial peptides of different structural classes [18–20]. NMR structural studies on potent MSI-78 and MSI-594 peptides are presented in the following section. It should also be mentioned that there are other antimicrobial peptides that do not have a specific amphipathic structure, but are still very active.

Atomic-level 3D structures of potent antimicrobial peptides in a membrane environment obtained from NMR studies

As the function of antimicrobial peptides is exerted at the cell membrane interface, it is essential to solve their structures in a biologically relevant membrane environment. At the same time, determining the high-resolution structure of membrane-associated peptides has been a major challenge to most biophysical techniques. Fortunately, recent NMR studies have shown that atomic-level 3D structure, membrane orientation and dynamics can be obtained by using a combination of NMR techniques and model membranes. Detergent micelles or near-isotropic bicelles are well suited for solution NMR spectroscopy, as they tumble sufficiently quickly to result in high-resolution spectral lines. The negatively charged SDS micelle has been considered to be a close mimic of the anionic lipid membranes of bacterial cells, whereas the zwitterionic dodecylphosphocholine (DPC) micelles could provide an environment akin to mammalian cell membranes [18–20]. As a complex of peptide and model lipid membrane is immobile in the NMR time scale, solid-state NMR techniques are used to determine the high-resolution structure of antimicrobial peptides [12,21]. In addition, solid-state NMR methods have been used to determine the orientation and dynamics of antimicrobial peptides in fluid membrane bilayers. Solid-state NMR measurements with a varying membrane composition have been used to determine oligomerization and the mechanism(s) of membrane permeation and disruption [22].

Solution NMR experiments were used to determine the 3D structures of antimicrobial peptides such as MSI-78 [23], MSI-594 [23], pardaxin [20], LL-37 [18], polyphemusin [19], and analogs of melittin [24]. These studies have also determined the location of an antimicrobial peptide in micelles, using paramagnetic relaxation effects. Whereas most peptides exist as monomers in micelles, MSI-78 (or pexiganan) and magainin-2 have been shown to exist as antiparallel helical dimers located near the head group region of micelles. As oligomerization is a key step in the antimicrobial activity of antimicrobial peptides, a recent study enhanced

the hydrophobicity of the dimeric helical interface by substituting with fluorinated amino acids. The resultant fluorinated MSI-78 (or fluorogainin) has been shown to be more potent than the nonfluorinated MSI-78 [25,26]. Vogel *et al*. have used high-resolution solution NMR spectroscopy to determine the 3D structures of antimicrobial peptides [27], and correlated structures of antimicrobial peptides with their functions [28].

A combination of static solid-state NMR experiments on mechanically aligned lipid bilayers [29] and magic angle spinning experiments on multilamellar vesicles was used to determine the backbone conformation and the membrane orientation of MSI peptides. Solid-state NMR experiments on lipid vesicles confirmed the helical conformation of these peptides as determined from solution NMR experiments on detergent micelles. Two-dimensional polarization inversion spin exchange at the magic angle [30,31] experiments on mechanically aligned bilayers revealed the membrane surface orientation of these peptides in lipid bilayers. Multilamellar vesicles with varying membrane composition were investigated to understand the effect of individual components on the structure and membrane orientation of antimicrobial peptides [32,33]. For example, samples with varying concentration ratios of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*sn*-glycerol) were used to determine the role of an anionic lipid in mammalian cell membranes; 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*sn*-glycerol) were used to determine the role of an anionic lipid in bacterial cell (inner) membranes; POPC and cholesterol were used to determine the role of cholesterol in mammalian cell membranes; and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) were used to investigate the role of hydrophobic thickness of the membrane bilayer. Rotational echo double resonance [34] magic angle spinning experiments suggested that the backbone helical conformation of MSI peptides does not depend on the variation in the membrane composition. Two-dimensional polarization inversion spin exchange at the magic angle experiments on mechanically aligned samples revealed that the membrane orientations of both MSI-78 and MSI-594 peptides do not vary within experimental errors with most of the above-mentioned samples [32]. Both peptides were stabilized by the lipid–peptide interactions near the head group region of the bilayer, with the helical axis nearly parallel to the bilayer surface. Interestingly, the tilt of the MSI-594 helix varied from 15° to 25° from the bilayer surface, whereas that of the MSI-78 helix varied from 5° to 10°. The difference in the tilt angle between these two peptides could be due to the difference in their oligomeric size: MSI-78 is a dimer and MSI-594 is a monomer [23]. The peptide–peptide interaction in MSI-78 could dominate and lead to a stabilized membrane orientation without the need for insertion into the hydrophobic region of the bilayer. On the other hand, the presence of cholesterol reduced the tilt of the MSI-594 helix to within 5° and that of the MSI-78 peptide to $< 5°$. This observation on the reduction in the tilt angle could be attributed to the cholesterol-induced ordering of the lipid bilayer, which considerably reduces the peptide insertion into the hydrophobic area of the lipid bilayer. Our solid-state NMR studies indicated that the membrane orientations of MSI and LL-37 [35,36] peptides do not significantly change with the hydrophobic thickness of the lipid bilayer; the membrane orientation of pardaxin was found to change from the transmembrane orientation in the thinner 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine bilayer to an orientation with its main helical axis close to the thicker POPC bilayer surface [20]. Other solid-state NMR studies on peptide starting with a glycine and ending with a leucine amide (PGLa) have reported a change in the membrane orientation of the peptide due to oligomerization [37]. Such high-resolution information on the membrane orientation of antimicrobial peptides provides insights into their mechanism and will also aid in the design of more potent antimicrobial peptides.

Various combinations of solid-state NMR experiments were used to determine the peptide-induced membrane permeation and disruption for these peptides [21,32,36–39]. Peptide-induced effects such as (a) the disorder in the lipid head group region of lipids, (b) change in the lipid head group conformation, (c) membrane curvature and (d) disorder in the hydrophobic region of bilayers were measured from fluid lipid bilayers with various membrane compositions under physiologically relevant experimental conditions. Both high-resolution spectra of aligned bilayers and powder pattern spectral line shapes of unaligned samples were used in these experiments. All MSI (MSI-78, MSI-594, and MSI-843) [32,40] and LL-37 [12,36] peptides were found to disrupt the membrane via carpet mechanisms at low concentrations. MSI peptides led to the formation of toroidal-type pores, whose geometry, as determined from solid-state NMR experiments, was also reported [32]. Interestingly, these MSI peptides also behaved like a detergent, and, after a certain time ($\sim$ 3 weeks), induced the formation of bicelles that spontaneously aligned in the magnetic field. Finally,

after 1 month, these samples consisted of micelles, as detected by the presence of isotropic ^{31}P chemical shifts [21,41]. Solid-state NMR experiments on bicelles revealed the detergent-like behavior of MSI-78 and functional peptide fragments of human β-defensin-3, as they preferred to be associated with the toroidal pores of bicelles.

Structures and mechanisms of antimicrobial peptides in a model outer membrane of bacteria

In addition to the inner phospholipid membrane, Gram-negative bacteria contain an outer membrane that acts as a permeability barrier against hydrophobic antibiotics, host defense antimicrobial peptides, and other detergent molecules [42–44]. The outer leaflet of the outer membrane consists of a specialized lipid called lipopolysaccharide (LPS). Chemically, LPS is organized into three distinct regions: a hydrophobic lipid A region, a variable polysaccharide moiety or O-antigen, and the core oligosaccharide region that covalently bridges the two [44]. The lipid A region is highly conserved among Gram-negative bacteria, and consists of a bis-phosphorylated diglucosamine backbone containing six to seven fatty acyl chains per molecule [44,45]. In order to gain access to the inner membrane or to the intracellular targets, antimicrobial peptides have to interact with the LPS layer. Recent studies have suggested that LPS is actively involved in controlling the binding and permeation of antimicrobial peptides into Gram-negative organisms [46–49]. Structures of antimicrobial peptides derived from model membranes composed of synthetic lipids often show poor correlation with their functions. Interactions of the antimicrobial peptides with LPS could constitute one of the determining factors. Moreover, LPS or endotoxin, a potent stimulator of innate immune systems, is the primary agent of septic shock syndromes [50,51]. Therefore, determination of structures of antimicrobial peptides in the context of LPS would be an important step towards understanding the mechanism of outer membrane permeabilization and the development of endotoxin-neutralizing molecules. We have determined 3D structures of antimicrobial peptides and designed peptides in complex with LPS, using NMR experiments, to gain insights into the peptide interactions with LPS [52–57]. LPS-bound structures of peptides and antimicrobial peptides are determined using transferred-NOE (Tr-NOE) effect spectroscopy. In the Tr-NOE method, NOEs from the bound ligands are observed in their free-state resonances, whereas ligand–macromolecule complexes undergo fast dissociation on the NMR time scale. Usually, Tr-NOE-based structure determination is applicable to macromolecule–ligand complexes with binding affinities ranging from micromolar to millimolar. Tr-NOE of LPS-bound peptide was first demonstrated in an analog of polymyxin B [58]. Since then, the method has met with notable successes in determining 3D structures of LPS-interacting peptides [59–61]. LPS forms large molecular mass micelles or bilayers in solutions at a significantly lower (≤ 1 μM) concentration [62]. The larger size of LPS micelles, coupled with rapid dissociation of LPS–peptide complexes, may generate a large number of Tr-NOE cross-peaks for the bound peptides. High-resolution structures of the LPS-bound states of peptides are determined on the basis of the distance constraints obtained from Tr-NOE analyses [52–57]. LPS, being a lipid of the outer membrane, provides a native environment for the folding of the peptides and antimicrobial peptides. In conjunction with Tr-NOE, we have employed a saturation transfer difference NMR method [63] to determine the proximity or localization of several antimicrobial and cytotoxic peptides in LPS micelles at residue-specific details [53,55,56].

Recently, the LPS-bound structure of the highly potent antimicrobial peptide MSI-594 was determined by us [56]. The 3D structure determination of MSI-594 in complex with LPS reveals a helix–loop–helix or a helical hairpin structure (Fig. 1A). The solution structure of MSI-594 in complex with LPS is determined by two segments of helices, Ile2–Lys10 and Ile13–Leu24, with an intervening loop maintained by two Gly residues (Fig. 1A). The two helices are stabilized by mutual packing interactions whereby the single aromatic residue Phe5 is found to be in proximity with a number of nonpolar residues, Ile2, Ile13, Leu17, and Leu20 (Fig. 1B) [56]. Determination of the LPS-bound structure of MSI-594 showed that all of its five positively charged Lys residues are situated at one face of the molecule and that nonpolar residues occupy the opposite surface (Fig. 1C). The saturation transfer difference NMR studies revealed that the aromatic ring of Phe5 and side chain methyl groups of Ile and Leu are in close contact with LPS. MSI-594 possesses a parallel orientation in LPS micelles, as inferred from the nitro oxide spin-labeled measurements. Interestingly, the NMR structure of MSI-594 derived from DPC micelles showed a straight helix without any long-range packing as observed in LPS. Therefore, it is likely that antimicrobial peptides could have different structural organizations at the outer membrane [53,56]. Such compact conformations may essentially help the

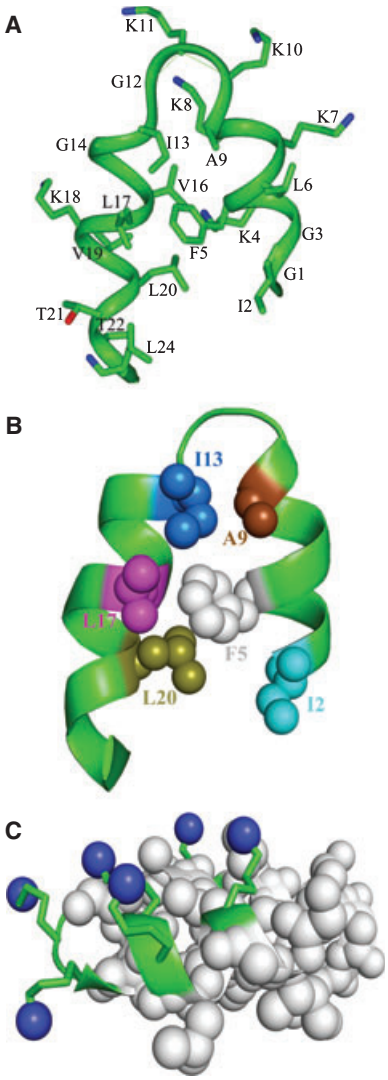

Fig. 1. Three-dimensional structure of MSI-594 in LPS. (A) The helix–turn–helix organization of the peptide, showing backbone and side chain orientation. (B) Space-filling representation of the interhelical interactions whereby aromatic residue Phe5 undergoes intimate packing with nonpolar residues Ile13, Leu17 and Leu20 from the long helix. (C) Unique disposition of the positively charged side chains of MSI-594 in the amphipathic helical hairpin structure. A 13 Å distance between the charged groups geometrically complements interphosphate distance of the lipid A moiety of LPS. The figure was generated using PYMOL (Protein Data Bank: 2K98).

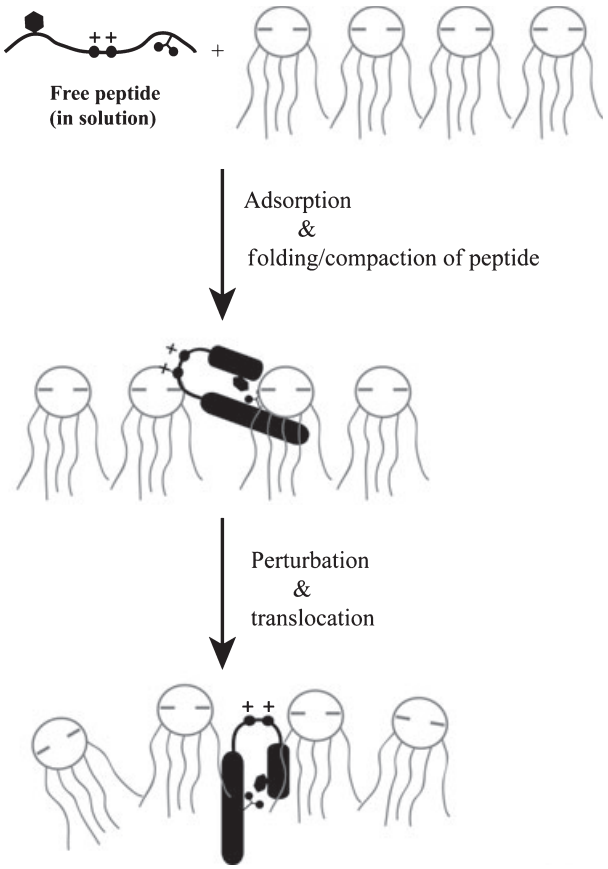

Fig. 2. A proposed model for the mechanism of permeation of MSI-594 through the LPS layer. Top panel: free MSI-594 is unstructured in solution. Middle panel: peptide binds to the LPS surface via electrostatic interactions, as charge and geometrical compatibility facilitate optimal adsorption; upon binding to LPS, MSI-594 folds into a compact helical hairpin structure, secluding some of the nonpolar residues. Bottom panel: binding could lead to further destabilization or perturbation of the LPS layer, whereby the peptide in its compact state may easily translocate towards the inner cell membrane.

peptides to translocate across the LPS bilayer (Fig. 2). A different mechanism may have been utilized by melittin, a hemolytic peptide from bee venom, to overcome the LPS barrier. Melittin adopted a partial helical structure restricted to the cationic C-terminus of the molecule in LPS micelles [53]. The relatively hydrophobic N-terminus of melittin was found to be unstructured and dynamic in LPS. It is likely that the folded C-terminus of melittin acts as an anchoring element and perturb LPS structures, enabling insertion of the hydrophobic N-terminus towards the inner membrane (Fig. 3). Our research shows not only helical conformations, but also that peptides may form β-strands and β-turn structures in LPS micelles [52,54,57]. With a set of designed peptides, the LPS-bound structures reveal multiple β-turn and β-strand structures (Fig. 4) of these antimicrobial and antiendotoxic peptides [51,53,57].

Our recent studies show that disruption of interhelical packing by mutating Phe5 of MSI-594 to Ala has severe consequences for the antimicrobial activity of MSI-594 (our unpublished result). These results clearly suggest that structure–function correlation of anti-

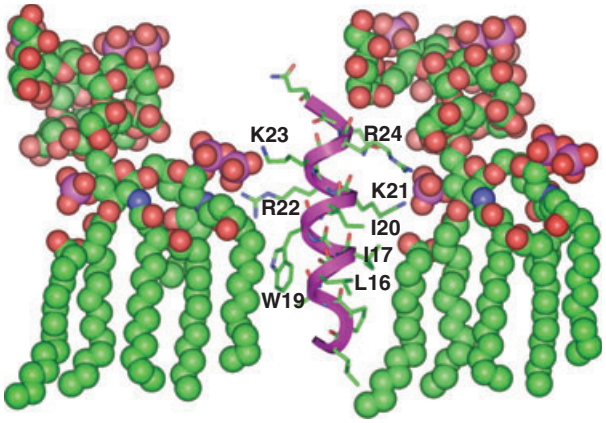

Fig. 3. Interactions of melittin with LPS. The bee venom peptide folds into a helical structure at its C-terminus in complex with LPS. The four positively charged residues, Lys21, Arg22, Lys23 and Arg24, at the C-terminus may stabilize the helical structure by inserting between two LPS molecules. The dynamic nonpolar N-terminus may drive the translocation of the peptide across the LPS layer. The figure was prepared using the INSIGHT II molecular modeling program.

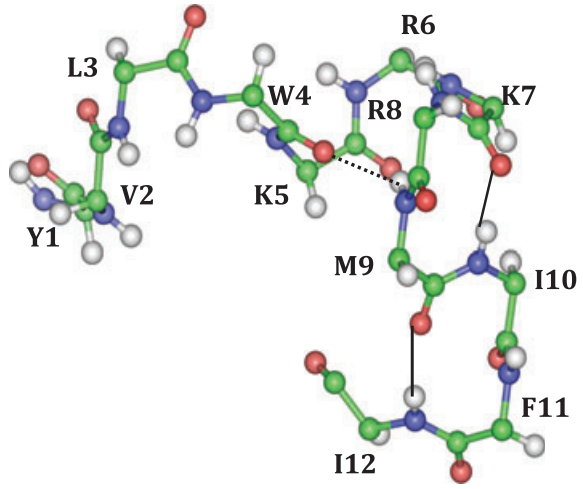

Fig. 4. Structure of a designed peptide in LPS. The designed peptide adopts multiple β-turns at the C-terminus, whereas the N-terminus has a β-strand-type structure. The plausible short-range and long-range hydrogen bonds stabilizing the folded structure are shown as solid and dotted lines, respectively. The figure was prepared using the INSIGHT II molecular modeling program (Protein Data Bank: 2o0S).

microbial peptides requires knowledge of interactions of peptides with outer membranes. In addition, NMR structure and dynamics have been reported for isotope-labeled ($^{15}N/^{13}C$) LPS solublized in detergent micelles [64,65]. Another study determined the binding sites of LPS in the presence of polymyxin antibiotic peptides, using DPC-solublized isotope-labeled LPS [66].

Remaining challenges

In order to form pores and disintegrate membrane components (inner and outer), the antimicrobial peptides might be required to form oligomeric assemblages. However, high-resolution structures of such higher-order states of antimicrobial peptides are not easily obtainable. MSI-78 and magainin showed a dimeric structure in DPC micelles and lipid vesicles, respectively. It is likely that currently used detergent micelles, SDS or DPC, may not stabilize such oligomeric structures. In particular, SDS is not known to disrupt noncovalent interactions in proteins. Therefore, alternative lipid environments need to be developed. Currently, small bicelles, containing a mixture of long-chain and short-chain phospholipids, are thought to constitute a close mimic of the bilayer of cell membranes [67–69]. Bicelles have been demonstrated to constitute a suitable medium for structural analysis of membrane proteins by NMR spectroscopy [68–71]. Larger bicelles have been found to be useful for solid-state NMR as an alignment medium [72,73]. Antimicrobial peptide studies in such lipid environments may prove to be useful for the determination of oligomeric structures. The presence of toroidal pores in lamellar-phase bicelles could be utilized in determining the mechanism of membrane disruption by antimicrobial peptides [73]. On the other hand, the LPS micelle has been shown to be a promising lipid system for the outer membrane that stabilizes not only secondary structures but also tertiary packing in antimicrobial peptides [56,57,60]. Even more recently, we were able to determine an oligomeric structure of an antimicrobial peptide belonging to the cathelicidin family from the chicken in LPS (Bhattacharjya, unpublished results). However, not all antimicrobial peptides will produce Tr-NOE in LPS, as a result of binding heterogeneity. Therefore, future studies on antimicrobial peptides and LPS will require the preparation of suitable outer membrane mimics. It will be interesting to see whether bicelles can be made using LPS and other short-chain phospholipids. It would also be interesting to investigate the intracellular action of certain antimicrobial peptides [74] using high-resolution NMR techniques.

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