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On the role of NMR spectroscopy for characterization of antimicrobial peptides

Fernando Porcelli[1,3], **Ayyalusamy Ramamoorthy**[4], **George Barany**[2], and **Gianluigi Veglia**[1,2,*]

[1]Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota – Minneapolis, 55455 - USA

[2]Department of Chemistry, University of Minnesota –Minneapolis, 55455 - USA

[3]Department for Innovation in Biological, Agro-food, and Forest Systems, University of Tuscia, Viterbo, 01100 – Italy

[4]Department of Biophysics and Department of Chemistry, University of Michigan, Ann Arbor, MI 48109-1055, USA

Summary

Antimicrobial peptides (AMPs) provide a primordial source of immunity, conferring upon eukaryotic cells resistance against bacteria, protozoa, and viruses. Despite a few examples of anionic peptides, AMPs are usually relatively short positively charged polypeptides, consisting of a dozen to about a hundred amino acids, and exhibiting amphipathic character. Despite significant differences in their primary and secondary structures, all AMPs discovered to date share the ability to interact with cellular membranes, thereby affecting bilayer stability, disrupting membrane organization, and/or forming well-defined pores. AMPs selectively target infectious agents without being susceptible to any of the common pathways by which these acquire resistance, thereby making AMPs prime candidates to provide therapeutic alternatives to conventional drugs. However, the mechanisms of AMP actions are still a matter of intense debate. The structure-function paradigm suggests that a better understanding of how AMPs elicit their biological functions could result from atomic resolution studies of peptide-lipid interactions. In contrast, more strict thermodynamic views preclude any roles for three-dimensional structures. Indeed, the design of selective AMPs based soley on structural parameters has been challenging. In this chapter, we will focus on selected AMPs for which studies on the corresponding AMP-lipid interactions have helped reach an understanding of how AMP effects are mediated. We will emphasize the roles of both liquid- and solid-state NMR spectroscopy for elucidating the mechanisms of action of AMPs.

Keywords

Antimicrobial peptides; solution NMR; solid-state NMR; lipid membranes

*To whom correspondence should be addressed: Dr. Gianluigi Veglia, Department of Chemistry and Department of Biochemistry, Molecular Biology, and Biophysics, 231 Church St. SE, Minneapolis, MN 55455. Telephone: (612) 625-0758. Fax: (612)-625-5780. vegli001@umn.edu.

1. Introduction

The increasing resistance of bacteria to conventional antibiotics represents a serious global emergency for human health that can only be addressed by the discovery and clinical implementation of new antimicrobial drugs (1–8). Over the past 40 years, however, only three new classes of antibiotics have been found that could be used as drugs: lipopeptides, oxazolidinones, and streptogramins (9). Since antimicrobial peptides (AMPs) are not susceptible to any of the common mechanisms whereby organisms acquire drug resistance, representatives of the class of AMPs offer an alternative to standard antimicrobial therapy.

AMPs constitute one of the first evolved chemical defense mechanisms of eukaryotic cells against bacteria, protozoa, fungi, parasites, and viruses (10–12). AMPs have been isolated from a variety of organisms, including humans, and are present in virtually all living species from bacteria to invertebrates to vertebrates (13). The first antimicrobial peptide, nisin, was isolated in 1947 (14, 15), but systematic research on AMPs started only in early 1980s, upon publication of Hans Boman's paper on the isolation, purification, sequencing, and specificity of two antibacterial peptides, cecropins A and B, involved in insect immunity (10). Since this pioneering research, more than 1800 AMPs have been identified and isolated from several organisms, and structural analogues have been synthesized and tested for clinical purposes (16–22). A comprehensive database on isolated or synthesized AMPs is available at http://aps.unmc.edu/AP/main.php (22).

Properties of AMPs in their role as antibiotics include a broad spectrum of antibacterial activity, high selectivity, and the ability to disrupt bacterial cell membranes (2, 11, 23, 24). In addition, several AMPs can modulate immune and inflammatory responses, inducing phagocytosis, promotion of immune cell recruitment, regulation of angiogenesis, and stimulation of prostaglandin release (13, 19, 20, 25–29)

AMPs can be grouped into diverse classes based on the pathways of their biosynthesis, their structural properties, and their biological activities. AMPs are either *ribosomally synthesized* oligopeptides, when produced by mammals, birds, amphibians, insects, plants, or certain microorganisms (30, 31) or *non-ribosomally synthesized* peptides when produced by bacteria and fungi, *i.e.*, bacteriocins. Additionally, AMPs can be subdivided as antiviral, antifungal, anticancer, antiparasital, insecticidal, spermicidal, anti-HIV, and/or as having chemotactic activity (22). Finally, AMPs can be classified on the basis of molecular structure as (a) linear amphipathic α-helical, e.g., cathelicidins, magainins, and cecropins (32–38), (b) amphiphilic β-sheet structures containing disulphide bonds, e.g., defensins (39–41), and (c) turns and extended structures, e.g. protegrins (42–45).

AMPs are usually cationic, relatively short (less than a hundred amino acid residues), and amphipathic, with approximately half of the residues hydrophobic so as to enable their associations with cell membranes (13, 46–54) In fact, to elicit their biological functions, it is necessary for AMPs to interact with the bacterial cytoplasmic membrane, under which conditions AMPs often adopt a secondary structure (48, 54–58). From a physical chemical standpoint, antibacterial activity of AMPs seems to be related to amino acid composition, which may also dictate their selectivity towards bacterial cell membranes with specific lipid

composition and structure (59, 60). However, a unified mechanism of action for AMPs has not been identified. It is possible that each class of AMPs has specific mechanism of action.

2. Proposed mechanisms of action

The biological actions of AMPs are not well understood. Researchers agree that the primary target for many AMPs is the outer cell membrane (61–64). Two main membrane interaction mechanisms have been proposed: (a) pore formation across the membrane and (b) a carpet-like mechanism (65, 66). These two models share some similarities and involve four major steps: attraction, attachment, insertion, and membrane permeation (13, 50). The initial *attraction* of the peptide toward the membrane is driven by electrostatic interactions between the positively charged residues (generally Arg and Lys) and the negative lipid headgroups of the bacterial membrane (67–69). The *attachment* step depends on the distribution of hydrophobic and hydrophilic residues of AMPs. After reaching the membrane, AMPs pass a concentration threshold after which cell membrane permeability is altered (48, 62, 70–73). At low peptide/lipid ratio (P/L) ratio, AMPs are positioned with their backbones parallel to the surface of the lipid bilayer, so that the AMPs interact with the lipid headgroups. With this topology, AMPs often insert into the membrane, modifying its thickness and curvature (74–80). As the P/L ratio increases, AMPs orient perpendicularly to the membrane and insert into the membrane bilayer while forming pores (81). Amino acid composition, charge, and amphipathicity drive AMP *insertion*. In addition, several studies show that membrane partitioning causes AMPs to adopt a regular secondary structure; this is referred to as the *folding-upon-binding* mechanism (82, 83). The latter effect causes a substantial reduction of the free energy of insertion, due to the formation of intramolecular hydrogen bonds (82, 84, 85). Within the lipid bilayer, AMPs can assume different architectures, including *toroidal*, *carpet-like* or *barrel stave* (65, 86–88). In the *toroidal model*, the polar faces of AMPs associate with lipid polar head groups and insert into the membrane in a manner that involves induced bending of the lipid monolayers and concomitant pore formation (50, 89). In the *carpet-like* model, AMPs coat the bilayer outer surface, just like a carpet; after reaching a certain concentration threshold, the AMPs disrupt the membrane in a detergent-like manner, causing membrane lysis (38, 50, 87, 90). In the *barrel-stave* model exemplified by alamethicin, AMPs aggregate to form a bundle of monomers (staves in a barrel), forming a pore in the membrane by thinning and bending of the inner and outer membrane leaflets (66, 91).

Disruption of the membrane dissipates the electrochemical gradients necessary for cells to thrive, while also causing the loss of essential cytoplasmic constituents. It has been reported the relative preference of barrel-stave versus toroidal mechanism depends on peptide length (57, 75, 92–94). Modifications of the peptide sequence involving changes in charge distribution, hydrophobicities, and surface areas of different amino acid side-chains have been shown to modulate the specificities and mechanisms of action for AMPs (49, 53, 70, 95, 96).

Over the past few years, a more thermodynamic approach has been applied to explain membrane permeabilization by AMPs. This viewpoint is based on the efflux of fluorescent dye observed with lipid vesicles, and precludes any structural factors (48, 62, 89, 97–101).

Such studies suggest two possible mechanisms for cell permeation: (a) *graded* or (b) *all-or-none* (89, 101–104). In the *graded* mode, all vesicles release part of their content, while in the *all-or-none* mode only a fraction of the vesicles release their contents while the remainder do not lose any dye. In essence, the *graded* mechanism can be understood in terms of a destabilizing detergent-like effect on all vesicles, whereas the *all-or-none* mode of depletion suggests the formation of multimeric pores or the disruption of the vesicle structure (89, 101–104).

To what extent do structures affect the activities and selectivities of AMPs? Is is possible that millions of years of evolution did not encode specificity and function into these peptides as for larger proteins? At this writing, the questions just posed remain unanswered. While there are clear examples of structure-function relationship for several classes of AMPs, the proponents of a more thermodynamic viewpoint invoke the many examples where AMPs with all D-amino acid residues or scrambled sequences still manifest antimicrobial activity. In favor of the structure-activity relationship is the possibility that the landscape of the AMPs structural propensities as well as their interactions with lipid membranes is still limited to selected cases.

3. Bacterial membrane composition and membrane mimicking systems for structural studies

The plasma membrane contains both neutral and zwitterionic lipids such as phosphatidylcholine (PC), or acidic lipid such as phosphatidylserine (PS), cardiolipin, and phosphatidylglycerol (PG) (65, 105, 106). While it is relatively easy to reproduce the relevant lipid composition with synthetic lipids, it is very challenging to mimic the complexity of cell membrane organization. The latter constitutes a significant concern for both functional and structural studies. For the latter, several different model systems have been employed. As a general guideline, the membrane mimetic systems should not only represent the natural environment as closely as possible, but it should also be compatible with structural techniques such as NMR, CD, and fluorescence measurements. Originally, NMR researchers used aqueous solutions of organic solvents such as trifluoroethanol (TFE) or hexafluoroisopropanol (HFIP). Under such conditions, most known AMPs are forced to adopt α-helical conformations, which often do not correlate with biological activities (107–110). To overcome this problem, detergent micelles, such as those formed from DPC (dodecylphosphocholine) plus SDS (sodium dodecylsulfate), have been used in order to better simulate the membrane interface. Micelles are definitively a superior medium to study AMPs, since they provide a water/lipid interface similar to the lipid membranes.

Usually, micelles are spherical monolayers with a diameter of ~ 3 nm that can assume elliptical or rod-like shapes, depending on detergent concentration and chain length (111, 112). Their small size and fast tumbling enable solution NMR spectroscopy of micelle-bound AMPs and small membrane proteins (34, 113–117). Detergent micelles, however, are only a rough approximation to a membrane bilayer. The monolayer structure and small curvature radius of micelles can cause peptides and proteins to adopt incorrectly folded conformations or to aggregate (118). Recently, discoidal micelles or bicelles have been used

in NMR spectroscopy of both membrane peptides and proteins (112, 118–122). Generally, bicelles are formed by long-chain phospholipids (DMPC, DMPG) and amphiphilic molecules such as CHAPSO, or a short-chain lipid, such as DHPC. The structures and shapes of bicelles depend on the lipid to detergent ratio (called q). At high detergent concentrations ($q \sim 0.1–0.8$), bicelles assume a discoidal shape and tumble rapidly in solution. At low detergent concentrations ($q \sim 2.8 – 6$), bicelles may resemble perforated bilayer sheets (123–125). While AMPs and membrane proteins can be analyzed by solution NMR techniques in isotropic bicelles, anisotropic bicelles align spontaneously in magnetic fields, enabling peptides and proteins to be analyzed using oriented solid-state NMR approaches (126–130). In addition to magnetically oriented bicelles, AMPs can be reconstituted in mechanically aligned membrane bilayers supported on glass plates (131–133). This method of sample preparation, originally developed by Seelig and co-workers (134–136), has been used widely to analyze membrane peptides and proteins (133, 137–142). This approach, however, is quite laborious and prone to artifacts since AMPs have a tendency to aggregate and disrupt the organization of lipid bilayers, making it difficult to discern the mechanism of action. Therefore, many studies are being carried out in lipid vesicles, which are amenable to magic angle spinning (MAS) NMR techniques (143, 144).

4. NMR spectroscopic approaches to study AMPs

Classical solution NMR techniques have been used successfully to determine three-dimensional structures of bioactive peptides in mixtures of water with fluorinated organic solvents (107, 110, 145, 146), in detergent micelles (34, 38, 115, 147, 148), and in isotropic bicelles (119, 149, 150). NOESY-based techniques have been used to measure distance restraints for structure calculations. In the case of recombinant-expressed peptides, weak alignment of peptides using acrylamide gels has been used to obtain residual dipolar couplings for orientational-dependent restraints (151–153). The positioning of peptides with respect to micelle surfaces has been inferred by paramagnetic relaxation enhancement mapping (34, 38, 147, 154, 155), water exposure measurements based on exchange peaks with the water signal (156–158), detection of NOEs between protonated micelles and peptides (159–162), or saturation transfer techniques (163–165).

Despite the results from solution NMR, accurate descriptions of interactions between any given peptide and the membrane within which it exerts bioactivity require use of lipid membranes (35, 70, 128, 129, 131, 141, 142, 166–177). Meeting this need, solid-state NMR techniques are well suited for studying membrane-embedded peptides and proteins. The two major approaches are: (a) static or oriented solid-state NMR (170, 178–182), wherein nuclear anisotropic interactions are obtained from aligning samples with respect to the direction of the static magnetic field, and (b) magic angle spinning (MAS) NMR, whereby samples are spun at the magic angle ($\theta \sim 54.7°$) to remove the effects of chemical shift anisotropy and dipolar couplings (70, 173, 183–185). With fast spinning at the magic angle, resonances can reach line widths similar to those observed in solution NMR spectra.

In the following, we will focus on selected antimicrobial peptides (magainins, pardaxins, distinctin, and cathelicidins) that our group has studied, and in so doing, highlight the role of NMR in understanding structure-function relationships.

4.1 Magainins

This class of AMPs is expressed by amphibians as a defense against microbes. Magainins were first identified by Zasloff in the early 1980's (36, 186). In general, magainins are helical and amphipathic, and exhibit a broad spectrum of antimicrobial activities (33, 34, 86, 107, 108, 172). Their structures and orientations with respect to membrane bilayers have been studied extensively, as has been reviewed by the Zasloff, Opella, and Bechinger groups (35, 108, 172, 187–192). In our laboratory, we determined the high-resolution structures and lipid interactions of two synthetic variants of magainins (MSI-78 and MSI-594) that were originally designed by Genaera Corporation for clinical use (193). MSI-78, an analog of magainin 2, is a 22-residue polypeptide known as pexiganan that is being tested in phase II and III clinical trials for treatment of diabetic foot ulcers. MSI-78 interacts strongly with model bacterial cell membranes, selectively inducing bacterial membrane disruption while leaving mammalian cells essentially unperturbed (194). MSI-594 is a 24-residue peptide with high efficacy against herpes simplex virus I (195). The primary sequences of MSI-78 and MSI-594 differ significantly only at the C-terminus (Figure 1). In dodecylphosphocholine (DPC) micelles, each peptide gives a high-resolution spectral fingerprint that enables the sequential assignment of nearly all of the backbone and side chain resonances. In DPC micelles, MSI-78 assumes a distorted α-helical conformation throughout the entire length of the polypeptide chain. A significant feature of MSI-78 is its ability to form stable antiparallel dimers, as defined by head to tail NOEs contacts. The latter may explain its resistance to proteolysis and its higher efficacy with respect to MSI-594. As it turns out, MSI-594 adopts a well-defined helical conformation, but does not dimerize, a property that may account for the significantly lower antibacterial activity displayed by this molecule. Dimerization is a common feature for the majority of magainins, as exemplified by magainin 2 which forms stable dimers in PC vesicles (196).

MSI-78 dimerization seems to be encoded in its primary sequence (Figure 1). In fact, the central hydrophobic core of the MSI-78 dimer is formed by a "phenylalanine zipper" that hold the two protomers together, with charged residues (e.g., lysines) pointing toward the bulk solvent. In contrast, MSI-594 lacks phenylalanine residues in position 13 and 16, preventing formation of a stable dimer. Moreover, the GIG motif located in the middle of the primary sequence enables MSI-78 to assume a curved structure, thereby inducing conformational dynamics (reported by broader NMR lines) that further hampers the dimerization process. The Ramamoorthy laboratory performed MAS solid-state NMR experiments on the magainin analogs reconstituted in POPC and 3:1 POPC/POPG MLVs. Peptides that had been ^{13}C-labeled at the α-carbonyl displayed isotropic chemical shift values of ~176–179 ppm that are characteristic of α-helical conformations (197), and confirmatory of the helical structure determined by solution NMR. These authors also found that the line widths of MSI-594 are broader than the corresponding resonances of MSI-78, signifying that conformational exchange dynamics are more accentuated for MSI-78. Again, solution and solid-state NMR data were consistent with each other, pointing towards the same conclusions.

Based on NMR studies both in micelles and in the solid-state, we proposed that differences observed for MSI-78 and MSI-594, in terms of both function and selectivity, may be due to

their differing tendencies to self-associate. These results support the *in vivo* antimicrobial activity of MSI-78, which has been proposed to oligomerize into toroidal-type structures that permeate bacterial cells. In contrast, the behavior of MSI-594 is completely different, suggesting an alternative mechanism of action that must await further studies for elucidation (34).

4.2 Pardaxins

This class of small shark-repellent AMPs are isolated from the sole fish of genus *Pardachirus* (198, 199). Pardaxins interact with cell membranes, causing disruption of ionic transport and presynaptic activity by forming voltage-dependent ion-selective channels (200–205). Pardaxins are proposed to follow the barrel-stave model, with an aggregation number of 6 (198). To test this hypothesis, we studied pardaxin 4 (Pa4), both in micelles and lipid bilayers, using a combination of solution and solid-state NMR spectroscopy (147). Fast tumbling in the DPC micelle/Pa4 complex enabled us to obtain high-quality solution NMR data and to assign the majority of resonances for both the backbone and the side chains. We found that Pa4 adopts a bend-helix-bend-helix secondary structure motif, with an angle of ~122° between the two helical domains. The topological orientation of Pa4 in micelles was assessed by paramagnetic quenching experiments, establishing that Pa4 lies on the surface of the micelle.

This model is supported by solid-state NMR data in lipid membranes. Specifically, ^{13}C-^{15}N rotational echo double resonance experiments (REDOR) carried out in vesicles of different composition (DMPC, POPC and POPE:POPG 3:1 mixture) containing 3% Pa4 are consistent with peptide helical structure when embedded in membranes (206, 207). ^{2}H and ^{31}P NMR experiments performed on d_{31}-POPC multilamellar vesicles (MLVs) in the presence and absence of Pa4 revealed that upon peptide binding, disorder was increased both in headgroups and in acyl chains located in the hydrophobic core of the lipid membrane (197). Interestingly, in DMPC, the C-terminal helix of Pa4 adopts a transmembrane orientation, while in POPC this domain is oriented with the helical axis approximately parallel to the bilayer normal. The latter suggest that membrane composition plays a pivotal role in the mechanism of action of Pa4. Taken together, the data from both solution and solid state NMR corroborate the hypothesized “barrel stave” mechanism of action of pardaxin, with the N-terminal domain involved in insertion into the bilayer, and the C-terminal helical portion involved in putative ion-channel formation.

4.3 Distinctin

This 47-residue peptide extracted from *Phyllomedusa distincta,* a tree frog from the Brazilian forests, interacts with negatively charged membranes and is active against both Gram-positive and Gram-negative bacteria. The primary sequence of distinctin comprises two linear chains of 22 (chain 1) and 25 (chain 2) residues, linked by a disulfide bridge between Cys19 of chain 1 and Cys23 of chain 2 (208–210). Unlike other antimicrobial peptides, distinctin adopts a well-folded conformation in aqueous environment, with a non-covalent parallel four-helical bundle that confers upon this peptide stability against proteolysis (211). Scaloni and co-workers also showed that distinctin forms voltage-dependent ion channels in POPC/POPE planar bilayers (211), which were modeled as

pentameric pores using molecular dynamic calculations (212). The mechanisms of pore formation and membrane permeabilization have also been investigated using electrochemical measurements with two different mercury-supported biomimetic membranes, i.e., a self assembled monolayer (SAM) and a tethered bilayer lipid membrane (tBLM). In SAM, distinctin forms selective ion channels for Tl^+ and Cd^{2+} ions; while the formation of K^+ permeable ion channels in tBLM occurred only at non-physiological potentials (209).

Using a combination of site-specific ^{15}N and ^{2}H NMR spectroscopy, Bechinger and co-workers showed that distinctin's helical domains orient approximately parallel to the surface of mechanically oriented POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) lipid bilayers. In a parallel study, we confirmed Bechinger's findings and showed that distinctin interacts more strongly with membranes containing charged lipid headgroups (PA, PS, and PG). We also discovered that both chains of distinctin adopt an approximately parallel orientation with respect to the membrane plane, with a slight angle between chains 1 and 2. However, in 1:1 POPC:DOPA lipid bilayers and 50:1 lipid to protein molar ratio, distinctin is unable to disrupt lipid bilayers. The chemical shift anisotropy and dipolar couplings from separated local field experiments were implemented in a hybrid simulated annealing protocol (213) to determine the orientation of the distinctin heterodimer more quantitatively. After energy minimization, the tilt angles with respect to the bilayer plane were calculated to be ~24° and ~5° for chains 1 and 2, respectively (129).

Our findings in mechanically oriented bilayers are supported by experiments carried out on distinctin reconstituted in DMPC/DHPC bicelles. Under these experimental conditions, distinctin adopts a dual topology for chain 2, indicating that the peptide can be either absorbed on the surface of the bilayer or exist in a trans-membrane topology (Verardi *et al.* in preparation). These new findings support the electrophysiological data, and explain how distinctin forms ion-conducting pores. In all, the data suggest that cell disruption may occur via formation in solution of stable tetramers that dissociate into monomers upon membrane interaction. Following an increase of concentration of the peptide on the membrane surface, distinctin organizes into ion-conducting pores. The propensity of distinctin to assume a transmembrane orientation even at low concentrations may suggest that stochastic formation of transient pores is also possible.

4.4 Cathelicidins

This well-known family of structurally different antimicrobial peptides (37, 44, 214, 215) includes peptides that comprise an N-terminal signal peptide that shares a highly conserved cathelin-like domain and a variable cationic C-terminal domain, which is responsible for the antimicrobial activity. LL-37 is the only cathelicidin-derived peptide found in humans (LL-37-hCAP18) (44, 216–218). LL-37-hCAP18 is expressed in epythelia, monocytes, and lymphocytes. During infection, inflammatory processes, or wound healing a 100-residues pro-peptide is expressed, containing the signal peptide, the cathelin domains, and the 37-residue mature antimicrobial peptide located at the C-terminal region (37, 214, 217, 219). LL-37 has a broad-spectrum of bactericidal activity, may play a role in cystic fibrosis remediation, and has been found to inhibit HIV-1 infection in vitro (37, 220, 221).

Using DPC micelles, we were able to obtain high-resolution spectra of the mature LL-37 peptide (38). The overall structure resembles that of Pa4, with a helix-break-helix motif and an angle between the two helical domains of ~120°. The N-terminal helix ends with a break at K12, and is more dynamic than the C-terminal helix (residues 13-33) and ends with a break at K12 (Figure 2). The kink starting at residue 12 may be due to a hydrophobic cluster of residues located in the concave face of the peptide (I13, F17 and I20) and appears to be facilitated by a groove created by G14. Dynamic light scattering measurements show that addition of LL-37 to DPC micelles does not change the overall organization of micelles, which display an average hydrodynamic radius of 24 ± 2 and 26 ± 2 Å in the absence and presence of LL-37, respectively. The latter result suggests that LL-37 associates on the micelle surface without changing the overall micellar shape. These data were supported by paramagnetic quenching experiments carried out with Gd^{3+} and by the detection of several peptide-to-micelle NOEs. The overall topology for LL-37 is common to other natural occurring helical AMPs, with the hydrophilic residues pointing toward the bulk solvent and the hydrophobic toward the inner hydrocarbon core of the micelles. The structural topology of LL-37 in micelle is reported in Figure 2. The peptide concave face containing the hydrophobic cluster points toward the micelle interior with the hydrophilic residues pointing outward. The helix-kink-helix motif recurs in other AMPs (*i.e.*, pardaxin), as already discussed. It has been hypothesized that the hinge region is required in order to confer structural flexibility for membrane insertion and pore formation (200, 202, 222, 223). The large curvature present in LL-37 has also been observed in the magainin-derived peptides (MSI-78 and MSI-594) as well as in other lysine-rich peptides (57, 95, 224). On the other hand, this curvature may be artificial and due to the interaction between the peptides and curved surfaces of the micelles. As described earlier in this article, micellar systems represent only a coarse approximation of membrane bilayers. Synthetic lipid bilayers such as vesicles or planar bilayers are preferable for testing lipid/peptide interactions. Despite structural similarities between LL-37 and pardaxins, their mechanisms of action appear to be different. Specifically, pardaxins are thought to disrupt cell membranes via a “barrel stave” mechanism, while LL-37 operates according to a “carpet-like” mechanism. The latter conclusion is supported by our helical model, as well as solid-state NMR studies from Ramamoorthy’s laboratory (197, 225, 226). Therefore, structure is important to modulate function, but amino acid composition is an important component that helps dictate the mechanism of action.

5. Conclusions and perspectives

Antimicrobial peptides (AMPs) are emerging therapeutics with considerable potential. However, progress in the rational design of AMPs as powerful and selective drugs has been slow. A significant problem continues to be how to define the structural determinants for activities and specificities of action. Until this is understood, identification of the sequence features important for antimicrobial activity cannot be done on anything other than a trial-and-error basis. NMR is clearly playing a fundamental role in understanding the role of structure for AMPs, as reported in the recent literature both from our laboratory (129, 209) and others (52, 227–230). The essential interactions of AMPs with membranes preclude the use of x-ray crystallography to determine high-resolution structural information. However,

structure does not seem to be the only determining factor to account for how AMPs elicit their biological functions. Primary sequence (amino acid content and nature), interactions with lipids, and flexibility are important factors to define specificity. Perhaps a most neglected aspect of research on AMPs is the characterization of their structural flexibilities. This is an area for which NMR is expected to have a special niche, pending the development of robust recombinant methods that will offer affordable combinations of isotope-labeled materials for solution and solid-state NMR.

Our working hypothesis is that AMPs are metamorphic polypeptides, capable of adopting different shapes. Thus, special structure may not be important *per se*. Instead, we suggest that the biological activities of AMPs may be encoded in their structural flexibility an ability to adopt several conformations and topologies upon homotropic (peptide-peptide) or heterotropic (peptide-lipid) interactions.

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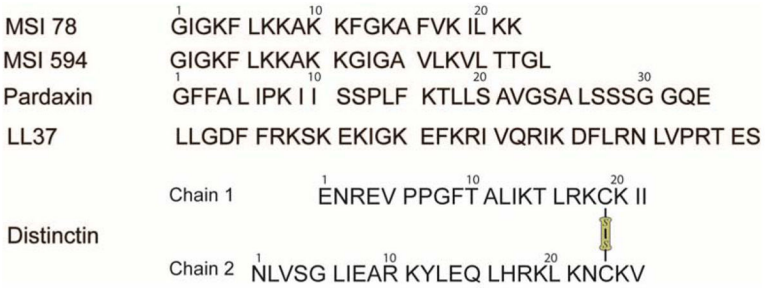

Figure 1.
Primary sequences of the helical AMPs studied by our groups.

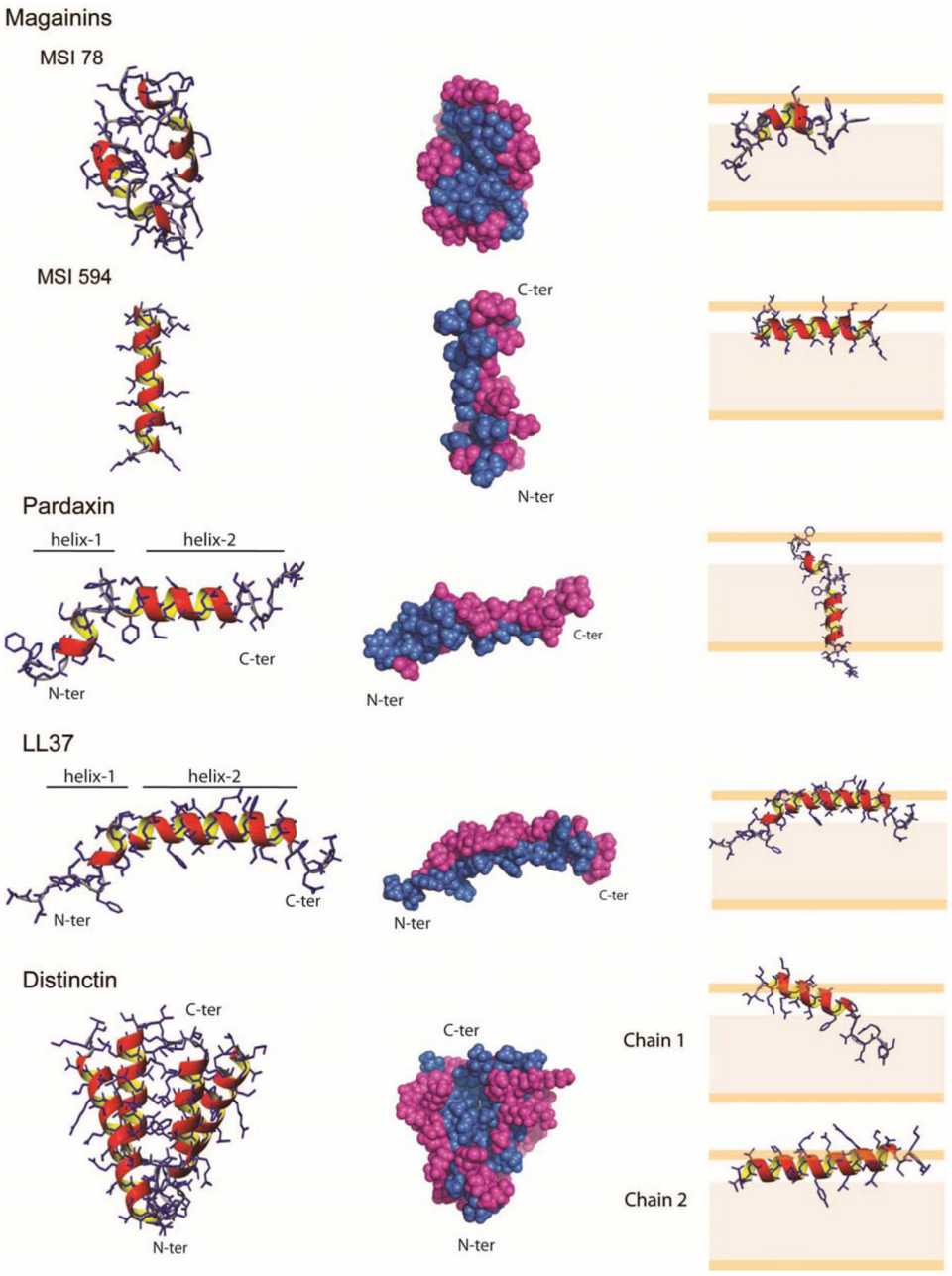

Figure 2.

Structures and membrane orientations (topology) of the helical AMPs studied by our groups. Note that the structures of MSI-78, MSI-594, Pa4, and LL-37 were obtained in detergent micelles, while the oligomeric structure of distinctin was obtained by in aqueous buffer. Left: backbone and side chain average structures of the AMPs from the NMR structural ensembles. Center: space filled model of the structures with the hydrophilic residues colored purple and the hydrophobic in blue. Right: topology of the AMPs deduced from ssNMR experiments carried out in lipid membranes.