

Review

# Diversity of antimicrobial peptides and their mechanisms of action

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## Abstract

Antimicrobial peptides encompass a wide variety of structural motifs. Many peptides have  $\alpha$ -helical structures. The majority of these peptides are cationic and amphipathic but there are also hydrophobic  $\alpha$ -helical peptides which possess antimicrobial activity. In addition, some  $\beta$ -sheet peptides have antimicrobial activity and even antimicrobial  $\alpha$ -helical peptides which have been modified to possess a  $\beta$ -structure retain part of their antimicrobial activity. There are also antimicrobial peptides which are rich in a certain specific amino acid such as Trp or His. In addition, antimicrobial peptides exist with thio-ether rings, which are lipopeptides or which have macrocyclic Cys knots. In spite of the structural diversity, a common feature of the cationic antimicrobial peptides is that they all have an amphipathic structure which allows them to bind to the membrane interface. Indeed, most antimicrobial peptides interact with membranes and may be cytotoxic as a result of disturbance of the bacterial inner or outer membranes. Alternatively, a necessary but not sufficient property of these peptides may be to be able to pass through the membrane to reach a target inside the cell. The interaction of these peptides with biological membranes is not just a function of the peptide but is also modulated by the lipid components of the membrane. It is not likely that this diverse group of peptides has a single mechanism of action, but interaction of the peptides with membranes is an important requirement for most, if not all, antimicrobial peptides. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Cytotoxic peptide; Peptide-lipid interaction; Membrane permeability; Peptide conformation; Lipopolysaccharide

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## Contents

1. Introduction . . . . .	12
2. Amphipathic and hydrophobic $\alpha$ -helices . . . . .	13
3. $\beta$ -Sheet peptides and small proteins . . . . .	14
4. Peptides with irregular amino acid composition . . . . .	16
5. Peptides with thio-ether rings . . . . .	16
6. Peptaibols . . . . .	17
7. Macrocyclic cystine knot peptides . . . . .	17

8. Role of membrane lipids . . . . .	18
9. The potential role of lipopolysaccharide (LPS) and the bacterial outer membrane . . . . .	18
10. Alternative mechanisms of action . . . . .	20
11. Biosynthesis of antimicrobial peptides . . . . .	21
12. Spectroscopic techniques to study peptide membrane interactions . . . . .	23
13. Summary . . . . .	25
Acknowledgements . . . . .	25
References . . . . .	25

## 1. Introduction

Organisms from throughout the phylogenetic tree, including animals [1], produce substances for protection against microbes. Many of these substances are peptides. With the growing problem of pathogenic organisms which are resistant to conventional antibiotics, there is increased interest in the pharmacological application of antimicrobial peptides to treat infection. Efforts are currently underway to increase the potency and specificity of these peptides so that they are toxic to microbes and not to mammals. In order to achieve this in an efficient manner, it is important to understand the mechanism of action of these agents and the reason for their selectivity against microbes.

Even limiting consideration of antimicrobial agents to peptides, there is still a large variety of structures known. This makes the task for designing improved agents more complex, but at the same time, it provides a range of opportunities for further development. The classification of antimicrobial peptides is somewhat arbitrary and there exist analogs with similar sequences but different conformational motifs which would fall into different classes, despite the similarity of their chemical structure and possibly also of their mechanism of action. Nevertheless, to simplify the problem and to illustrate the range of structures of peptides with antimicrobial activities, we have divided these peptides into groups. These groups include linear peptides which form amphipathic and hydrophobic helices, cyclic peptides and small proteins which form  $\beta$ -sheet structures, pep-

tides with unique amino acid compositions, cyclic peptides with thio-ether groups in the ring, lipopeptides terminating in an amino alcohol and macrocyclic knotted peptides. We will discuss what is known about the structure and the mechanism of action of each of these classes of antimicrobial peptides individually.

In general, the mechanism of action of any of these agents is not very well established. For many of these peptides, there is evidence that one of the targets for the peptide is the lipid bilayer of the membrane. This is because these peptides can often increase the rate of leakage of the internal aqueous contents of liposomes. In addition, most of the antimicrobial peptides are cationic and their interaction with anionic phospholipids would provide a ready explanation for their specificity for bacterial membranes. In Gram-negative bacteria, both the outer leaflet of the plasma membrane as well as the outer membrane contain anionic molecules oriented towards the exterior of the cell. This is not the case for mammalian membranes. Hence, the cationic antimicrobial peptides will preferentially bind to the exposed negative charges of bacterial membranes, but not to the zwitterionic amphiphiles present in the extracellular monolayer of mammalian plasma membranes. This specificity for anionic membrane components is also mimicked in model liposome studies. There is uncertainty, however, about how these peptides perturb the membrane and whether this membrane perturbation is related to the antimicrobial activity of these peptides. It has recently been shown that there is not always a correlation between the

ability of peptides to permeabilize membranes and their antimicrobial activity [2]. It is possible that the membrane effects of these peptides are not directly related to their mechanism of cytotoxic action but rather simply the manner by which they enter the cell to reach an alternative target [2].

With regard to the mechanism by which the peptide breaks down the membrane permeability barrier, it is possible that the peptide induces complete lysis of the organism by rupture of the membrane or that it perturbs the membrane lipid bilayer, which allows for leakage of certain cellular components as well as dissipating the electrical potential of the membrane. For example, the amphipathic helical peptide, cecropin, will dissipate a transmembrane electrochemical gradient at a low peptide concentration but requires a higher concentration to affect the release of an encapsulated fluorescent probe [3]. This peptide is cytotoxic to Gram-negative bacteria at low concentrations which dissipate ion gradients but which are not sufficient to cause the release of cytoplasmic contents [3]. Some of these possible mechanisms will be discussed in more detail below.

## 2. Amphipathic and hydrophobic $\alpha$ -helices

Perhaps one of the larger and better studied classes of bacteriostatic peptides are those that form cationic amphipathic helices [4]. However, there are also  $\alpha$ -helical peptides that are hydrophobic or even slightly anionic. Peptides which are not cationic exhibit less selectivity towards microbes compared with mammalian cells. An example of a well-studied hydrophobic and negatively charged cytotoxic peptide is alamethicin. This helical peptide forms clusters of helices that traverse the bilayer and surround an aqueous pore which can transport ions [5]. Another peptide that is hydrophobic and forms a helical transmembrane structure is gramicidin A. In membranes, it forms a cation-selective right-handed helix that traverses the membrane as a single-stranded head-to-head dimer [6] (Fig. 1). Both alamethicin and gramicidin are synthesized by microorganisms by a mechanism that does not involve ribosome synthesis. Since these peptides exhibit little selectivity for microbial membranes, their usefulness as specific pharmacological agents is limited. The majority of the cytotoxic am-

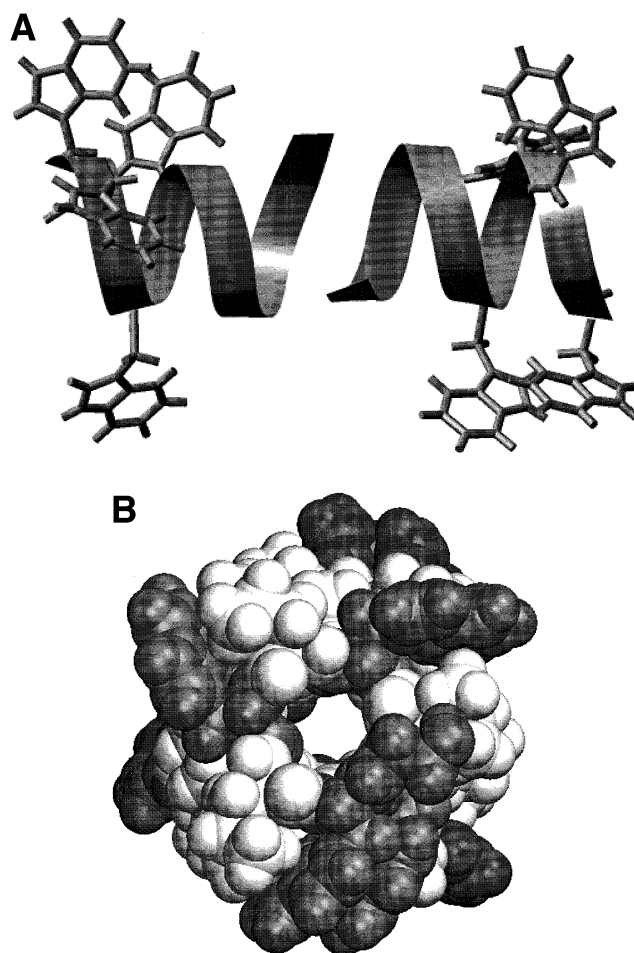


Fig. 1. Structure determined by solid state NMR for the peptide gramicidin A [6] in oriented membranes. A shows a peptide dimer traversing the membrane, with the four Trp side-chains anchoring the peptide to the membrane interface. B shows a space-filling structure, a 90° rotation compared to A reveals the hole down the center of the peptide (figures drawn with the program SETOR [152] from the protein database (PDB) entry IMAG).

phipathic helical peptides are cationic and they do exhibit selective toxicity for microbes.

One of the most studied of the cationic, antimicrobial, amphipathic helical peptides is magainin. This 23 amino acid peptide is secreted on the skin of the African clawed frog, *Xenopus laevis* [7]. The properties of this peptide have recently been reviewed [8,9]. From these studies, the concept of a lytic pore has developed. This pore differs in a number of respects from the type of pore formed by helical clusters of peptides, such as that of alamethicin. In the case of

magainin, the pore is larger and it does not have discrete open and closed states as conducting pores do. At the same time, the formation of this kind of pore does not result in complete lysis of the membrane. For example, magainin does not allow for the passage of trypsin, a protein of 24 kDa [10]. In addition, unlike the alamethicin pore which is lined with only peptide, the wall of the pore formed with magainin contains both lipid and peptide. Evidence for this comes from the fact that magainin stimulates the transbilayer movement of both peptide and lipid. The promotion of positive membrane bilayer curvature would be expected to facilitate the formation of a peptide- and lipid-lined pore and this is what has been found with magainin [11,12]. However, it has also been observed that the model peptide 18L, which has a consensus sequence for lytic peptides, promotes negative curvature, as does the wasp venom peptide mastoparan [13]. The cyclic peptide, gramicidin S, promotes the formation of structures which give rise to isotropic  $^{31}\text{P}$  NMR spectra, which may include the formation of inverted cubic phases [14]. At least for zwitterionic membranes, leakage caused by peptides that promote negative curvature is more rapid with bilayers having a negative curvature strain [15]. Current models of the large membrane pores formed by some antimicrobial peptides propose that the pore is lined with both peptide and lipid. The phospholipid in these pores would have a positive curvature and therefore, peptides that facilitated formation of structures with this curvature would facilitate pore formation. However, the mechanism by which peptides promoting negative curvature induce leakage is less well established. It could simply be explained by the pore which is formed being of a small diameter. The dependence of the curvature of a toroidal pore on its diameter is a consequence of the fact that there are two curvatures to consider. One is along the bilayer normal, which predominates in large pores, and the other is in the plane of the bilayer, which is of opposite sign and which predominates in small pores.

The importance of the amphipathic helical conformation (Fig. 2) to the cytotoxic activity of magainin was indicated by the finding that replacement of several amino acid residues in the peptide by their D-isomers resulted in loss of helicity and of antimicrobial activity [16]. However, the importance of an

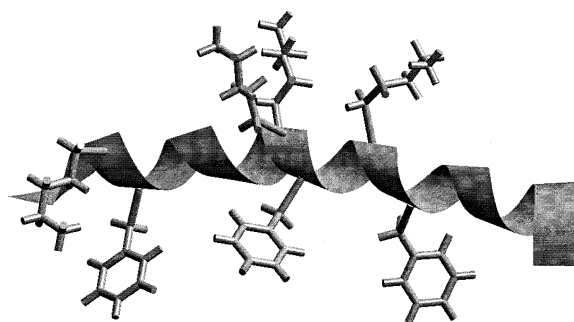


Fig. 2. Structure of the antimicrobial frog skin peptide magainin as determined by NMR spectroscopy in the presence of SDS micelles [125]. The structure was generated using the program SETOR [152] from the protein database entry 2MAG. The side-chains of Lys and Phe residues are indicated, illustrating the cationic amphipathic nature of the structure.

amphipathic helical conformation to the cytotoxic action of peptides was questioned in similar studies with the peptide pardaxin. Pardaxin is an amphipathic helical peptide that shows lytic activity with both microbial and mammalian cells. Incorporation of some D-amino acid residues into pardaxin converts the peptide conformation from an  $\alpha$ -helix to a  $\beta$ -structure [17]. The modified pardaxin with  $\beta$ -structure lost hemolytic activity but retained antimicrobial activity [17]. Thus, the conformational property of an amphipathic  $\alpha$ -helix is not required for the antimicrobial activity.

### 3. $\beta$ -Sheet peptides and small proteins

Helical peptides can exist in a membrane environment as a monomer with the hydrogen-bonding capacity of the amide groups fulfilled. In order for this to occur with  $\beta$ -structure peptides, a monomer must aggregate or form a bend to allow for an intramolecular anti-parallel  $\beta$ -structure to form. The latter situation is not likely to occur with small peptides because the loss in entropy would not be compensated by the favorable bonding interactions that would result. In addition, such a monomeric  $\beta$ -structure with only two interacting peptide segments would still have half the hydrogen-bonding potential of the amide groups unfulfilled. Many of the antimicrobial peptides which form a  $\beta$ -structure are able to do so because they are cyclical peptides and hence,

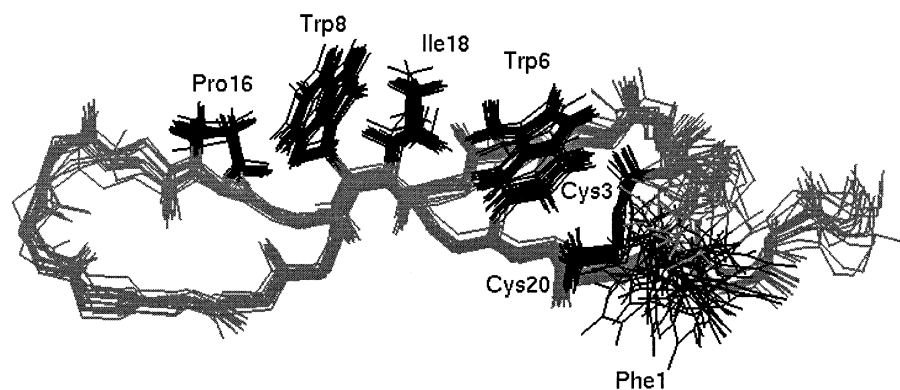


Fig. 3. The 20 best structures determined by proton NMR for the irregular  $\beta$ -sheet structure of lactoferricin B in aqueous solution [105]. The Trp, Phe, Ile and Pro residues, form the hydrophobic face of the amphipathic structure. The Lys and Arg sidechains on the opposite face of the structure (not shown) make up the positively charged surface. The position of the single disulfide bond is also indicated. These structures were generated using INSIGHTII (Molecular Simulations), from the protein database entry 1LFC.

there is less entropy loss on formation of a  $\beta$ -structure. The ring structure is formed either by disulfide bonds, as in the case of the tachyplesins, protegrins and lactoferricin (Fig. 3), or by cyclization of the peptide backbone, as in the case of gramicidin S, polymyxin B or the tyrocidines. The interaction of gramicidin S with membranes is reviewed elsewhere in this issue. Less is known about the mechanism by which this class of antimicrobial peptides produces membrane damage. A study of the orientation of protegrin in membranes using oriented circular dichroism has demonstrated that there are two different states of insertion of the peptide into a membrane that depend on the peptide concentration, on the nature of the lipid and on the extent of hydration [18]. The peptide tachyplesin, which has a cyclic anti-parallel  $\beta$ -sheet structure held together by two disulfide bonds, permeabilizes both bacterial and artificial lipid membranes. It also translocates across lipid bilayers and this translocation is coupled with transient pore formation [19], suggesting that it acts on the cytoplasmic membrane in a similar manner as the  $\alpha$ -helical magainin. The critical parameter associated with the antimicrobial action of this peptide appears to be the maintenance of a certain hydrophilic-hydrophobic balance [20].

The effect of substitution of D-amino acid residues in a cyclic  $\beta$ -structure peptide, based on the sequence of gramicidin S, was determined [21]. The  $\beta$ -structure conformation adopted by this peptide in aqueous solution was disrupted by the substitution of D-amino acid residues. The amphipathic nature of the pep-

tide analogues was assessed by their retention time in reversed phase high performance liquid chromatography. Peptides with a high amphipathicity exhibited high hemolytic activity but low antimicrobial activity. Those with a low amphipathicity, past a certain threshold, exhibited higher antimicrobial activity. Improvement in the therapeutic index as much as 10000-fold was noted for some of these analogues [21]. These results suggest that optimizing peptide incorporation into the membrane may not always produce the most effective antimicrobial agents, but that the interaction of the positive charges on the peptide with the negative charges on the bacterial membranes is important for antimicrobial activity. This is similar to the findings with paradaxin quoted above [17].

Defensins are a group of small proteins of about 50 amino acids that have a potent antimicrobial activity. They are produced and stored in the granules of neutrophils, where they form a part of the innate immune system. They have been described in mammals, insects and plant tissues. In the latter case, they are commonly referred to as thionines. They have a high number of disulfide bonds that help to maintain their structural integrity. The structures of several of these small proteins have been solved and all possess a  $\beta$ -sheet structure. The plant and insect defensins also contain an additional  $\alpha$ -helical structure motif (see Fig. 4). All defensins have amphipathic structures that probably allow them to act on bacterial membranes in a similar manner as the shorter amphipathic peptides [22].

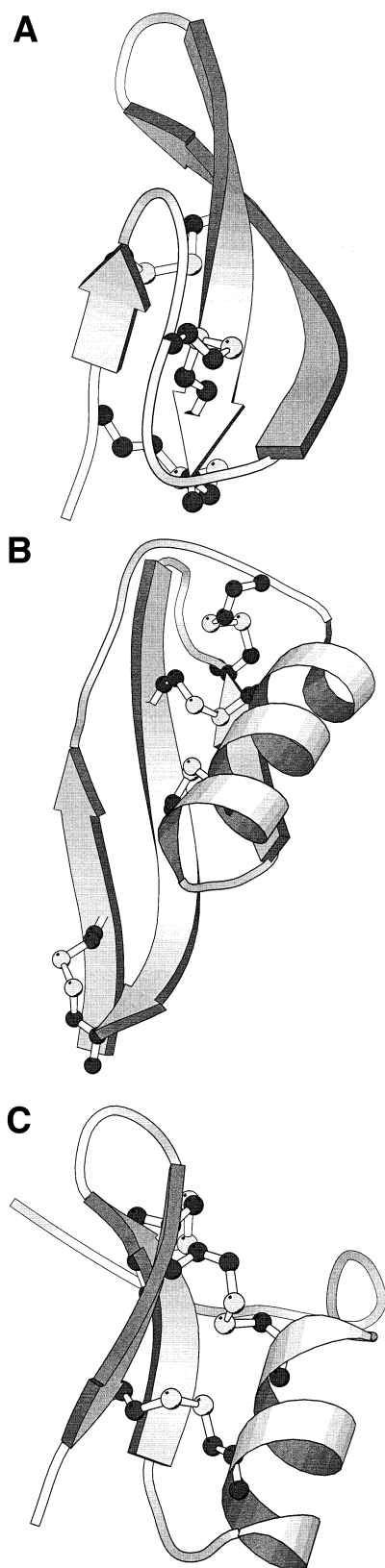


Fig. 4. Structures determined for several defensins ribbon diagrams are shown, with disulfide linkages in ball and stick representations. (A) Defensin HNP-3, (B) purothionine, (C) insect defensin A (PDB: 1ICA). This figure is taken from [117] with permission from NRC Press.

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#### 4. Peptides with irregular amino acid composition

Certain antimicrobial peptides have an unusual amino acid composition, having a sequence which is rich in one or more specific amino acids. For example, the peptide histatin, which is produced in saliva, is extremely rich in His residues [23–25]. Other peptides are produced by porcine neutrophils which are very rich in proline and arginine or proline and phenylalanine. These are called PR-39 and propeninin, respectively, and they are part of the cathelicidin family of antimicrobial peptides (see below).

Tryptophan is generally not an abundant amino acid residue in peptides or proteins. This amino acid is of particular interest with regard to the partitioning of peptides into membranes because of its propensity to position itself near the membrane/water interface [26,27]. Examples of antimicrobial peptides which are rich in Trp include tritrypticin (VRRFPWWPFLRR) [28] and indolicidin (ILPWKWPWPWRR-amide) [29]. Indolicidin appears to permeabilize bacteria without lysing them [30] and this peptide can form conductance channels [31]. The formation of conductance channels suggests that indolicidin can self-associate and that the hemolytic activity of this peptide is associated with the concentration required for its self-association [32].

#### 5. Peptides with thio-ether rings

There are several peptides made by bacteria which contain small ring structures enclosed by a thio-ether bond. This group of peptides is called lantibiotics and their structure and properties have recently been reviewed [33,34]. One of the lantibiotics, nisin, is currently used as an antimicrobial agent for food preservation. The properties of this peptide are reviewed in this issue [35]. It has been found that nisin rapidly induces leakage of ions and small metabolites from bacteria [36] and opens electrical conductance channels in black lipid membranes [37,38]. The mo-

lecular details of the mechanism by which nisin breaks down the membrane permeability barrier is not well understood [39]. However, there have been a number of studies which indicate the groups which are important for the antimicrobial action of this peptide and for the insertion of this peptide into a membrane. Unlike most antimicrobial peptides, which discriminate among lipids largely on the basis of charge interactions, certain lantibiotic peptides, such as cinnamycin, have specificity for phosphatidylethanolamine [40]. Electrostatic interactions are important, however, for the initial binding of nisin to membranes containing anionic lipids [41]. The positively charged C-terminal domain of the peptide is important for this binding. This conclusion is supported by experiments on the fluorescence properties of Trp substituted in this region of the molecule [42,43]. Other regions of the peptide are also inserted into the membrane, suggesting an initial parallel orientation of the peptide to the membrane surface [43]. This is also supported by NMR evidence, suggesting that the peptide remains at the surface of dodecylphosphocholine micelles [44]. However, results of experiments with trypsin entrapped within vesicles suggest that there is a transient insertion of the C-terminal region of the peptide [45]. It was found that the pH dependence of this insertion paralleled the permeabilization of the membrane, suggesting that a membrane pore was formed as a consequence of the insertion of this region of the peptide into a membrane [45]. Conductivity studies indicate that the insertion of the peptide into the membrane follows surface association [46]. It has also been observed that a higher pH leads to higher leakage rates of liposomal contents as a result of a small number of nisin molecules being required to induce leakage [47]. This may be a consequence of less charge on the peptide at a high pH, facilitating greater self-association. In this and other cases, there may be a difference between the position of the peptide in a membrane in its most stably inserted form and the arrangement which is responsible for its membrane activity.

## 6. Peptaibols

These peptides are characterized by having a high

proportion of  $\alpha$ -amino-isobutyric acid (Aib) residues. These residues are conformationally restricted and favor the formation of  $\alpha$ -helical and in particular,  $3_{10}$  helical structures, even in the case of the solution conformation of the short antimicrobial peptide trichogin [48]. In addition, these peptides are lipopeptides which are acylated at the N-terminus, thus favoring their partitioning into membranes. (Attachment of short fatty acid chains occurs more frequently in peptides produced by microorganisms: note that polymyxin B is also acylated.) This group of peptides derives their name of 'peptaibols' from the fact that they have a 1,2-amino alcohol at the C-terminus. There have been many studies on the peptaibol alamethicin, which forms membrane channels through self-association into a ring of transmembrane helices. Another, less studied peptaibol is trichogin GA IV, which has the sequence *N*-octanoyl-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Lol. The membrane is a likely site of action of trichogin, since this lipopeptide is sparsely soluble in water, contains an octanoyl anchor and is devoid of charged groups. Two of the ways this lipopeptide can insert into membranes are with the helix axis either largely perpendicular or largely parallel to the plane of the bilayer. Channel-forming cytotoxic peptides, such as alamethicin, form clusters of helices parallel to the bilayer normal. This is not a likely motif for the interaction of trichogin with membranes. X-ray diffraction studies of trichogin crystals show that the length of the helix is only about 16 Å [49]. Trichogin is thus too short and would not span a membrane bilayer in a helical conformation. However, there may be a transient, higher energy state of the peptide which is more deeply inserted into the membrane and which causes leakage.

## 7. Macrocyclic cystine knot peptides

As indicated before, several antimicrobial peptides have a cyclic peptide structure, with usually less than 15 amino acids involved. Recently, four macrocyclic end-to-end 30 amino acid residue cyclic peptides from plants of the *Rubiaceae* family were found to possess potent antimicrobial activity [50]. These four proteins (kalata, circulin A and B and cyclopsycho-tride) also have a cystine knot motif, where the knot-

ted motif arises because one disulfide bond is threaded through the other two. This structural motif is also found in protease inhibitors and toxins and, together with the cyclic backbone, confers a high rigidity to the structure. Structures for two of these proteins have been reported [51,52]. Circulin A and B have been shown to have anti-viral activity as well [53] and may play a role as an anti-HIV drug.

## 8. Role of membrane lipids

Biological membranes contain a large variety of lipids, hence most of our knowledge of the roles of specific lipid properties comes from studies of model membranes. One property of these membranes that has been associated with antimicrobial specificity is their negative charge. Several antimicrobial peptides are cationic and preferentially bind to anionic lipids. This can provide a potential mechanism for microbial specificity, since most of the anionic lipids of mammalian membranes are sequestered on the cytoplasmic side of the membrane while they are exposed to the external medium with microbial membranes. In addition to the aspect of microbial specificity, it is possible that the interaction of cationic peptides with membranes causes a preferential sequestering of the anionic lipids, leading to less regularly packed boundaries between domains rich in anionic lipids and the remainder of the membrane. Studies with fluorescence-labelled lipids demonstrated that a model cationic peptide can attract small clusters of anionic lipid [54], although it is unclear at present whether this clustering phenomenon plays a role in their antibacterial effects. It has been shown that liposomal leakage induced by magainin is very sensitive to the nature of the anionic lipid [11]. It is interesting that this peptide is much more effective in inducing leakage in liposomes of phosphatidylglycerol, a lipid with a high abundance in microbial membranes, than in liposomes of phosphatidylserine, the major anionic lipid of mammalian membranes. These findings also suggest that other properties, in addition to membrane charge, determine the rate of leakage caused by these peptides. One of these factors is membrane curvature [11,15,55]. However, knowledge of the effects of antimicrobial peptides on curvature is not sufficient to predict the potency or antimicrobial spe-

cificity of these peptides, since peptides that increase positive membrane curvature as well as those that increase negative curvature can be lytic. However, membranes with intrinsic negative curvature tend to be lysed more readily by peptides that promote additional negative curvature [55].

## 9. The potential role of lipopolysaccharide (LPS) and the bacterial outer membrane

All Gram-negative bacteria have LPS in the outer leaflet of their outer membrane. LPS can be subdivided in three specific regions, the O-antigen, the core and lipid A. Both O-antigen and the core con-

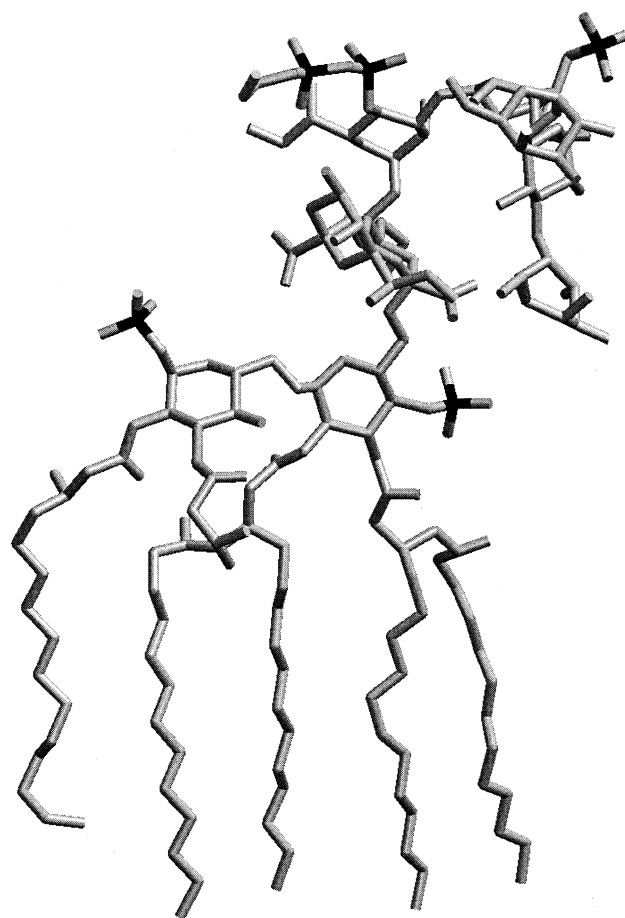


Fig. 5. Structure of a LPS molecule, as determined by X-ray crystallography. A single LPS molecule was found non-covalently bound to the outer membrane ferrichrome transporter Fhu A in its crystal structure [153].



sist of polysaccharide chains and protrude outwards from the membrane surface. The major membrane component, lipid A, is a complex molecule consisting primarily of fatty acids and phosphate groups bonded to a carbohydrate backbone (see Fig. 5). The charge on the lipid A molecules is negative and the outer membrane leaflet structure is held together in part by the binding of divalent cations. Because electrostatic interactions are effective over relatively long distances, the charges on the bacterial Gram-negative outer membrane make it an ideal target for binding cationic peptides. Many studies, including theoretical quantum chemical calculations [56], have shown that Arg side-chains and phosphate groups in particular can form relatively stable interactions.

Several studies of the interactions between cationic antimicrobial peptides, such as magainin, cecropin and polymyxin, and LPS have been reported. In some of the earliest work, a correlation between the affinity of magainin for mutant strains of *Salmonella typhimurium* and the depth of lesions in the rough LPS was shown [57]. Also, the diphosphoryl moiety of lipid A was shown to play a major role in the binding of cecropin [58]. Moreover, it was demonstrated by infrared spectroscopy that binding of magainin can lead to disorder in the LPS [59]. More recently, similar results have been reported for polymyxin binding to purified LPS [60,61] and for magainin binding to defined vesicles containing LPS [62], as well as LPS derived from *Yersinia enterocolitica* [63]. Based on these results, it seems likely that the binding of cationic antimicrobial peptides to the outer membrane could play a major role in their toxicity towards the microorganisms. However, heterogeneity amongst various LPS preparations makes it difficult to directly compare the various studies performed to date. Better defined and standardized preparations would allow for more detailed biophysical studies of these interactions, which would lead to a deeper understanding of the role of LPS. For example, it has been suggested that cationic peptides could displace the divalent cations, hence leading to destabilization of the outer membrane. To date, direct evidence for such a mode of action is lacking, however. In some studies, serious disruption of the outer membrane (e.g. membrane blebbing) has been observed by electron microscopy [64–67] upon addi-

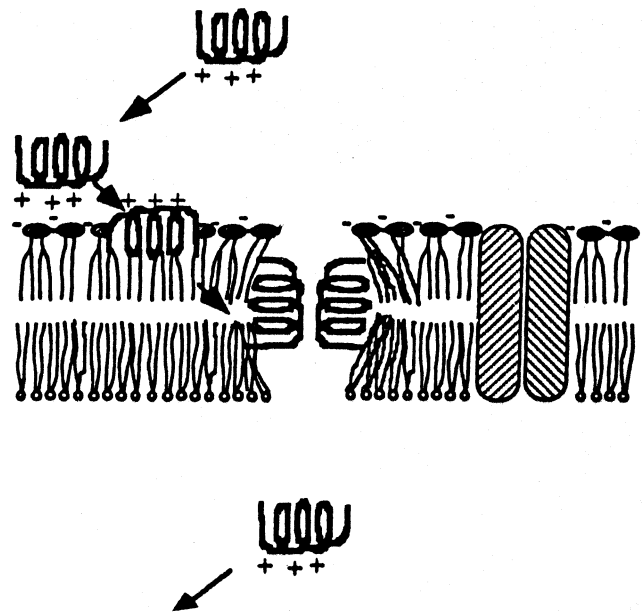


Fig. 6. Mechanism proposed for the transport of antimicrobial peptides across the outer membrane of Gram-negative bacteria. The initial recognition involves the negatively charged LPS on the outer leaflet and the cationic peptide. Once entered into the periplasmic space, the peptide can move to the cytoplasmic membrane (adapted from [68]).

tion of cationic peptides such as polymyxin B, suggesting that this may be sufficient to explain the antibacterial action. However, not all antimicrobial peptides appear to have such a dramatic effect on the morphology of the outer membrane (see below). Be that as it may, even if the direct bacteriostatic action of a cationic antimicrobial peptide is at the level of the cytoplasmic membrane, peptides would still have to find a way to cross the outer membrane to gain access to the periplasmic space, from where they can act on the cytoplasmic membrane. Given the limited size of the opening in outer membrane porin proteins, the most likely transfer mechanism across the outer membrane involves direct binding to LPS and subsequent transfer to the periplasm [68,69] (Fig. 6).

In several molecular genetics studies, *S. typhimurium* mutants have been uncovered that have an altered sensitivity to cationic antimicrobial peptides. Increased resistance to polymyxin B was shown to correlate with extensive modification of LPS, in particular  $^{31}\text{P}$  NMR studies showed that modification of the phosphate groups of LPS occurred, which gave it a reduced negative charge [70–73]. Additional resist-

ant mutants have been described where the level of acylation of lipid A was reduced and it was suggested that this could block transfer of peptide across the outer membrane [68,74]. These results again underscore the importance of the lipid A portion of LPS in the bacteriostatic action of cationic antimicrobial peptides. They also show that it is possible to select for resistant bacteria, suggesting that, like commonly used antibiotics, resistance could eventually develop against some of the bacteriostatic peptides as well. It seems likely that binding to LPS plays a different role with distinct cationic peptides. Enterobacteria that had a defective outer membrane, and had become resistant to polymyxin B, were still completely susceptible to cecropin B, indicating that both peptides have different targets [75]. Likewise, defensins were also fully active in strains with defective outer membranes [76], suggesting that they do not act at the outer membrane but at other structures such as the cytoplasmic membrane.

Gram-positive bacteria such as *Staphylococcus aureus* do not have an outer membrane or LPS, but instead they possess a peptidoglycan as a component of the surface membrane which is rich in teichoic acid. Mutations leading to an increased negative surface charge in this structure also lead to an increased sensitivity towards cationic antimicrobial peptides [77], again illustrating the importance of electrostatic interactions in their mechanism of action.

Finally, it should be pointed out that clinically, LPS and in particular lipid A play an important role in the pathophysiology of Gram-negative bacterial sepsis which leads to endotoxic shock, often culminating in the death of the patient. In the USA alone, the annual mortality is approximately 100 000 patients, a significant problem. In principle, agents that can bind LPS which have been released from bacterial cell walls in the bloodstream can neutralize its toxic action. Indeed, vertebrates as well as invertebrates have proteins in their circulatory system which can bind to LPS and neutralize its endotoxin activity. The X-ray structures of some of these proteins have recently been reported [78,79]. Since various cationic antimicrobial peptides are known to bind to LPS, they may have a role in treating sepsis, providing yet another important rationale for future biophysical studies in this area [60,61].

## 10. Alternative mechanisms of action

Studies with synthetic all-D antimicrobial peptides are widely quoted as proof that many peptides act directly on the membrane lipid bilayer, rather than on a receptor protein. Indeed, the original studies with  $\alpha$ -helical peptides such as magainin, cecropin and melittin showed that the all-D peptides were equipotent to the naturally occurring all-L peptides [80–83], making it unlikely that a highly stereospecific target, such as a membrane-bound protein receptor or a cytoplasmic enzyme, would be required to mediate their bacteriostatic effects. However, subsequent studies with various other peptides have shown that all-L and all-D enantiomer peptides are frequently not of equal activity [82], moreover, the results appear to be strongly species dependent. For example, the ratio of lethal concentrations of L/D-isomers for the proline-arginine-rich PR-39 peptide was shown to be high for *Pseudomonas aeruginosa* (L/D = 66) and *S. aureus* (L/D > 1000), while no chiral selectivity was observed with *Escherichia coli* or *Bacillus subtilis* [82]. Also the enantiomers of the  $\alpha$ -helical porcine cecropin P peptide showed differences in the sensitivity with these bacterial species [82]. In the case of short 11 residue lactoferricin analogs, the activity of the all-D peptide was markedly increased over its naturally occurring analog when tested against various bacteria [84]. On the other hand, D-enantiomers of lysozyme-derived peptides were equipotent when tested against four bacterial strains, but a lot less active than the L-derivative with five others [85]. Perhaps, the different effects on these bacterial strains are related to differences in their membrane compositions. However, such results could also point towards a stereospecific complementarity between the peptide and a bacterial ‘receptor’ molecule, at least in certain bacterial species. The nature of such molecules has remained elusive to date. However, since many antimicrobial peptides seem to be capable of spontaneously traversing bacterial outer and inner membranes, the idea that they can act on an intracellular target has some merit. There have been suggestions made that DNA could be the target. Indeed, the basic N-terminal region of lactoferrin, which contains the antimicrobial peptide lactoferricin, has been shown to bind to specific regions of DNA, where it may act to regulate transcription [86,87]. For PR-39,

no lysis of *E. coli* has been observed, leading to the suggestion that DNA and protein synthesis may be terminated by this peptide [88]. In the case of the lactic acid bacterial peptide leucocin and many of its related peptides, the requirement for a membrane-bound receptor protein as one potential key molecular recognition event for antimicrobial activity has been discussed [89]. Hopefully, such proteins, if they exist, will soon be isolated, to help us to understand their role in the mechanism of action of antimicrobial peptides. Clearly, there could be different mechanisms of action for different peptides and the mode of action may, for a given peptide, also vary among bacterial species.

### 11. Biosynthesis of antimicrobial peptides

Antimicrobial peptides in multicellular organisms are found on external surfaces such as the skin or the lungs or they are sequestered in granules of neutrophils, from where they can be released to attack a pathogenic bacterium. Their synthesis is often induced in response to an infection [90]. As such, these peptides form an integral part of the innate immune response [91]. The large majority of antimicrobial peptides synthesized by multicellular organisms are encoded by the genome. They are produced through regular processes of gene transcription and ribosomal translation, often followed by further proteolytic processing of the gene product. Many antimicrobial peptides produced by microorganisms contain uncommon amino acids. Several of these are not ribosomally synthesized or they are ribosomally synthesized and subsequently modified. These are highly specific processes and we will not discuss the latter group any further (for reviews, see [92,93]).

Magainins are synthesized as a long preproprotein containing six copies of the peptide. Proteolytic processing leads to the release of the individual magainin peptides [6,94]. Likewise, the 35–37 residues insect cecropins are synthesized as preproproteins of 62 amino acids and processing involves several protease activities [95,96]. Moreover, in many cationic antimicrobial peptides such as the cecropins, the negative charge of the carboxyl terminus is removed by an amidation process [97].

An interesting group of antimicrobial peptides are

the cathelicidins [98]. These all have a conserved pro-region of about 100 amino acids resembling the protein cathelin, a member of the cysteine proteinase inhibitor superfamily. Cathelicidins are stored in the granules of leukocytes and they are released upon activation of the leukocyte. However, they have a remarkably different C-terminal antimicrobial domain, encoding either  $\beta$ -sheet proteins such as protegrin or proline-arginine-rich and proline-phenylalanine-rich antimicrobial proteins (PR-39 and propenin) or short 13 residue tryptophan-rich peptides (e.g. indolicidin and tritripticin) or  $\alpha$ -helical peptides (CAMP OR LL37). The bovine, porcine and ovine cathelicidins make up a multigene family. However, in humans, the group only appears to have one member, the  $\alpha$ -helical LL37 peptide [98–101]. The latter gene is also induced during inflammatory disorders [101].

Also the dermaseptins, a group of antimicrobial peptides found in the skin of a variety of frog species, constitute a family with a common preproregion and a highly variable carboxy-terminal antimicrobial domain. The peptides produced are either linear amphipathic  $\alpha$ -helices or disulfide-linked or short hydrophobic peptides [102]. Despite the diversity in the structures of the antimicrobial peptides, they all have very similar preproregions with a rather acidic proregion following a regular signal peptide. The high conservation of the precursor prepropart indicates that this region must have an important function [102], as has also been suggested for the cathelicidins [98].

More recently, it has become apparent that several cationic antimicrobial peptides can be released by cleavage of intact proteins that may have no or limited antibacterial activity themselves. This has been demonstrated first for the milk protein lactoferrin [103]. Proteolytic cleavage of the intact bovine protein by pepsin under acidic conditions releases a 25 residue peptide (lactoferricin B), which shows a markedly increased bacteriostatic potency compared to the intact protein [103]. This region of the protein contains many positively charged residues [104]. A similar but less dramatic effect is observed upon hydrolysis of the human lactoferrin protein [103]. It has been shown that the increase in antimicrobial potency of the peptide is probably related to a change in the secondary structure giving rise to an amphi-

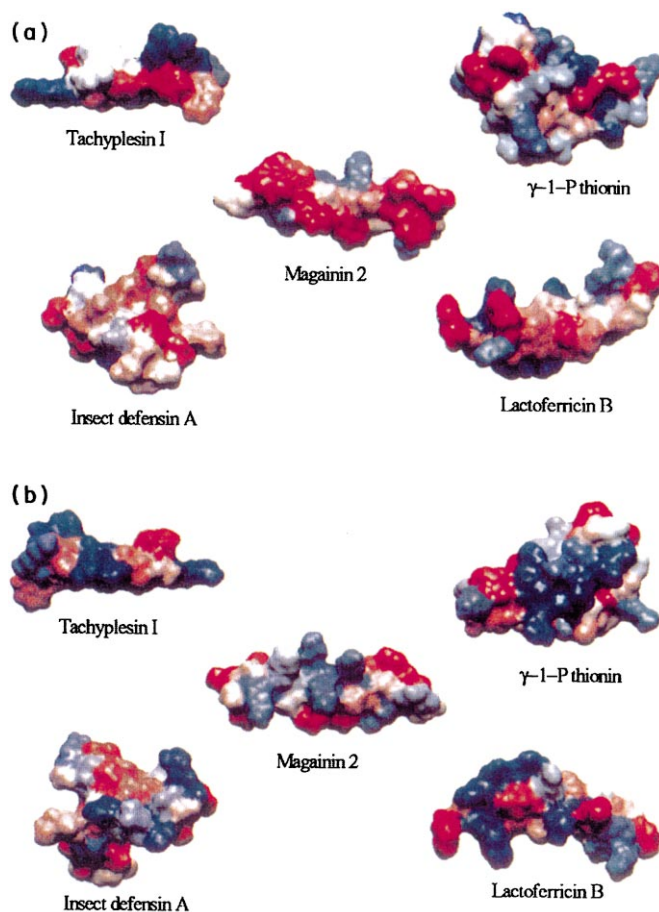


Fig. 7. Panel of structures showing the amphipathic nature of many antimicrobial peptides (reproduced with permission from NRC Press from [117]). Connolly surface diagrams of several different antimicrobial peptides. The peptides are colored according to a hydrophobicity scale: blue represents the least hydrophobic residues (charged residues), whereas red represents the most hydrophobic residues. (a) The hydrophobic peptide surfaces. (b) The cationic surfaces.

pathic structure (Fig. 3), that seems ideally suited for binding to the surface of negatively charged membrane surfaces [105]. The alteration in the secondary structure is rather dramatic, changing from a largely  $\alpha$ -helical structure in the protein to a distorted  $\beta$ -sheet in the peptide [105]. When the peptide binds to membrane mimetics, such as sodium dodecyl sulfate micelles, further changes occur in its structure [106,107]. Somewhat surprisingly, most of the antimicrobial activity of the lactoferrin-derived peptide resides in a short hexapeptide comprising three Arg and two Trp residues [108]. This result is consistent with the outcome of combinatorial chemistry studies of hexapeptides, which also revealed a high potency for Trp and Arg-rich hexapeptides [109]. Such short peptides could be of commercial interest and hence,

many short analogs of lactoferricin B have been synthesized and tested for activity [84,110].

Other examples of antimicrobial peptides derived from food proteins through proteolysis have been reported. For example, it has been shown that heat denaturation of hen egg white lysozyme generated an antimicrobial activity in the unfolded protein that was not related to its enzymatic activity [111]. Subsequently, the bactericidal domain was isolated as a peptide encompassing residues 98–112 of the intact protein [85]. Also, the related viral T<sub>4</sub> lysozyme has an amphipathic C-terminal region that appears to have membrane disrupting properties [112]. The cecropin-like antimicrobial peptides produced by *Helicobacter pylori*, the bacteria which colonizes the human stomach, were shown to be N-terminal

fragments of the ribosomal L1 protein [113]. Finally, it should be pointed out that the highly cationic antimicrobial peptides buforin I and parasin I are directly derived from the N-terminal domain of histone H<sub>2</sub>A, a protein normally associated with the nucleosome [114,115]. This, taken together with the fact that highly cationic nuclear protamines also bind and dissolve the bacterial outer membrane [116], may explain why in Japan, soft fish roe, a by-product of the fish industry, can effectively be used as an antimicrobial substance to help in the preservation of certain rice products.

## 12. Spectroscopic techniques to study peptide membrane interactions

Amongst the experimental techniques used to study the structure of antimicrobial peptides, high resolution NMR spectroscopy occupies a prominent position (for reviews, see [117,118]). With the exception of the defensins and thionines, for which the crystal structure can be solved by X-ray methods (e.g. [119]), the majority of the peptides are small and flexible and hence, they resist attempts at crystallization. Moreover, X-ray crystallography is generally not suitable for studying peptide-membrane interactions, although some progress is being made in this area [120]. Neutron diffraction can also be used to glean information about peptide-membrane interactions [121–123]. However, this technique does not yet provide high resolution structural information. This leaves NMR spectroscopy as a major technique that can be utilized to obtain insight into the three-dimensional structure of antimicrobial peptides in solution or bound to membrane mimetics (Fig. 7) [117,124]. Solid state NMR studies aimed at solving the secondary structure and the orientation of peptides with respect to oriented model membranes are described by B. Bechinger in this issue and will not be further discussed here.

The relatively small size of the antimicrobial peptides makes them in principle ideal targets for high resolution NMR studies. Generally, application of routine two-dimensional proton NMR methods [124], followed by structure calculations, is sufficient to provide insight into the structure. The NMR spectra are relatively simple and can be assigned using

proton total correlation experiments. Subsequently, as many nuclear Overhauser effect crosspeaks as possible are analyzed to provide distance restraints. These can be supplemented with restraints for bond angles as derived from NMR coupling constants. Generally, distance geometry or restrained molecular dynamics simulated annealing protocols are used to calculate the three-dimensional structure.

For many shorter peptides, it was shown that in an aqueous environment, these peptides have no defined structure. For example, typical amphipathic  $\alpha$ -helical peptides such as magainins and cecropins are unstructured in aqueous solution [125–127]. Similar results are obtained with bacterially synthesized peptides such as nisin and leucocin [128,89]. In contrast, small  $\beta$ -sheet peptides, such as tachyplesin, protegrin and lactoferricin B, seem to already form a more or less defined amphipathic sheet structure in aqueous solution. The presence of one or two disulfide linkages may help to stabilize these structures [129–131]. Other peptides, such as the proline-arginine-rich and proline-phenylalanine-rich peptides, have resisted detailed structural analysis by NMR to date. Their spectra suffer from serious spectral overlap because of their unusual amino acid compositions.

The structure of peptides in solution can be manipulated by adding organic co-solvents such as trifluoroethanol or hexafluoroisopropanol [132]. These agents induce a secondary structure in the peptide that is thought to resemble that of the peptide bound to the membrane [125,126]. Ideally, one would solve the structure of a membrane-bound peptide directly, but this is not possible with current solution NMR methods. Membranes move very slow on the time scale of the NMR experiment, leading to broad unresolvable resonances in the spectra. Therefore, it has been customary to resort to the use of small membrane mimetics, such as micelles, to study the structure of peptides bound to membranes [133,134]. Micelles are formed spontaneously by putting detergent-like molecules such as sodium dodecyl sulfate (SDS) or dodecyl phosphorylcholine (DPC) in aqueous solution. They are known to be able to induce a secondary structure in bound peptides [135]. They have a highly curved surface, making them not ideal substitutes for a relatively flat bilayer surface. Larger vesicles would be preferred, but these again would give NMR lines that are too broad to analyze. The

recently introduced bicelles may offer an alternative [136], but so far, no detailed studies of bicelle-bound antimicrobial peptides have been reported. Hence, the determination of all high resolution NMR structures of membrane-bound antimicrobial peptides has relied on the use of SDS and/or DPC micelles. Because of its overall negative charge, SDS may be a good mimetic for bacterial membranes, while DPC is related to the zwitterionic headgroups that make up the majority of the outer leaflet of mammalian membranes. To date, to our knowledge, no NMR studies of peptides bound to LPS have been reported. Given the importance of such interactions, as described above, more work in this area is warranted in the future.

It is important to recall that the structures of many antimicrobial peptides differ when bound to a membrane mimetic or in solution. In fact, many of them only acquire a well-defined three-dimensional structure upon binding to a membrane, including magainin, cecropins [125–126] or Trp-rich peptides such as indolicidin and tritrypticin (unpublished data). The membrane-bound structures are all clearly amphipathic with the hydrophobic amino acid side-chains sequestered into the hydrophobic fatty acid portion of the membrane, whereas the positively charged Arg and Lys side-chains can interact with negatively charged phosphodiester groups in the membrane interface region (Fig. 2). Uniformly, all studies show that all the cationic peptides bind in a parallel orientation to the membrane surface, which has implications for their mode of action. While some information can be obtained, it is difficult to define exactly the position of the peptide in the membrane. Solid state NMR REDOR measurements have been used to show that the peptide backbone amides of magainin are bound near the phospholipid head groups [137]. Also, nitroxide spin-labelled fatty acids have been used. By varying the position of the nitroxide along the fatty acid chain, one can place the nitroxide to a different depth in the membrane. This perturbs NMR spectra in a predictable manner, giving information on the position of the peptide [106,138]. Most studies indicate a surface location for bound peptides.

In addition to NMR, other lower resolution spectroscopic techniques, such as circular dichroism and Fourier transform infrared spectroscopy, are useful

in studying the secondary structures of antimicrobial peptides in solution and bound to membranes [139,140]. Circular dichroism is particularly useful to study the formation of an  $\alpha$ -helical structure, because the prominent negative ellipticity observed at 208 and 222 nm that is associated with this structure is easily observed. Infrared spectroscopy is often a better choice for  $\beta$ -sheet structures, as these give a signal that is generally more easy to distinguish from random coils than  $\alpha$ -helices. An advantage of these spectroscopic techniques, as compared to NMR, is that it is often possible to study more realistic membrane mimetics, such as vesicles. Clearly, a combined spectroscopic approach can provide the most detailed information.

Another magnetic resonance method that has long been used to study peptide-lipid interactions is electron paramagnetic resonance. For this type of system, this method requires the introduction of a paramagnetic species. This has generally been in the form of a stable, sterically hindered nitroxide covalently linked to one of the components of the lipid or peptide molecule. The first applications of this method have used nitroxide-labelled phospholipids to determine the number of lipids whose motional properties are perturbed by the presence of a protein or peptide [141]. The relative effects of peptides in ordering different locations along the acyl chain can be monitored by introducing spin labels at different positions in the acyl chain. Spin labels have also been introduced into specific residues in proteins and peptides. Often, the reactivity of cysteine residues has been used to achieve this specific labelling. Such studies have been applied to determine the partitioning of peptides between aqueous and lipid environments [142], as well as to obtain information about the nature of peptide and protein insertion into membranes [143,144].

Fluorescence spectroscopy can be used to study the insertion of intrinsic fluorophores such as tryptophan into membranes. Generally, a blue shift and an increase of the quantum yield is obtained when the Trp moves from a flexible, water-exposed position to a more restricted position in the hydrophobic region of the membrane [145]. Fluorescence quenching with molecules such as iodide can provide insight into the depth of Trp insertion into the membrane [145]. Also, the quenching effect of nitroxide-labelled phos-

pholipids on the Trp fluorescence can be used to gain some insight into the depth of insertion of the peptide [146,147]. Studies have shown that the Trp residues of antimicrobial peptides are generally bound close to the membrane head groups, in agreement with its role as a membrane interface anchor [26,27]. Kinetic information about the rate of the peptide binding process can also be obtained by fluorescence spectroscopy [148]. A technique that is gaining in prominence is surface plasmon resonance, which makes it possible to study complex formation and its on and off rates in one direct measurement [148].

Finally, it should be pointed out in this section that NMR spectroscopy and infrared spectroscopy both can be used to directly study the properties of the membrane lipid bilayer in the presence and absence of the peptide. Such studies may help us to gain an understanding of the changes in membrane stability that accompany the binding and transport of cationic antimicrobial peptides through the membrane.

### 13. Summary

There are a wide variety of peptides with different chemical structures and different peptide conformations which all exhibit antimicrobial activity. These peptides, however, have certain properties in common. They all have affinity for membrane lipids and their specificity for microbial membranes in many cases has been shown to be related to the positive charge on the peptide favoring interaction with the exposed anionic lipids of microorganisms.

The mechanism by which these peptides kill cells is less well understood in most cases. In particular, the role of the membrane lipid bilayer in the biological action of these peptides has become controversial. There are several ways in which membranes may be involved in the action of these peptides. The peptides may form pores in the membrane allowing for leakage of ions and other materials from the cell. Alternatively, some of these peptides, such as alamethicin and gramicidin A, may form specific ion channels through the membrane. In addition, the peptide may disrupt membranes by a ‘carpet-like’ mechanism, by which the association of the peptide

with lipid head groups destabilizes the membrane [149]. However, the breakdown of the permeability barrier in bacteria is not always closely correlated with cytotoxic activity [2]. There are also other membrane-dependent processes that may be involved in the action of some of these peptides, including the translocation of the cytotoxic peptide across the membrane to the cytoplasm or the facilitation of transbilayer lipid diffusion leading to loss of lipid asymmetry in the membrane. In addition, alternative mechanisms have been proposed to explain the cytotoxic action of these peptides, including their stimulation of autolytic enzymes [150], interference with DNA and/or protein synthesis [30,80] or their binding to DNA [151]. However, one would anticipate that many of these interactions would be similar in mammalian cells and in microbes. Therefore, the antimicrobial specificity of these peptides must come from another source, such as differences in membrane interactions resulting in different rates of transport of the peptides into cells. Given the diversity of antimicrobial peptides and of target microorganisms, it is not likely that there is one unique mechanism for their mode of action. It would appear that their interaction with membranes is an important feature of these agents.

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