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# Peptide–membrane interactions and mechanisms of membrane destruction by amphipathic α-helical antimicrobial peptides

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**Abstract**

Antimicrobial peptides (AMPs) have received considerable interest as a source of new antibiotics with the potential for treatment of multiple-drug resistant infections. An important class of AMPs is composed of linear, cationic peptides that form amphipathic α-helices. Among the most potent of these are the cecropins and synthetic peptides that are hybrids of cecropin and the bee venom peptide, mellitin. Both cecropins and cecropin-mellitin hybrids exist in solution as unstructured monomers, folding into predominantly α-helical structures upon membrane binding with their long helical axis parallel to the bilayer surface. Studies using model membranes have shown that these peptides intercalate into the lipid bilayer just below the level of the phospholipid glycerol backbone in a location that requires expansion of the outer leaflet of the bilayer, and evidence from a variety of experimental approaches indicates that expansion and thinning of the bilayer are common characteristics during the early stages of antimicrobial peptide–membrane interactions. Subsequent disruption of the membrane permeability barrier may occur by a variety of mechanisms, leading ultimately to loss of cytoplasmic membrane integrity and cell death.


*Keywords:* Antimicrobial peptide; Cecropin; Mellitin; Magainin; Spin label; EPR

## 1. Introduction

The ever increasing prevalence of multiple-drug resistant pathogens is a major health concern throughout the world. As of 2003, over 57% of *Staphylococcus aureus* infections in US intensive care units were resistant to multiple antibiotics [1], and there is an emerging resistance to vancomycin [2–4]—a drug that has often been considered the last line of defense. Thus, the need for the development of new antibiotics cannot be overstated. During the past two decades a large number of peptides with potent antibacterial, antiviral, and antifungal properties have been identified from a wide range of both vertebrate and invertebrate species. These antimicrobial peptides (AMPs) form an essential part of the "innate" arm of host resistance, serving as a first line of defense against infection. Importantly, AMPs are believed to have a mechanism of action entirely distinct from those of current clinically-used antibiotics, and so there is great interest in their development for treatment of drug-resistant infections [5–8].

AMPs can be broadly classified based on secondary structure and composition [5–7]. Ribosomally-synthesized AMPs, containing only natural amino acids, may be grouped into linear, α-helical peptides (such as cecropins, magainins, and mellitin), peptides characterized by enrichment in one or two amino acids (proline arginine-rich PR39, indolicidin), and peptides containing disulfide bonds (e.g., defensins, protegrins). There are also a large number of peptides with potent antimicrobial activity that are synthesized extra-ribosomally or contain substantial post-translational modifications, for example lipopeptides (polymyxin, dermaseptin) and the lantibiotics [9], which contain non-native amino acids and/or non-peptide backbone structures. In addition, a wide variety of synthetic AMPs have been developed utilizing either a combinatorial synthesis approach [10–12] or through modification of existing AMPs [13]. These include peptides such as the potent bactericidal–fungicidal decapeptide, KSL [12], the ornithine-based lipopeptide MSI-843 [14], and cyclic peptides containing a combination of *D*- and *L*-amino acids [15–17].

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Antimicrobial peptide selectivity for prokaryotic cells is believed to be based on recognition of general properties of the cell membrane. This is supported by a number of lines of evidence, including the sequence diversity of AMPs and the fact that synthetic, all *D*-amino acid analogs retain full functional activity [18–20]. AMPs bind strongly to lipid bilayers and binding is enhanced by increasing amounts of anionic lipids [21–25]. AMPs dissipate transmembrane ionic gradients [21,26–32], and release entrapped solutes from liposomes [21,23,33,34]. Prokaryotic cell membranes have a much more negative surface potential than eukaryotic membranes [25,35], promoting electrostatic interactions with the cationic peptides and providing at least a partial explanation for specificity. Although studies have shown that some antimicrobial peptides interact with intracellular targets [36–38], these peptides still must traverse the cell membrane to reach their site of action. Consequently, an understanding of peptide–membrane interactions is essential for the improved design and development of AMP antibiotics. In this review we will consider the membrane-bound structure, peptide–membrane interactions, and mechanism of action for the linear, amphipathic α-helical class of AMPs, with particular emphasis on a group of peptides that are synthetic hybrids of cecropins and mellitin.

## 2. Cecropins

Cecropins were among the first antibacterial peptides identified. Initially isolated from the silk moth *Hyalophora cecropia* [39–41], cecropin analogs have now been isolated from a variety of insect species [13], and a mammalian (porcine) version has also been found [42]. As a family, cecropins contain 31–39 amino acids with an amphipathic, basic N-terminal domain and a hydrophobic C-terminal domain [43–45] (Table 1). Numerous synthetic analogs of the naturally-occurring cecropins have been used to elucidate sequence–activity relationships. Substitution of proline residues in the N-terminal domain significantly decrease activity, indicating the importance of the amphipathic α-helix [43]. A conserved aromatic residue at position 2 is essential for full antimicrobial activity, with analogs containing the native tryptophan being more active than those containing phenylalanine [43]. Studies using modular constructs have indicated that a general organization of an amphipathic N-terminal helix connected to a hydrophobic C-terminal helix by a flexible hinge region is required for strong, broad-spectrum antimicrobial activity [45]. These studies led to the synthesis of a chimeric cecropin A–cecropin D construct with enhanced activity [44].

Table 1
Representative amino acid sequences of naturally-occurring cecropins

| | |
|---|---|
| cecropin A: | *KWKLFKKIEK*[10] *VGQNIRDGIIKAGP*[24] AVAVVGQATQIAK[37]*CONH*$_2$ |
| cecropin B: | *KWKVFKKIEK*[10] *MGRNIRNGIVKAGP*[24] AIAVLGEAKAL[35]*CONH*$_2$ |
| cecropin D: | *WNPFKELEK*[9] *VGQRVRDAVISAGP*[23] AVATVAQATALAK[36]*CONH*$_2$ |
| cecropin B1: | *RWKIFKKIEK*[10] *MGRNIRDGIVKAGP*[24] AIEVLGSAKAI[35]*CONH*$_2$ |
| cecropin B2: | *WNPFKELER*[9] *AGQRVRDAVTSAAP*[23] AVATVGQAAAIAR[36]*CONH*$_2$ |
| cecropin C: | *GW-LKKLGKR*[9] *IERIGQHTRDATIQGLG*[26] IAQQAANVAATAR[39]*CONH*$_2$ |
| cecropin P1: | *SW-LSKTAKK*[9] *LENSAKKRISEG*[21] IAIAIQGGPR[31]*COOH* |

Cecropins A, B, and D are found in *Hyalophora* sp. [39,41], cecropin B1 is from *Bombyx mori* [13], cecropin B2 is from *Manduca sexta* [129], cecropin C from *D. melanogaster* [13], and cecropin P1 is found in the intestinal mucosa of the pig [42]. The cationic, amphipathic N-terminal domain is shown in italics, and the predominantly hydrophobic C-terminal domain is underlined.

Cecropins posses broad antibacterial activity against essentially all Gram-negative bacteria and some Gram-positive bacteria [43–45], but notably are relatively inactive against *Staphylococcus aureus* [46–48]. Cecropins also show good activity against the protozoan parasite, *Leishmania* [49], the yeast *Candida albicans* [50], and the malarial parasite *Plasmodium falciparum* [47]. Transgenic higher plants engineered to express cecropins have been developed to enhance resistance against bacterial and fungal phytopathogens [51]. Cecropins have also shown selective activity in vitro against a number of tumor cell lines [52,53]. Cecropins exhibit low toxicity toward normal eukaryotic cells and relatively weak hemolytic activity [46–48]. In a rat model of Gram-negative bacterial sepsis, treatment with cecropin B alone or in combination with β-lactams significantly reduced plasma levels of tumor necrosis factor-α (TNF-α) and endotoxin (measures of septic shock), decreased infectious load, and improved survival [54].

Circular dichroism (CD) studies show that cecropins are unstructured in aqueous solution, but form a high percentage of α-helical secondary structure in the presence of hexafluoroisopropanol (HFIP) [43,55], vesicles of lipopolysaccharide (LPS) [56], SDS micelles [57], or liposomes [57]. Synthetic *all-D* enantiomers have been shown to form left-handed helices with mirror-image CD spectra, are protease resistant, and to have the same level and range of antibacterial activities as the naturally occurring *L*-enantiomers [18,33,56]. Solution NMR studies of cecropin A in 15% HFIP indicated the presence of two well-defined helices, encompassing residues 5–21 and residues 24–37 and corresponding to the cationic N-terminal and hydrophobic C-terminal domains, respectively [58]. The two helices were oriented at an angle of 70–100°, consistent with the presence of a Gly[23]–Pro[24] hinge. Solution-phase NMR of cecropin P1 in 30% HFIP also indicated adoption of helical secondary structure, in this case a single α-helix spanning almost the full length of the peptide [59].

## 3. Mellitin

Mellitin, a 26-residue peptide found in the venom of the honey bee *Apis mellifera*, also contains distinct hydrophilic and hydrophobic domains but in reverse order relative to cecropins, with four cationic amino acids sequestered near the C-terminus (Table 2). Mellitin displays strong, broad-spectrum antimicrobial activity, but is also highly hemolytic [46,47]. In solution, mellitin undergoes a concentration-dependent equilibrium between random-coil monomer and a predominantly α-helical

Table 2
Amino acid sequences of cecropin A, mellitin, and cecropin-mellitin hybrid peptides first identified by Andreau et al. [46]

| | |
|---|---|
| Cecropin A: | Ac-KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK |
| C(1-7), M(2-9): | Ac-KWKLFKK----------IGAVLKVL |
| C(1-7), M(3-10): | Ac-KWKLFKK----------GAVLKVLT |
| C(1-7), M(4-11): | Ac-KWKLFKK----------AVLKVLTT |
| C(1-7), M(5-12): | Ac-KWKLFKK----------VLKVLTTG |
| C(1-8), M(1-12): | Ac-KWKLFKKI----------GIGAVLKVLTTG |
| C(1-13) M(1-13): | Ac-KWKLFKKIEKVGQGIGAVLKVLTTGL |
| Mellitin: | GIGAVLKVLTTGLPALISWIKRKRQQ |

The cecropin-mellitin hybrids incorporate the cationic N-terminal domain of cecropin A (red box) and mostly hydrophobic amino acids from the N-terminal of mellitin (blue box).

tetramer [60]. A number of studies indicate that in the presence of bilayers mellitin forms N-terminal and C-terminal α-helices separated by a flexible hinge (reviewed in 61, 62). At high lipid: peptide ratios (e.g., >200:1) membrane-bound mellitin is monomeric with its helical axis parallel to the membrane surface [61,62], with each of its lysine residues exposed to the aqueous phase [63]. As the concentration of membrane-bound mellitin is increased, the peptide undergoes a dynamic reorientation relative to the membrane normal and eventually produces a complete destruction (micellization) of the membrane bilayer [61,62].

## 4. Cecropin-mellitin hybrids

In order to reduce the size of full-length cecropins to facilitate their solid-phase synthesis, Bowman, Merrifield, and co-workers synthesized and evaluated the antimicrobial activity of a large group of peptides that were hybrids of cecropin A and mellitin. These chimeric peptides were constructed from various combinations of the hydrophilic domain of cecropin with the hydrophobic domain of mellitin, or vice versa. Initial studies examined peptides containing from 18 to 37 amino acids, with net charges of +5 to +7 [47,48]. Many of these constructs displayed antibacterial activity comparable to full-length cecropins, and yet lacked the hemolytic properties associated with mellitin. Interestingly, and in contrast to full-length cecropins, several of the hybrids displayed good antibacterial activity against Gram-positive organisms, including *Staphylococcus aureus* [46–48]. Those studies were followed by a further reduction in size, with the identification of several active constructs having only 15 amino acids [46]. These minimal peptides all contain the first 7–8 residues of cecropin A with various combinations of mostly hydrophobic amino acids from near the N-terminus of mellitin (Table 2). Lethal concentrations against a panel of bacteria ranged from 0.1 to 15 μM, while hemolytic concentrations were >300 μM [46].

Hancock and co-workers have carried out extensive studies on a cecropin-mellitin (CM) hybrid consisting of the first 8 residues of cecropin A and the first 18 residues of mellitin, C(1–8)M(1–18), which they designate CEME [64–66]. This construct demonstrates strong antimicrobial activity against both Gram-negative and Gram-positive bacteria [65–68]. CEME has demonstrated high affinity for lipopolysaccharide (LPS), and enhances both outer and cytoplasmic membrane permeability [65, 67]. CEME and an analog with two additional cationic residues near the C-terminus also inhibited LPS-induced TNF-α production by isolated macrophages, and improved survival in a murine model of acute septic shock [69]. These peptides also bind lipoteichoic acid, a cell-wall component of Gram-positive bacteria, and were able to reduce a number of physiological effects (e.g., TNF-α and cytokine production) related to Gram-positive sepsis [70].

An even smaller CM hybrid consisting of the first seven residues of cecropin A and residues 2–9 of mellitin, C(1–7)M(2–9) (which we designate CM15, Table 2) has also received particular attention due to its broad-spectrum antimicrobial efficacy [46]. Both CM15 and CEME have been shown to be active against the protozoan parasite *Leishmania* [49]. Activity of CM15 against *Leishmania* is enhanced by N-terminal substitution with fatty acids up to 12 carbons, but acylation of the Lysine 7 ε-amino group significantly reduced both bactericidal and leishmanicidal activities [49]. The N-octanoyl derivative of CM15 has been shown to be safe and effective in treatment of naturally-acquired canine leshmaniasis [71].

As with cecropin A [18], the *all-D* enantiomer of CM15 retains full biological activity against a broad panel of bacterial species [20], indicating that interaction with cellular targets occurs in a non-stereospecific manner. Interestingly, the reverse ("retro") and *all-D* reverse ("retroenantio") sequences were active against some bacterial strains (e.g., *E. coli, Bacillus subtilis, Streptococcus pyogenes)*, but inactive against either *S. aureus* or *Pseudomonas aeruginosa* [20]. Thus it consistently appears that chirality is not a factor in determining peptide efficacy, although in some cases it may be important to consider sequence order and the direction of amide bonds.

CM hybrids, like their parent peptides, are members of the linear, amphipathic α-helical class of AMPs. CM hybrids exist in a random coil configuration in solution, and only adopt their α-helical secondary structure in the presence of membranes or helix-promoting organic solvents [46]. Solution NMR of a 26-residue CM hybrid, C(1–13)M(1–13), in 30% HFIP indicated the formation of two α-helices separated by a flexible linker [72], similar to the structural motif found for cecropin A. A predominantly α-helical solution NMR structure has also been reported for C(1–8)M(1–12) in 50% HFIP [73].

Upon folding, CM hybrids are highly amphipathic, with an almost ideal distribution of polar and non-polar amino acids (Fig. 1). CM hybrids retain the domain structure of full-length cecropins, with a strongly cationic N-terminal domain and a

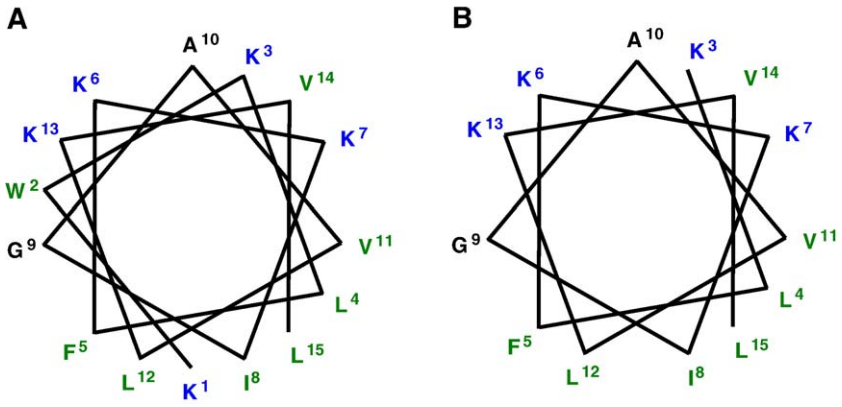


Fig. 1. Helical wheel display of the cecropin-mellitin hybrid, CM15. (A) Full sequence, (B) representation with the omission of Lys1–Trp2. It has been suggested that the first two amino acids may not be included in the α-helical secondary structure. Note that, in either case, the polar and non-polar side chains are sequestered on opposite faces of the helix.

predominantly hydrophobic C-terminal domain. Several CM hybrids also contain a centrally-located glycine, similar to the gly-pro hinge that has been shown to be functionally important in full-length cecropins [45]. However this glycine does not appear to be a required for antimicrobial activity in CM hybrids since it is absent in a number of effective analogs [46]. Indeed, the 15-residue C(1–7)M(3–10) construct, in which isoleucine 8 is replaced by glycine, exhibits significantly diminished antibacterial activity [74]. Substitution of Ile8 with serine also strongly reduced activity, suggesting the requirement of a hydrophobic amino acid at this position [74]. When folded into a membrane-bound α-helix, Ile8 is buried deeply in the bilayer (discussed below), and so substitution of a polar amino acid at this site may prevent membrane insertion. Decreasing the length of CM15 by one residue to give the 14-mer, C(1–7)M(2–8), reduced its antibacterial activity, while adding an additional residue, giving C(1–7)M(2–10), slightly enhanced activity [74].

## 5. Peptide–membrane interactions

Despite extensive study, the precise mechanisms of peptide–membrane interaction and cell killing have not been firmly established for many antimicrobial peptides. It is generally agreed that, upon addition to a bacterial cell suspension, AMPs undergo "self-promoted uptake" to cross the outer membrane or cell wall [64,67], followed by disruption of the cytoplasmic membrane as the lethal event leading to bacterial cell death [30,32,75]. It is proposed that the initial binding of AMPs to the outer membrane displaces divalent cations, destabilizing the cell envelope and resulting in the subsequent uptake of the peptide [64]. Several studies have shown that the magainins [32] and CM hybrids [65] disrupt the outer membrane permeability barrier. Although the molecular mechanisms involved in crossing the outer membrane remain poorly defined at a molecular level, most studies have focused on the events associated with binding to and disruption of the cytoplasmic membrane. This may occur through a detergent-like "carpet" mechanism [29,33,76], or the formation of discrete pores that dissipate ion gradients [74,75,77].

It can be imagined that the initial association of an α-helical AMP with a lipid bilayer could occur in any of three general orientations: parallel to the membrane surface, along the membrane normal, or at an oblique angle (Fig. 2). There are precedents in the literature for all three cases. Peptides based on the myristoylated alanine-rich C-kinase substrate (MARKS) for protein kinase C [78] and model peptides composed entirely of lysine and phenylalanine [79] bind parallel to the membrane surface with varying depths of penetration depending on overall hydrophobicity. Peptides designed to model transmembrane helices [80], transmembrane helices of integral membrane proteins, and certain peptide ionophores such as alamethicin [81] align more or less vertically across the bilayer; while the membrane-insertion sequences of SNARE proteins [82,83] and viral fusion peptides [84,85] integrate into the bilayer at an oblique angle.

Both full-length cecropins and CM hybrids appear to be monomeric in solution. Using a spin-labeled analog of cecropin AD we found no evidence of aggregation in either physiological or low ionic strength solutions up to a peptide concentration of 200 μM [22,86]. Although aggregation of cecropin AD was observed upon addition of low concentrations (5–10%) of HFIP, upon titration with higher HFIP concentrations the peptide reverted to a folded monomer—consistent with a model in which peptide binding and secondary structure formation are a concerted process [86]. Sedimentation equilibrium studies indicate that cecropin A is also monomeric in solution [21]. Thus, the initial event in peptide–membrane interaction is the

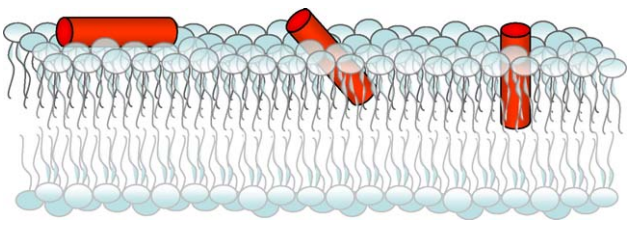

Fig. 2. Association of amphipathic α-helical peptides (cylinders) with a lipid bilayer can occur in three general orientations: parallel to the membrane surface, at an oblique angle, or perpendicular to the membrane surface (i.e., along the bilayer normal).

association of monomeric peptide with a large excess of phospholipid. This is a situation that can be readily modeled using artificial membrane bilayers.

Solid-state NMR of full-length cecropin A selectively $^{15}$N-labeled at either Val11 or Ala27 and incorporated into oriented planar bilayers indicated that the amide bonds at these two sites were oriented parallel to the membrane surface [87], inferring that both the N- and C-terminal helical domains lie along the surface of the bilayer. Consistent with these results, Silvestro and Axelson used internal reflection Fourier-transform infrared (FTIR) spectroscopy to demonstrate that upon initial membrane association cecropin A adopted secondary structure that was primarily α-helical, with the longitudinal helical axis preferentially oriented parallel to the membrane surface [88]. The peptide expanded the membrane surface upon insertion, but did not alter its structure or orientation. Attenuated total reflection (ATR)-FTIR studies indicate that cecropin P1 also orients almost parallel to the membrane surface upon initial binding to PE (phosphatidylethanolamine)–PG (phosphatidylglycerol) membranes that mimic the Gram-negative bacterial inner membrane [89]. Similarly, numerous studies have indicated that members of the magainin family of peptides initially bind to membranes with their long helical axis parallel to the bilayer surface [61,62]. We have used site-directed spin labeling (SDSL) to characterize the initial interaction of CM15 with liposomes that mimic the bacterial inner membrane [24]. In the SDSL approach, peptides are synthesized or expressed with a single cysteine residue at any given desired location (or, typically, at all possible locations, Fig. 3). The cysteine residue is then covalently modified with a sulfhydryl-specific nitroxide spin label (Fig. 4), allowing study of the peptide structure and dynamics by electron paramagnetic resonance (EPR) spectroscopy. One advantage of SDSL relative to other molecular probe techniques is that the labeled side chain is relatively small – about the same molecular volume as tryptophan (Fig. 4) – so that the physical properties of the peptide are not significantly perturbed. Additionally, EPR is unaffected by the optical properties of the sample so that one can conduct studies across a wide range of lipid concentrations without concern for light-scattering or inner filter effects. SDSL provides access to two particularly important parameters: the rotational dynamics of the labeled side chain, which is sensitive to local structure and intermolecular interactions, and the accessibility of the nitroxide—which can be used to determine the depth of the labeled site in a lipid bilayer (for reviews of SDSL methods and applications, see references 90,91).

| | |
|---|---|
| KWKLFKKIGAVLKCL | C14 |
| KWKLFKKIGAVLCVL | C13 |
| KWKLFKKIGAVCKVL | C12 |
| KWKLFKKIGACLKVL | C11 |
| KWKLFKKIGCVLKVL | C10 |
| KWKLFKKICAVLKVL | C9 |
| KWKLFKKCGAVLKVL | C8 |
| KWKLFKCIGAVLKVL | C7 |
| KWKLFCKIGAVLKVL | C6 |
| KWKLCKKIGAVLKVL | C5 |
| KWKCFKKIGAVLKVL | C4 |
| KWCLFKKIGAVLKVL | C3 |

Fig. 3. Single-cysteine analogs of CM15 used for the selective attachment of spin labels or other sulfhydryl-specific probes. In this study the N- and C-termini were avoided since they are typically unstructured. The tryptophan at position 2 was also retained throughout, both because it is highly conserved (see Table 1) and as a chromophore for quantitation of peptide concentration.

To characterize the structure and localization of membrane-bound CM15, we carried out a "nitroxide-scanning" study using several of the single-cysteine analogs shown in Fig. 3 [24]. These peptides were spin-labeled and mixed with large unilamellar vesicles (LUVs) composed of *E. coli* inner membrane lipids at a high lipid:peptide ratio, designed to mimic conditions of initial peptide binding. The resulting EPR spectra indicated that under these conditions the peptides adopt a folded conformation and are fully membrane-bound [24]. Bilayer depth was determined for each spin-labeled analog, giving the sequence-related pattern shown in Fig. 5. As can be seen, the experimental data fit well to an idealized α-helix with 3.6 residues/turn. This is in agreement with CD results showing that CM15 is essentially 100% α-helical in the presence of liposomes (Fig. 6). In addition, the data in Fig. 5 show that all of the residues along the hydrophobic face of the peptide (i.e., F5, I8, L12) reside at approximately the same depth—about 12.5 Å below the bilayer surface, while all of the side chains along the hydrophilic face lie about 2.5 Å above the membrane. This pattern clearly indicates that upon initial binding CM15 intercalates parallel to the membrane surface, with its central helical axis at a depth of ~5 Å (Fig. 7). Such localization is well-suited to bury non-polar side chains in the hydrophobic core of the membrane, while positioning lysine residues to interact with lipid phosphates. Importantly, essentially the entire molecular volume of the peptide must be accommodated by the membrane, which may be an important factor in promoting destabilization and eventual disruption of the bilayer.

## 6. Mechanisms of membrane disruption

Mechanisms of membrane disruption by AMPs have been intensely studied. In general, membrane disruption is believed to occur either via a detergent-like carpet mechanism [33,76,89], through induction of non-lamellar lipid phases [92], or through formation of discrete pores [77]. There is good experimental evidence for each of these processes, and it may be that different peptides utilize different mechanisms to ultimately disrupt the microbial membrane. These mechanisms also need not be mutually exclusive, one process may represent an initial or intermediate step and another may be its consequence. Additional factors such as lipid-to-peptide ratio and target membrane composition may also be involved.

Many studies of membrane disruption by AMPs have utilized model membranes (lipid monolayers, liposomes,

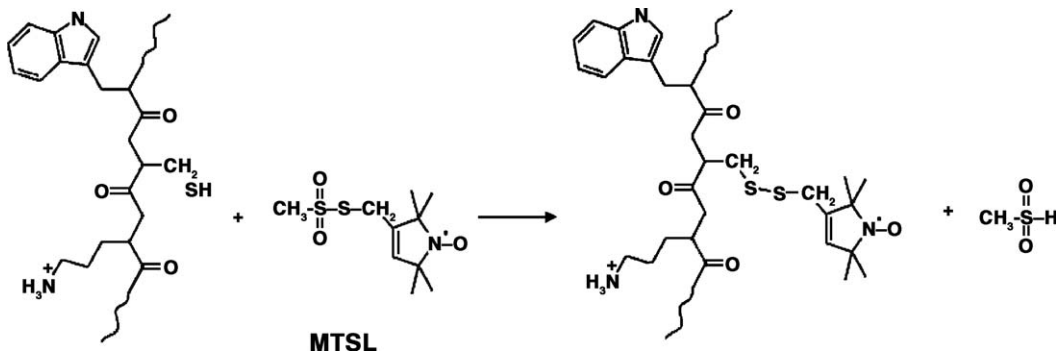


Fig. 4. Cysteine labeling with the methiosulfonate spin label, MTSL. Note that the nitroxide side chain has about the same molecular volume as tryptophan.

micelles, etc) to simulate the inner membrane of bacteria, which as discussed above is a key target of AMPs. The use of model membranes allows rigorous control and experimental manipulation of membrane composition and lipid–peptide ratio. Upon the addition of AMPs to these artificial membranes, AMP monomers bind to the outer leaflet of the phospholipid bilayer as a first step in the processes of membrane lysis. As discussed above, in the presence of a large excess of lipid (i.e., at a low peptide/lipid ratio) the initial binding state of most α-helical AMPs is parallel to the lipid bilayer surface. For example, CM15 [24], cecropin A [87,88], cecropin P1 [89], and magainin [93,94] all form their initial association with the membrane in this fashion. It has been suggested that such binding induces positive membrane curvature [92, 95], increasing the surface area of the outer leaflet, leading to a decrease in the thickness of hydrophobic core [77] and thinning of the lipid bilayer [96–100]. An NMR study of a magainin analog, MSI-78, demonstrated that peptide binding to the bilayer surface disorders phospholipid acyl chains, which may be either a cause or a reflection of bilayer thinning [98]. AMP concentration-dependent membrane thinning (proportional to the peptide/lipid ratio) has also been detected by X-ray diffraction [97,99]. Although the initial association of amphipathic α-helical AMPs with the membrane surface increases the volume and surface area of the outer leaflet, the inner leaflet is little affected and this mismatch may contribute to peptide insertion and/or aggregation [88]. Thus, changes in the physical properties of the lipid bilayer induced via peptide binding appear to facilitate further penetration of AMPs, which in turn may lead to the formation of transmembrane pores.

A pore formation model of AMP activity was first suggested by Baumann and Mueller based on their studies of alamethicin [101]. Huang and coworkers have subsequently developed a two-state model for membrane pore formation by α-helical AMPs [77]. In this model, AMPs initially associate parallel to the bilayer surface; when the peptide concentration reaches a critical threshold, membrane-bound AMPs reorient and penetrate or insert into the hydrophobic core of bilayer with the formation of transmembrane pores [77]. Evidence for this general mechanism has come largely from studies that employ oriented CD [99,102], as well as neutron [100–104] and X-ray scattering [97,99,105,106]. Pore formation mechanisms are also supported by studies showing the differential leakage of solutes of different sizes and patch-clamp experiments demonstrating

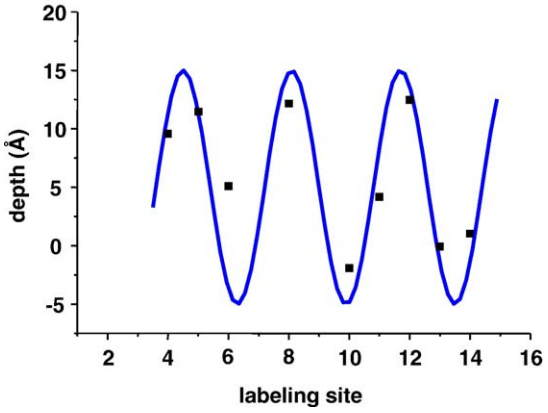


Fig. 5. Bilayer depth as a function of sequence position for membrane-bound CM15, determined using SDSL. Single-cysteine analogs of CM15 were spin labeled with MTSL and added to large unilamellar vesicles composed of *E. coli* inner membrane lipids (PE:PG:CL, molar ratio 68:26:6) at a lipid:peptide ratio of 200:1. The position determined is that of the spin labeled side chain relative to the bilayer surface. Squares represent experimental data and the solid line is a sine function with a periodicity of 3.6, characteristic of an idealized α-helix. Some of the data were published previously [24].

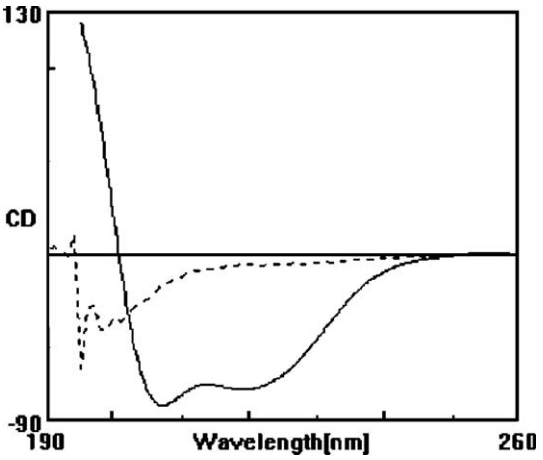


Fig. 6. Circular dichroism spectra of CM15 in aqueous solution (dotted line) and in the presence of PE:PG (8:2 molar ratio) unilamellar vesicles. Analysis of the spectrum in the presence of liposomes using the K2D algorithm [130] indicates nearly 100% α-helical secondary structure.

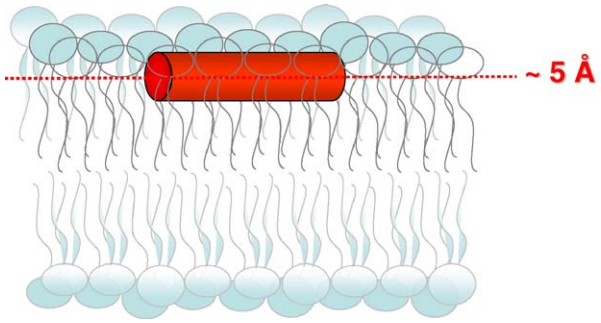


Fig. 7. Localization of membrane-bound CM15. This model is based on SDSL data obtained at a lipid:peptide ratio of 200:1. The peptide forms a single, uninterrupted α-helix with its helical axis parallel to the membrane surface approximately 5 Å below the aqueous interface (defined as the level of the phospholipid glycerol backbone). This positioning buries non-polar side chains in the hydrophobic core of the bilayer and allows lysine residue on the polar face of the helix to hydrogen bond and/or salt bridge with the lipid head groups. Incorporation of the peptide at this location would require expansion of the outer leaflet of the bilayer.

the formation of ion channels. For example, cecropin A dissipates membrane potentials at concentrations much lower than those required for release of calcein [21], and Christensen et al. found that cecropin A and its analogues formed time-variant, voltage-dependent ion channels in planar lipid membranes [75]. Ion channel formation with discrete open and closed states has also been demonstrated for CM15, where a correlation between channel activity, pore size, and peptide concentration was observed with larger pores being formed at higher peptide concentration [74].

Two distinct channel architectures have been described: a barrel-stave structure that involves the formation of transmembrane ion channels, and a torroidal-pore model in which the transmembrane channel is composed of both AMPs and membrane lipids [77] (Fig. 8).

### 6.1. "Barrel stave" model

Many investigators have suggested that certain AMPs self-associate to form a transmembrane pore [26,74,75,102,107–109]. The pore acts as a conductance channel that disrupts transmembrane potential and ion gradients, leading to a leakage of cell components and cell death. Dissipating the transmembrane electrochemical gradient causes a loss of the bacterial cell's ability to synthesize ATP, and the increase in water and ion flow that accompanies loss of the permeability barrier leads to cell swelling and osmolysis. In the barrel-stave model, amphipathic peptides align with their hydrophobic sides facing the phospholipid acyl chains and their hydrophilic surfaces lining a water-filled channel, much like the staves of a barrel (Fig. 8). This model requires peptides to be sufficiently long to traverse the hydrophobic core of the bilayer, and implies direct contact between peptides upon channel formation.

A prototype for the formation of barrel-stave channels is the fungal peptide, alamethicin. This 20-residue, hydrophobic peptide from the fungus *Trichoderma veridae* contains eight residues of the unusual amino acid α-methylalanine (α-aminoisobutyric acid). Although alamethicin has little selectivity for bacterial or eukaryotic membranes, it has been intensely studied due to its formation of voltage-gated channels in bilayer systems [110–112]. In contrast to amphipathic peptides, alamethicin monomers insert into the bilayer and then aggregate in the membrane to form a barrel-stave pore [77]. Membrane insertion of alamethicin was well established using oriented circular dichroism and the change in peptide orientation was observed reversibly and continuously [96]. An in-plane neutron

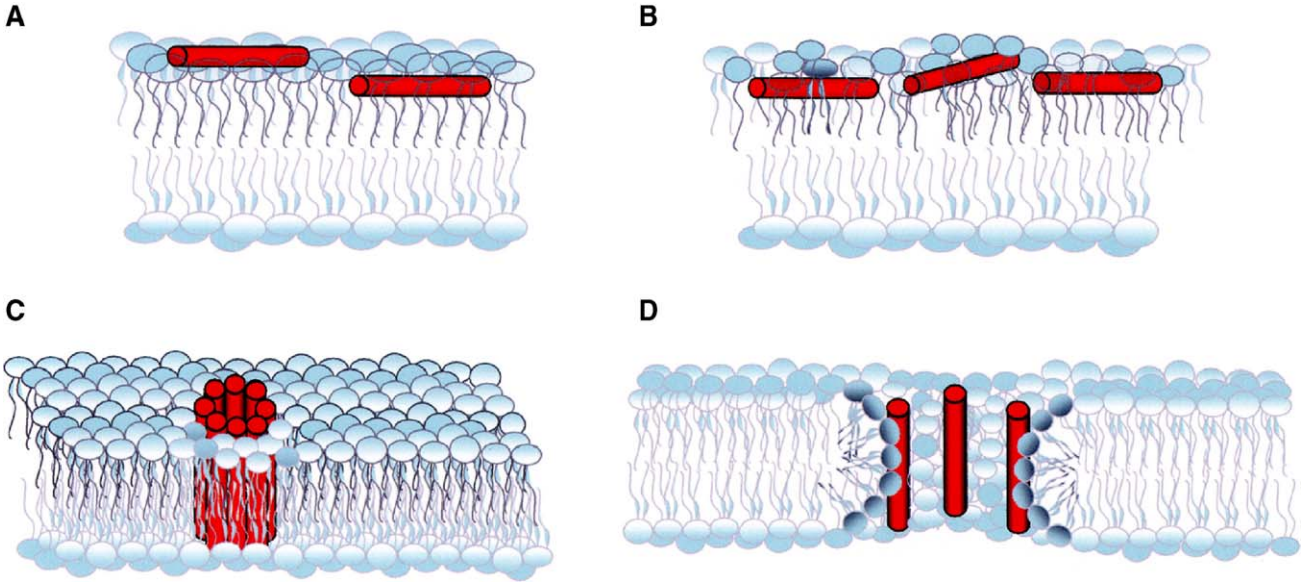


Fig. 8. Models of transmembrane channel formation. (A) Peptide α-helices (cylinders) initially associate parallel to the membrane surface, either superficially (left) or embedded just below the aqueous interface. (B) Peptides continue to accumulate at or near the bilayer surface, disrupting lipid packing and causing membrane thinning. This step may or may not involve peptide–peptide aggregation. Once a critical peptide/lipid ratio is reached, peptides either (C) insert into the membrane as a barrel-stave type pore, or (D) induce the localized formation of toroidal pores.

scattering study demonstrated that alamethicin formed stable aqueous pores at high peptide concentration, based on the difference in the scattering lengths of $H_2O$ and $D_2O$ [100]. This neutron scattering study suggested a barrel-stave model of the alamethicin channel with 8 or 9 monomer α-helices per channel and a pore diameter of ~18–26 Å, depending of lipid composition and degree of hydration [96,104,113]. This is in good agreement with studies on black lipid membranes, which indicate 8–10 monomers per channel [101].

### 6.2. "Torroidal-pore" model

In this model, AMP-induced expansion of the lipid head group region results in a bending of the bilayer back on itself followed by connection of the outer and inner leaflets, and the pore is composed of both peptide and phospholipid [77,92,102,114,115] (Fig. 8). Positive curvature of the membrane surface, resulting from accumulation of AMP at the bilayer interface, facilitates the bending of phospholipids and toroidal pore formation [92,95]. The energetically-favorable insertion of peptide into the membrane may also produce changes in the bilayer structure and facilitate toroidal pore formation [115].

The toroidal model was first proposed based on studies with magainin peptides [115,116]. Magainin is a cationic amphipathic α-helical peptide that forms transient ion channels [116]. The magainin pore size has been estimated at approximately 30 to 50 Å, formed with 4–7 peptides and 90 phospholipids per pore [77,95,102,115]. $^{31}$P-NMR and differential scanning calorimetry studies of MSI-78, an optimized, synthetic analog of magainin, also support toroidal pore formation [92]. Recent oriented CD and neutron scattering studies of melittin have suggested that it also forms a toroidal-pore type channel, with a pore diameter of ~44 Å [102]. The size of pores induced by melittin were estimated at around 25–30 Å in diameter based on the release of differential sizes of fluorescence-labeled dextran markers [117], which agrees well with the pore size (20–30 Å) detected by osmotic protection of erythrocytes [118]. The differences in these estimates are not unreasonable, given the disparity in experimental methods, peptide/lipid ratios, and degrees of hydration.

The structure of the toroidal pore model addresses the fact that many AMPs are simply too small to span an unperturbed phospholipid bilayer in an α-helical conformation. For an alpha-helix, ~22 amino acids are required to transverse the bilayer, the width of which is typically 32–38 Å [35,100,119]. This is clearly beyond the limits of CM15 and many other AMPs. For example, the crystal structure of trichogin indicated that the length of α-helix is ~16 Å [120]. In the toroidal pore model, channels are composed of a mixture of peptides and lipids and thus are not required to span the complete bilayer. Toroidal pores are proposed to form subsequent to peptide-induced membrane thinning [77,97,99,105], which may also allow the penetration of short peptides to form a peptide/lipid pore.

#### 6.2.1. Pore formation and osmotic stress

Regardless of the precise channel structure, once even a transient pore is formed across the membrane osmotic gradients will induce cell swelling and thus facilitate further thinning of the lipid bilayer. Hypo-osmotic conditions were shown to enhance melittin-induced lysis even though the binding of the peptide was not affected [121]. Interestingly, in lipid vesicles a low concentration of cecropin A was able to dissipate ion gradients across the membrane, while a higher peptide concentration was required to release an encapsulated fluorescent marker—indicating that the mechanism of membrane disruption may change with peptide/lipid ratio [21]. Low concentrations of cecropin A dissipate the inner membrane potential and kill *E. coli* cells, while much higher concentrations are required to release β-galactosidase [21,30]. Similarly, magainin 2a and its analogs induce a rapid release of intracellular potassium at peptide concentrations that are cytotoxic, without the release of intracellular β-galactosidase [32]. These data suggest that prior to frank cytolysis, channel formation by AMPs disrupts membrane potential and pH gradients. This in turn will uncouple ATP synthesis and generate an osmotic shock.

Osmotic swelling can increase cell surface area by 5 to 25% [122,123] and increases lipid alkyl chain disorder [121], which may promote peptide interaction with the hydrophobic core of the bilayer [121]. This may be particularly important for phospholipids with a small head group (e.g. PE), and many studies have demonstrated that membrane lipid composition has an important influence on AMP interactions that extends beyond simple electrostatic effects [25,109,124,125,126]. Such changes in the physical properties of the membrane may also enhance peptide binding—creating a disruptive cycle that eventually leads to cell lysis. It is noteworthy that, because of the rigidity of the cell wall, Gram-positive bacteria are able to withstand turgor pressures 3 to 25 times higher than those tolerated by Gram-negative bacteria [127] and this (along with other influences of cell wall composition) may be one of the reasons Gram-positive bacteria are typically more resistant to antimicrobial peptides.

### 6.3. Detergent-like "carpet" mechanism

A conceptually different model for the way in which AMPs disrupt membranes is the carpet mechanism (Fig. 9). In this model, peptides accumulate at the bilayer surface like a carpet and, above a threshold concentration of monomers, the membrane is permeated and disintegrated in a detergent-like manner without the formation of discrete channels [7,29,33,76,89]. This mechanism was first proposed by Steiner et al. based on the observation that, at the concentration needed to obtain 50% cell killing, cecropin A was present in sufficient amounts to completely cover the bacterial cell surface [33]. Subsequent studies on the porcine cecropin P1 were also interpreted in terms of a carpet mechanism [29]. Cecropin P1 studies using ATR-FTIR spectroscopy indicated that it incorporated parallel to the surface of the PE/PG membrane and did not change the order parameters of the acyl chains, suggesting the peptide did not translocate into the hydrocarbon core [89].

In some instances, it can be quite difficult to distinguish between carpet and toroidal pore mechanisms. For example,

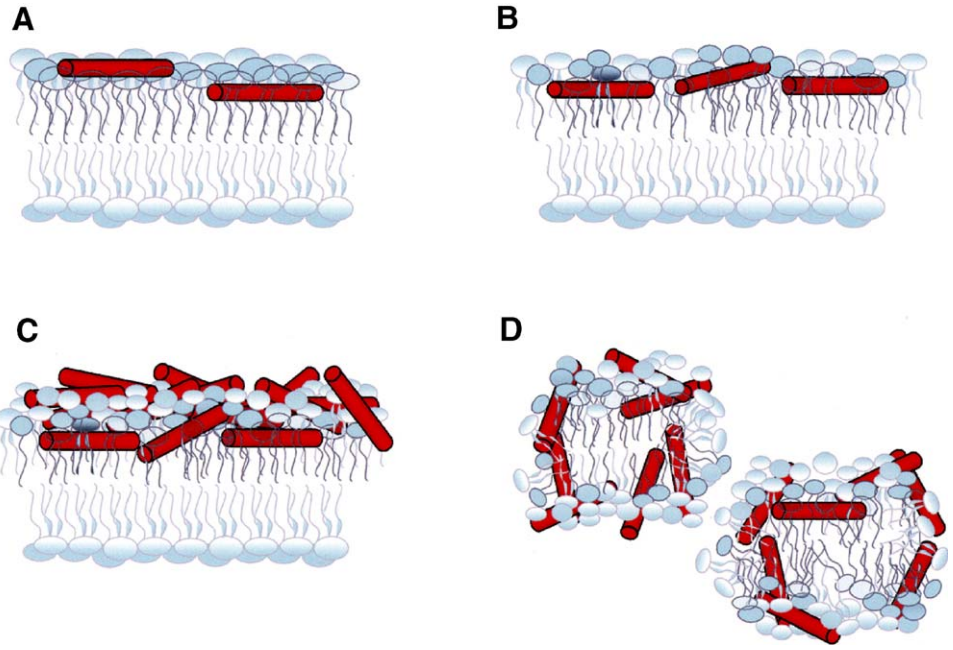


Fig. 9. Model of membrane disruption by the carpet mechanism. As in channel formation, peptide α-helices (cylinders) initially (A) bind and (B) accumulate in an orientation parallel to the membrane surface. (C) Continued accumulation of membrane-bound peptide associated with the phospholipid head-groups, eventually covering (i.e., carpeting) the bilayer. (D) Detergent-like membrane disintegration.

the human AMP LL-37 intercalates parallel to the membrane surface as a tightly-bound α-helix that does not reorient along the bilayer normal (as occurs during toroidal pore formation by mellitin and magainin, discussed above), even at relatively high peptide/lipid ratios [128]. However, $^{31}$P-NMR shows no evidence of an isotropic lipid component as would be required in some stage of the carpet mechanism [128].

Although the carpet and channel-forming models have a number of differences, they also share some common characteristics. Both types of mechanisms begin with AMP association parallel to the membrane surface, followed by peptide accumulation to some critical concentration. In the carpet model, peptides remain associated with the phospholipid head groups throughout the membrane disintegration process [76]. This is similar to the transition that occurs in the toroidal-pore model, and it has been suggested that the formation of "holes" or toroidal pores may occur as an early step in membrane disintegration, i.e. toroidal pores are regarded as an intermediate state prior to micellization [29,76]. It seems likely that most AMPs, by virtue of their amphipathic character, will act as detergents at sufficiently high concentrations. Nonetheless, in many cases (e.g., magainin, MSI-78) it is clear that pore formation is sufficient to induce membrane lysis, without progression to complete membrane destruction.

## 7. Conclusions

The intensive study of antimicrobial peptide–membrane interactions has given rise to molecular models of membrane disruption that include both the formation of discrete channels and more non-specific detergent-like processes. Although there continues to be a debate regarding the particular mechanism used by a given peptide, some general principles are beginning to emerge. There is a general consensus that, at least for the amphipathic α-helical class of AMPs, peptides initially associate parallel to the bilayer surface. There is also general agreement that for most peptides this orientation persists until enough peptide accumulates to achieve a critical membrane-bound concentration. Peptide accumulation leads to a thinning of the bilayer, which in turn leads to conditions allowing a localized collapse of the lipid bilayer to form a lipid–peptide toroidal pore, peptide self-association and insertion into the bilayer in a barrel-stave arrangement, or simply a detergent-like disintegration of the bilayer structure. The precise mechanism almost certainly depends on the particular AMP involved, and likely on the composition and physical properties of the target bilayer as well. Peptide accumulation in the outer leaflet of the bilayer is a critical phase in the process of membrane disruption, and for many peptides it is becoming increasingly clear that perturbation of the membrane near the hydrophobic–hydrophilic interface leads to a thinning of the lipid bilayer that precedes loss of the permeability barrier. Further advances in our understanding of the molecular events that lead to membrane disruption will facilitate improvements in peptide design, and aid in the development of new antibiotics based on AMP structures and mechanistic principles.

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## References


[1] National Nosocomial Infections Surveillance (NNIS) System Report, Data summary from January 1992 through June 2003, issued August 2003, Am. J. Infect. Control 31 (2003) 481–498.

[2] S.B. Levy, The challenge of antibiotic resistance, Sci. Am. 278 (1998) 46–53.

[3] R. Novak, B. Henriques, E. Charpentier, S. Normark, E. Tuomanen, Emergence of vancomycin tolerance in *Streptococcus pneumoniae*, Nature 399 (1999) 590–593.

[4] R.J. Willems, J. Top, M. van Santen, D.A. Robinson, T.M. Coque, F. Baquero, H. Grundmann, M.J. Bonten, Global spread of vancomycin-resistant *Enterococcus faecium* from distinct nosocomial genetic complex, Emerg. Infect. Dis. 11 (2005) 821–828.

[5] R.E. Hancock, Peptide antibiotics, Lancet 349 (1997) 418–422.

[6] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature 415 (2002) 389–395.

[7] K.A. Brogden, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev., Microbiol. 3 (2005) 238–250.

[8] R.E. Hancock, M.G. Scott, The role of antimicrobial peptides in animal defenses. [Review] [47 refs], Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 8856–8861.

[9] L. Xie, L.M. Miller, C. Chatterjee, O. Averin, N.L. Kelleher, W.A. van der Donk, Lacticin 481: in vitro reconstitution of lantibiotic synthetase activity, Science 303 (2004) 679–681.

[10] R.A. Houghten, C. Pinilla, S.E. Blondelle, J.R. Appel, C.T. Dooley, J.H. Cuervo, Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery, Nature 354 (1991) 84–86.

[11] S.E. Blondelle, E. Takahashi, P.A. Weber, R.A. Houghten, Identification of antimicrobial peptides by using combinatorial libraries made up of unnatural amino acids, Antimicrob. Agents Chemother. 38 (1994) 2280–2286.

[12] S.Y. Hong, J.E. Oh, M. Kwon, M.J. Choi, J.H. Lee, B.L. Lee, H.M. Moon, K.H. Lee, Identification and characterization of novel antimicrobial decapeptides generated by combinatorial chemistry, Antimicrob. Agents Chemother. 42 (1998) 2534–2541.

[13] W.L. Maloy, U.P. Kari, Structure–activity studies on magainins and other host defense peptides, Biopolymers 37 (1995) 105–122.

[14] S. Thennarasu, D.K. Lee, A. Tan, U. Prasad Kari, A. Ramamoorthy, Antimicrobial activity and membrane selective interactions of a synthetic lipopeptide MSI-843, Biochim. Biophys. Acta 1711 (2005) 49–58.

[15] L.H. Kondejewski, M. Jelokhani-Niaraki, S.W. Farmer, B. Lix, C.M. Kay, B.D. Sykes, R.E. Hancock, R.S. Hodges, Dissociation of antimicrobial and hemolytic activities in cyclic peptide diastereomers by systematic alterations in amphipathicity, J. Biol. Chem. 274 (1999) 13181–13192.

[16] S. Fernandez-Lopez, H.S. Kim, E.C. Choi, M. Delgado, J.R. Granja, A. Khasanov, K. Kraehenbuehl, G. Long, D.A. Weinberger, K.M. Wilcoxen, M.R. Ghadiri, Antibacterial agents based on the cyclic D, L-alpha-peptide architecture, Nature 412 (2001) 452–455.

[17] R.M. Kohli, C.T. Walsh, M.D. Burkart, Biomimetic synthesis and optimization of cyclic peptide antibiotics, Nature 418 (2002) 658–661.

[18] D. Wade, A. Boman, B. Wahlin, C.M. Drain, D. Andreu, H.G. Boman, R. B. Merrifield, All-D amino acid-containing channel-forming antibiotic peptides, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 4761–4765.

[19] R. Bessalle, A. Kapitkovsky, A. Gorea, I. Shalit, M. Fridkin, All-D-magainin: chirality, antimicrobial activity and proteolytic resistance, FEBS Lett. 274 (1990) 151–155.

[20] R.B. Merrifield, P. Juvvadi, D. Andreu, J. Ubach, A. Boman, H.G. Boman, Retro and retroenantio analogs of cecropin-melittin hybrids, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 3449–3453.

[21] L. Silvestro, K. Gupta, J.N. Weiser, P.H. Axelsen, The concentration-dependent membrane activity of cecropin A, Biochemistry 36 (1997) 11452–11460 (erratum appears in Biochemistry 1999 Mar 23;38 (12):3850).

[22] H.S. Mchaourab, J.S. Hyde, J.B. Feix, Binding and state of aggregation of spin-labeled cecropin AD in phospholipid bilayers: effects of surface charge and fatty acyl chain length, Biochemistry 33 (1994) 6691–6699.

[23] J.M. Mancheno, M. Onaderra, A. Martinez del Pozo, P. Diaz-Achirica, D. Andreu, L. Rivas, J.G. Gavilanes, Release of lipid vesicle contents by an antibacterial cecropin A-melittin hybrid peptide, Biochemistry 35 (1996) 9892–9899.

[24] K. Bhargava, J.B. Feix, Membrane binding, structure, and localization of cecropin-mellitin hybrid peptides: a site-directed spin-labeling study, Biophys. J. 86 (2004) 329–336.

[25] S.E. Blondelle, K. Lohner, M. Aguilar, Lipid-induced conformation and lipid-binding properties of cytolytic and antimicrobial peptides: determination and biological specificity, Biochim. Biophys. Acta 1462 (1999) 89–108.

[26] H.V. Westerhoff, D. Juretic, R.W. Hendler, M. Zasloff, Magainins and the disruption of membrane-linked free-energy transduction, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 6597–6601.

[27] P. Diaz-Achirica, S. Prieto, J. Ubach, D. Andreu, E. Rial, L. Rivas, Permeabilization of the mitochondrial inner membrane by short cecropin-A-melittin hybrid peptides, Eur. J. Biochem. 224 (1994) 257–263.

[28] M. Hugosson, D. Andreu, H.G. Boman, E. Glaser, Antibacterial peptides and mitochondrial presequences affect mitochondrial coupling, respiration and protein import, Eur. J. Biochem. 223 (1994) 1027–1033.

[29] E. Gazit, A. Boman, H.G. Boman, Y. Shai, Interaction of the mammalian antibacterial peptide cecropin P1 with phospholipid vesicles, Biochemistry 34 (1995) 11479–11488.

[30] L. Silvestro, J.N. Weiser, P.H. Axelsen, Antibacterial and antimembrane activities of cecropin A in *Escherichia coli*, Antimicrob. Agents Chemother. 44 (2000) 602–607.

[31] M. Wu, E. Maier, R. Benz, R.E. Hancock, Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*, Biochemistry 38 (1999) 7235–7242.

[32] K. Matsuzaki, K. Sugishita, M. Harada, N. Fujii, K. Miyajima, Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria, Biochim. Biophys. Acta 1327 (1997) 119–130.

[33] H. Steiner, D. Andreu, R.B. Merrifield, Binding and action of cecropin and cecropin analogues: antibacterial peptides from insects, Biochim. Biophys. Acta 939 (1988) 260–266.

[34] K. Matsuzaki, O. Murase, K. Miyajima, Kinetics of pore formation by an antimicrobial peptide, magainin 2, in phospholipid bilayers, Biochemistry 34 (1995) 12553–12559.

[35] R.B. Gennis, Biomembranes Molecular Structure and Function (1989).

[36] G. Kragol, R. Hoffmann, M.A. Chattergoon, S. Lovas, M. Cudic, P. Bulet, B.A. Condie, K.J. Rosengren, L.J. Montaner, L. Otvos Jr., Identification of crucial residues for the antibacterial activity of the proline-rich peptide, pyrrhocoricin, Eur. J. Biochem. 269 (2002) 4226–4237.

[37] G. Kragol, S. Lovas, G. Varadi, B.A. Condie, R. Hoffmann, L. Otvos Jr., The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding, Biochemistry 40 (2001) 3016–3026.

[38] L. Otvos Jr., I. O, M.E. Rogers, P.J. Consolvo, B.A. Condie, S. Lovas, P. Bulet, M. Blaszczyk-Thurin, Interaction between heat shock proteins and antimicrobial peptides, Biochemistry 39 (2000) 14150–14159.

[39] H. Steiner, D. Hultmark, A. Engstrom, H. Bennich, H.G. Boman, Sequence and specificity of two antibacterial proteins involved in insect immunity, Nature 292 (1981) 246–248.

[40] D. Hultmark, H. Steiner, T. Rasmuson, H.G. Boman, Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*, Eur. J. Biochem. 106 (1980) 7–16.

[41] D. Hultmark, A. Engstrom, H. Bennich, R. Kapur, H.G. Boman, Insect immunity: isolation and structure of cecropin D and four minor antibacterial components from *Cecropia pupae*, Eur. J. Biochem. 127 (1982) 207–217.

[42] J.Y. Lee, A. Boman, C.X. Sun, M. Andersson, H. Jornvall, V. Mutt, H.G. Boman, Antibacterial peptides from pig intestine: isolation of a

mammalian cecropin, Proc. Natl. Acad. Sci. U. S. A 86 (1989) 9159–9162.
[43] D. Andreu, R.B. Merrifield, H. Steiner, H.G. Boman, N-terminal analogues of cecropin A: synthesis, antibacterial activity, and conformational properties, Biochemistry 24 (1985) 1683–1688.
[44] J. Fink, R.B. Merrifield, A. Boman, H.G. Boman, The chemical synthesis of cecropin D and an analog with enhanced antibacterial activity, J. Biol. Chem. 264 (1989) 6260–6267.
[45] J. Fink, A. Boman, H.G. Boman, R.B. Merrifield, Design, synthesis and antibacterial activity of cecropin-like model peptides, Int. J. Pept. Protein Res. 33 (1989) 412–421.
[46] D. Andreu, J. Ubach, A. Boman, B. Wahlin, D. Wade, R.B. Merrifield, H.G. Boman, Shortened cecropin A-melittin hybrids. Significant size reduction retains potent antibiotic activity, FEBS Lett. 296 (1992) 190–194.
[47] H.G. Boman, D. Wade, I.A. Boman, B. Wahlin, R.B. Merrifield, Antibacterial and antimalarial properties of peptides that are cecropin-melittin hybrids, FEBS Lett. 259 (1989) 103–106.
[48] D. Wade, D. Andreu, S.A. Mitchell, A.M. Silveira, A. Boman, H.G. Boman, R.B. Merrifield, Antibacterial peptides designed as analogs or hybrids of cecropins and melittin, Int. J. Pept. Protein Res. 40 (1992) 429–436.
[49] C. Chicharro, C. Granata, R. Lozano, D. Andreu, L. Rivas, N-terminal fatty acid substitution increases the leishmanicidal activity of CA(1–7)M (2–9), a cecropin-melittin hybrid peptide, Antimicrob. Agents Chemother. 45 (2001) 2441–2449.
[50] J. Andra, O. Berninghausen, M. Leippe, Cecropins, antibacterial peptides from insects and mammals, are potently fungicidal against *Candida albicans*, Med. Microbiol. Immunol. (Berl) 189 (2001) 169–173.
[51] W.B. Borth, V.P. Jones, D.E. Ullman, J.S. Hu, Effects of synthetic cecropin analogs on in vitro growth of *Acholeplasma laidlawii*, Antimicrob. Agents Chemother. 45 (2001) 1894–1895.
[52] S.Y. Shin, J.H. Kang, K.S. Hahm, Structure-antibacterial, antitumor and hemolytic activity relationships of cecropin A-magainin 2 and cecropin A-melittin hybrid peptides, J. Pept. Res. 53 (1999) 82–90.
[53] H.M. Chen, W. Wang, D. Smith, S.C. Chan, Effects of the anti-bacterial peptide cecropin B and its analogs, cecropins B-1 and B-2, on liposomes, bacteria, and cancer cells, Biochim. Biophys. Acta 1336 (1997) 171–179.
[54] R. Ghiselli, A. Giacometti, O. Cirioni, F. Mocchegiani, F. Orlando, G. D'Amato, V. Sisti, G. Scalise, V. Saba, Cecropin B enhances betalactams activities in experimental rat models of gram-negative septic shock, Ann. Surg. 239 (2004) 251–256.
[55] H. Steiner, Secondary structure of the cecropins: antibacterial peptides from the moth *Hyalophora cecropia*, FEBS Lett. 137 (1982) 283–287.
[56] J.M. Bland, A.J. De Lucca, T.J. Jacks, C.B. Vigo, All-D-cecropin B: synthesis, conformation, lipopolysaccharide binding, and antibacterial activity, Mol. Cell Biochem. 218 (2001) 105–111.
[57] W. Wang, D.K. Smith, K. Moulding, H.M. Chen, The dependence of membrane permeability by the antibacterial peptide cecropin B and its analogs, CB-1 and CB-3, on liposomes of different composition, J. Biol. Chem. 273 (1998) 27438–27448.
[58] T.A. Holak, A. Engstrom, P.J. Kraulis, G. Lindeberg, H. Bennich, T. A. Jones, A.M. Gronenborn, G.M. Clore, The solution conformation of the antibacterial peptide cecropin A: a nuclear magnetic resonance and dynamical simulated annealing study, Biochemistry 27 (1988) 7620–7629.
[59] D. Sipos, M. Andersson, A. Ehrenberg, The structure of the mammalian antibacterial peptide cecropin P1 in solution, determined by proton-NMR, Eur. J. Biochem. 209 (1992) 163–169.
[60] C. Altenbach, W.L. Hubbell, The aggregation state of spin-labeled melittin in solution and bound to phospholipid membranes: evidence that membrane-bound melittin is monomeric, Proteins 3 (1988) 230–242.
[61] B. Bechinger, Structure and functions of channel-forming peptides: magainins, cecropins, melittin and alamethicin, J. Membr. Biol. 156 (1997) 197–211.
[62] B. Bechinger, The structure, dynamics and orientation of antimicrobial peptides in membranes by multidimensional solid-state NMR spectroscopy, Biochim. Biophys. Acta 1462 (1999) 157–183.
[63] C. Altenbach, W. Froncisz, J.S. Hyde, W.L. Hubbell, Conformation of spin-labeled melittin at membrane surfaces investigated by pulse saturation recovery and continuous wave power saturation electron paramagnetic resonance, Biophys. J. 56 (1989) 1183–1191.
[64] K.L. Piers, R.E. Hancock, The interaction of a recombinant cecropin/melittin hybrid peptide with the outer membrane of *Pseudomonas aeruginosa*, Mol. Microbiol. 12 (1994) 951–958.
[65] K.L. Piers, M.H. Brown, R.E. Hancock, Improvement of outer membrane-permeabilizing and lipopolysaccharide-binding activities of an antimicrobial cationic peptide by C-terminal modification, Antimicrob. Agents Chemother. 38 (1994) 2311–2316.
[66] C. Friedrich, M.G. Scott, N. Karunaratne, H. Yan, R.E. Hancock, Salt-resistant alpha-helical cationic antimicrobial peptides, Antimicrob. Agents Chemother. 43 (1999) 1542–1548.
[67] M.G. Scott, H. Yan, R.E. Hancock, Biological properties of structurally related alpha-helical cationic antimicrobial peptides, Infect. Immun. 67 (1999) 2005–2009.
[68] J.G. Sawyer, N.L. Martin, R.E.W. Hancock, Interaction of macrophage cationic proteins with the outer membrane of *Pseudomonas aeruginosa*, Infect. Immun. 56 (2003) 693–698.
[69] M. Gough, R.E. Hancock, N.M. Kelly, Antiendotoxin activity of cationic peptide antimicrobial agents, Infect. Immun. 64 (1996) 4922–4927.
[70] M.G. Scott, M.R. Gold, R.E. Hancock, Interaction of cationic peptides with lipoteichoic acid and gram-positive bacteria, Infect. Immun. 67 (1999) 6445–6453.
[71] J. Alberola, A. Rodriguez, O. Francino, X. Roura, L. Rivas, D. Andreu, Safety and efficacy of antimicrobial peptides against naturally acquired leishmaniasis, Antimicrob. Agents Chemother. 48 (2004) 641–643.
[72] D. Sipos, K. Chandrasekhar, K. Arvidsson, A. Engstrom, A. Ehrenberg, Two-dimensional proton-NMR studies on a hybrid peptide between cecropin A and melittin. Resonance assignments and secondary structure, Eur. J. Biochem. 199 (1991) 285–291.
[73] D. Oh, S.Y. Shin, J.H. Kang, K.S. Hahm, K.L. Kim, Y. Kim, NMR structural characterization of cecropin A(1–8) - magainin 2(1–12) and cecropin A (1–8) - melittin (1–12) hybrid peptides, J. Pept. Res. 53 (1999) 578–589.
[74] P. Juvvadi, S. Vunnam, E.L. Merrifield, H.G. Boman, R.B. Merrifield, Hydrophobic effects on antibacterial and channel-forming properties of cecropin A-melittin hybrids, J. Pept. Sci. 2 (1996) 223–232.
[75] B. Christensen, J. Fink, R.B. Merrifield, D. Mauzerall, Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 5072–5076.
[76] Y. Shai, Mode of action of membrane active antimicrobial peptides, Biopolymers 66 (2002) 236–248.
[77] H.W. Huang, Action of antimicrobial peptides: two-state model, Biochemistry 39 (2000) 8347–8352.
[78] J.F. Ellena, M.C. Burnitz, D.S. Cafiso, Location of the myristoylated alanine-rich C-kinase substrate (MARCKS) effector domain in negatively charged phospholipid bicelles, Biophys. J. 85 (2003) 2442–2448.
[79] K.G. Victor, D.S. Cafiso, Location and dynamics of basic peptides at the membrane interface: electron paramagnetic resonance spectroscopy of tetramethyl-piperidine-N-oxyl-4-amino-4-carboxylic acid-labeled peptides, Biophys. J. 81 (2001) 2241–2250.
[80] S. Ozdirekcan, D.T. Rijkers, R.M. Liskamp, J.A. Killian, Influence of flanking residues on tilt and rotation angles of transmembrane peptides in lipid bilayers. A solid-state 2H NMR study, Biochemistry 44 (2005) 1004–1012.
[81] M. Barranger-Mathys, D.S. Cafiso, Membrane structure of voltage-gated channel forming peptides by site-directed spin-labeling, Biochemistry 35 (1996) 498–505.
[82] C.S. Kim, D.H. Kweon, Y.K. Shin, Membrane topologies of neuronal SNARE folding intermediates, Biochemistry 41 (2002) 10928–10933.
[83] D.H. Kweon, Y. Chen, F. Zhang, M. Poirier, C.S. Kim, Y.K. Shin, Probing domain swapping for the neuronal SNARE complex with electron paramagnetic resonance, Biochemistry 41 (2002) 5449–5452.
[84] J.C. Macosko, C.H. Kim, Y.K. Shin, The membrane topology of the

fusion peptide region of influenza hemagglutinin determined by spin-labeling EPR, J. Mol. Biol. 267 (1997) 1139–1148.
[85] X. Han, J.H. Bushweller, D.S. Cafiso, L.K. Tamm, Membrane structure and fusion-triggering conformational change of the fusion domain from influenza hemagglutinin, Nat. Struct. Biol. 8 (2001) 715–720.
[86] H.S. Mchaourab, J.S. Hyde, J.B. Feix, Aggregation state of spin-labeled cecropin AD in solution, Biochemistry 32 (1993) 11895–11902.
[87] F.M. Marassi, S.J. Opella, P. Juvvadi, R.B. Merrifield, Orientation of cecropin A helices in phospholipid bilayers determined by solid-state NMR spectroscopy, Biophys. J. 77 (1999) 3152–3155.
[88] L. Silvestro, P.H. Axelsen, Membrane-induced folding of cecropin A, Biophys. J. 79 (2000) 1465–1477.
[89] E. Gazit, I.R. Miller, P.C. Biggin, M.S. Sansom, Y. Shai, Structure and orientation of the mammalian antibacterial peptide cecropin P1 within phospholipid membranes, J. Mol. Biol. 258 (1996) 860–870.
[90] J.B. Feix, C.S. Klug, Site-directed spin labeling of membrane proteins and peptide-membrane interactions, in: L.J. Berliner (Ed.), Biological Magnetic Resonance, vol. 14, Plenum Press, New York, 1998, pp. 252–281.
[91] W.L. Hubbell, D.S. Cafiso, C. Altenbach, Identifying conformational changes with site-directed spin labeling, Nat. Struct. Biol. 7 (2000) 735–739 (Review).
[92] K.J. Hallock, D.K. Lee, A. Ramamoorthy, MSI-78, an analogue of the magainin antimicrobial peptides, disrupts lipid bilayer structure via positive curvature strain, Biophys. J. 84 (2003) 3052–3060.
[93] K. Matsuzaki, O. Murase, H. Tokuda, S. Funakoshi, N. Fujii, K. Miyajima, Orientational and aggregational states of magainin 2 in phospholipid bilayers, Biochemistry 33 (1994) 3342–3349.
[94] A. Ramamoorthy, F.M. Marassi, M. Zasloff, S.J. Opella, Three-dimensional solid-state NMR spectroscopy of a peptide oriented in membrane bilayers, J. Biomol. NMR 6 (1995) 329–334.
[95] K. Matsuzaki, Y. Mitani, K.Y. Akada, O. Murase, S. Yoneyama, M. Zasloff, K. Miyajima, Mechanism of synergism between antimicrobial peptides magainin 2 and PGLa, Biochemistry 37 (1998) 15144–15153.
[96] W.T. Heller, K. He, S.J. Ludtke, T.A. Harroun, H.W. Huang, Effect of changing the size of lipid headgroup on peptide insertion into membranes, Biophys. J. 73 (1997) 239–244.
[97] F.Y. Chen, M.T. Lee, H.W. Huang, Evidence for membrane thinning effect as the mechanism for peptide-induced pore formation, Biophys. J. 84 (2003) 3751–3758.
[98] A. Mecke, D.K. Lee, A. Ramamoorthy, B.G. Orr, M.M. Banaszak Holl, Membrane thinning due to antimicrobial peptide binding—An atomic force microscopy study of MSI-78 in lipid bilayers, Biophys. J. (2005).
[99] M.T. Lee, F.Y. Chen, H.W. Huang, Energetics of pore formation induced by membrane active peptides, Biochemistry 43 (2004) 3590–3599.
[100] K. He, S.J. Ludtke, D.L. Worcester, H.W. Huang, Neutron scattering in the plane of membranes: structure of alamethicin pores, Biophys. J. 70 (1996) 2659–2666.
[101] G. Baumann, P. Mueller, A molecular model of membrane excitability, J. Supramol. Struct. 2 (1974) 538–557.
[102] L. Yang, T.A. Harroun, T.M. Weiss, L. Ding, H.W. Huang, Barrel-stave model or toroidal model? A case study on melittin pores, Biophys. J. 81 (2001) 1475–1485.
[103] K. He, S.J. Ludtke, W.T. Heller, H.W. Huang, Mechanism of alamethicin insertion into lipid bilayers, Biophys. J. 71 (1996) 2669–2679.
[104] K. He, S.J. Ludtke, H.W. Huang, D.L. Worcester, Antimicrobial peptide pores in membranes detected by neutron in-plane scattering, Biochemistry 34 (1995) 15614–15618.
[105] W.T. Heller, A.J. Waring, R.I. Lehrer, T.A. Harroun, T.M. Weiss, L. Yang, H.W. Huang, Membrane thinning effect of the beta-sheet antimicrobial protegrin, Biochemistry 39 (2000) 139–145.
[106] A. Spaar, C. Munster, T. Salditt, Conformation of peptides in lipid membranes studied by X-ray grazing incidence scattering, Biophys. J. 87 (2004) 396–407.
[107] H. Duclohier, G. Molle, G. Spach, Antimicrobial peptide magainin I from *Xenopus* skin forms anion-permeable channels in planar lipid bilayers, Biophys. J. 56 (1989) 1017–1021.
[108] B.L. Kagan, M.E. Selsted, T. Ganz, R.I. Lehrer, Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 210–214.
[109] F. Porcelli, B. Buck, D.K. Lee, K.J. Hallock, A. Ramamoorthy, G. Veglia, Structure and orientation of pardaxin determined by NMR experiments in model membranes, J. Biol. Chem. 279 (2004) 45815–45823.
[110] P. Mueller, D.O. Rudin, Action potentials induced in biomolecular lipid membranes, Nature 217 (1968) 713–719.
[111] D.S. Cafiso, Alamethicin: a peptide model for voltage gating and protein–membrane interactions [Review], Annu. Rev. Biophys. 23 (1994) 141–165.
[112] M. Barranger-Mathys, D.S. Cafiso, Collisions between helical peptides in membranes monitored using electron paramagnetic resonance: evidence that alamethicin is monomeric in the absence of a membrane potential, Biophys. J. 67 (1994) 172–176.
[113] V. Rizzo, S. Stankowski, G. Schwarz, Alamethicin incorporation in lipid bilayers: a thermodynamic study, Biochemistry 26 (1987) 2751–2759.
[114] K. Matsuzaki, O. Murase, N. Fujii, K. Miyajima, An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation, Biochemistry 35 (1996) 11361–11368.
[115] S.J. Ludtke, K. He, W.T. Heller, T.A. Harroun, L. Yang, H.W. Huang, Membrane pores induced by magainin, Biochemistry 35 (1996) 13723–13728.
[116] K. Matsuzaki, O. Murase, N. Fujii, K. Miyajima, Translocation of a channel-forming antimicrobial peptide, magainin 2, across lipid bilayers by forming a pore, Biochemistry 34 (1995) 6521–6526.
[117] A.S. Ladokhin, M.E. Selsted, S.H. White, Sizing membrane pores in lipid vesicles by leakage of co-encapsulated markers: pore formation by melittin, Biophys. J. 72 (1997) 1762–1766.
[118] T. Katsu, C. Ninomiya, M. Kuroko, H. Kobayashi, T. Hirota, Y. Fujita, Action mechanism of amphipathic peptides gramicidin S and melittin on erythrocyte membrane, Biochim. Biophys. Acta 939 (1988) 57–63.
[119] Y.P. Zhang, R.N. Lewis, R.S. Hodges, R.N. McElhaney, Peptide models of helical hydrophobic transmembrane segments of membrane proteins: II. Differential scanning calorimetric and FTIR spectroscopic studies of the interaction of Ac-K2-(LA)12-K2-amide with phosphatidylcholine bilayers, Biochemistry 34 (1995) 2362–2371.
[120] C. Toniolo, C. Peggion, M. Crisma, F. Formaggio, X. Shui, D.S. Eggleston, Structure determination of racemic trichogin A IV using centrosymmetric crystals, Nat. Struct. Biol. 1 (1994) 908–914.
[121] T. Benachir, M. Lafleur, Osmotic and pH transmembrane gradients control the lytic power of melittin, Biophys. J. 70 (1996) 831–840.
[122] E. Hantz, A. Cao, J. Escaig, E. Taillandier, The osmotic response of large unilamellar vesicles studied by quasielastic light scattering, Biochim. Biophys. Acta 862 (1986) 379–386.
[123] F.R. Hallett, J. Marsh, B.G. Nickel, J.M. Wood, Mechanical properties of vesicles. II. A model for osmotic swelling and lysis, Biophys. J. 64 (1993) 435–442.
[124] K.J. Hallock, D.K. Lee, J. Omnaas, H.I. Mosberg, A. Ramamoorthy, Membrane composition determines pardaxin's mechanism of lipid bilayer disruption, Biophys. J. 83 (2002) 1004–1013.
[125] K.A. Henzler-Wildman, G.V. Martinez, M.F. Brown, A. Ramamoorthy, Perturbation of the hydrophobic core of lipid bilayers by the human antimicrobial peptide LL-37, Biochemistry 43 (2004) 8459–8469.
[126] R.F. Epand, A. Ramamoorthy, R.M. Epand, Membrane lipid composition and the interaction of pardaxin: the role of cholesterol, Protein Pept. Lett. 13 (2006) 1–5.
[127] A.L. Koch, M.F. Pinette, Nephelometric determination of turgor pressure in growing gram-negative bacteria, J. Bacteriol. 169 (1987) 3654–3663.
[128] K.A. Henzler Wildman, D.K. Lee, A. Ramamoorthy, Mechanism of lipid bilayer disruption by the human antimicrobial peptide, LL-37, Biochemistry 42 (2003) 6545–6558.
[129] L. Dickinson, V. Russell, P.E. Dunn, A family of bacteria-regulated, cecropin D-like peptides from *Manduca sexta*, J. Biol. Chem. 263 (1988) 19424–19429.
[130] L. Whitmore, B.A. Wallace, DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data, Nucleic Acids Res. 32 (2004) W668–W673.