Review

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Insights into the mechanisms of action of host defence peptides from biophysical and structural investigations‡

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In order to better understand the mechanisms of action of linear cationic host defense peptides, biophysical and structural investigations of their interactions with membranes and with other biomacromolecules are reviewed. In particular, an extensive overview will be given of the topological studies of magainins in a number of different lipid environments. Furthermore, amphipathic helices have been designed in such a manner to allow the easy control of their membrane alignment. These peptides not only exhibit potent antimicrobial and transfection activities, but their investigation has also provided important insights into mechanistic aspects of their biological functions. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: magainin; alamethicin; solid-state NMR; uniaxially oriented membranes; membrane topology; hydrophobic mismatch; cecropin; in-plane orientation; transmembrane alignment; peptide-lipid interactions; PGLa; peptaibol; membrane pore; membrane macroscopic phase; equlibria

The continuously increasing resistance of pathogens against many of the commonly used antibiotics imposes new challenges to human health [1,2]. Possible alternatives can be found by exploring natural resources, and interesting antimicrobial compounds have indeed been discovered in the plant [3,4] and in the animal kingdoms [5,6]. During the past decades, a large variety of different host defence peptides have been identified that are produced when infections occur and/or which are stored in exposed tissues of animals and plants. These molecules protect the respective organisms by establishing a defence system that can react in a fast and efficient manner [7,8]. More recently they have also been shown to exhibit immunomodulatory functions and in order to take into account their extended spectrum of activities, they are now often referred to as 'host defense peptides' [9–11]. These naturally occurring bacteriocidal and fungicidal molecules can serve as a templates for the development of more easy to produce and/or more potent analogues [12–16]. To this end structure–function studies and biophysical investigations are performed that result in a better understanding of their mechanisms of action [12,17–21].

Early on, peptides from frogs and insects were among the first to be identified and the ones investigated most thoroughly to date are probably the members of the magainin and cecropin families [22–26] (*cf* Table 1 for the amino acid sequences of the peptides discussed in this article). These cationic linear peptides exhibit a broad-range of antimicrobial activities when at the same time their detailed spectrum of antimicrobial action varies with their sequence. Interestingly, it has been demonstrated that several host defence peptides, including magainins, also exhibit virucidal, spermicidal and anti-cancer activities [6,15,27–30].

Magainins, cecropins and related sequences carry an overall positive charge and are characterized by pronounced interactions with phospholipid membranes, where many of them adopt amphipathic α-helical conformations [20,31–35]. By interacting with phospholipid bilayers they have been shown to disrupt the bilayer integrity, to cause openings, a decrease in ohmic resistance and a concomitant collapse of the transmembrane electrochemical gradients [36,37]. Therefore, it is believed that their main mechanism of killing bacterial and fungal cells is the formation of pores and the concomitant effects on cellular respiration [38], events which deprive the affected organisms of their source of energy [39].

The notion that the membranes are the main target of these antimicrobial compounds is supported by investigations of enantiomers such as all-D-magainins, all-D-cecropins, cecropins with inverted sequences (retro) or inversed D-cecropins (retroenantio), which all possess the high antibiotic and pore-forming activities of the parent L-sequence. These observations suggest that the cell-killing activities of these peptides are related to direct interactions with, for example, phospholipid membranes rather than through specific, chiral receptor interactions [40]. A number of models have been suggested to explain the pore forming and antimicrobial properties of these catonic amphipathic peptides. These are reviewed in some detail in Ref. 41 and illustrated within Figure 1. The models include the formation of torrodial pores where the

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Abbreviations used: *DMPC, 1, 2-dimyristoyl-sn-glycero-3-phosphocholine; DMPG, 1, 2-dimyristoyl-sn-glycero-3- phospho-(1′-rac-glycerol); IP, in-plane; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol); r.h., relative humidity; TM, transmembrane.*

Journal of PeptideScience

Biography

Burkhard Bechinger obtained his PhD in 1989 at the Biocenter of the University of Basel, Switzerland on the investigation of electrostatic interactions within lipid bilayers using membrane biophysical approaches, in particular solid-state NMR spectroscopy. During his postdoctoral stay at the University of Pennsylvania he used and developed the latter technique during the investigation of membrane-associated polypeptides (1990–93). He started his own research group in 1993 at the Max Planck Institute of molecular physiology, Dortmund Germany and expanded size of his team when being nominated head of an Independent Junior Research Group at the MPI of Biochemistry in Martinsried, Germany (1995–2001). Since 2001 he is full professor at the chemistry department of the University of Strasbourg, France where his team designs and studies peptides with different biological activities as well as membrane proteins using NMR spectroscopy and a variety of other biophysical approaches.

peptides together with the lipid assemble into a supramolecular arrangement of high curvature thereby transiently forming well-defined pores [42,43]. A second model suggests that the peptides accumulate at the membrane at an alignment parallel to the surface [44]. Once this 'carpet' becomes too dense the membrane disintegrates and opens. Third, at lower peptide concentrations stochastic fluctuations of the in-planar peptides within the membrane surface can explain the transient and step-wise increases in membrane conductivity that have been observed experimentally [41]. Finally a unifying view integrates all these different arrangements into a phase diagram as shown schematically in Figure 1 [41,45,46], a model which will be discussed in more detail below.

It should be noted that more recent evidence also points to the existence of intracellular targets albeit also in this case it

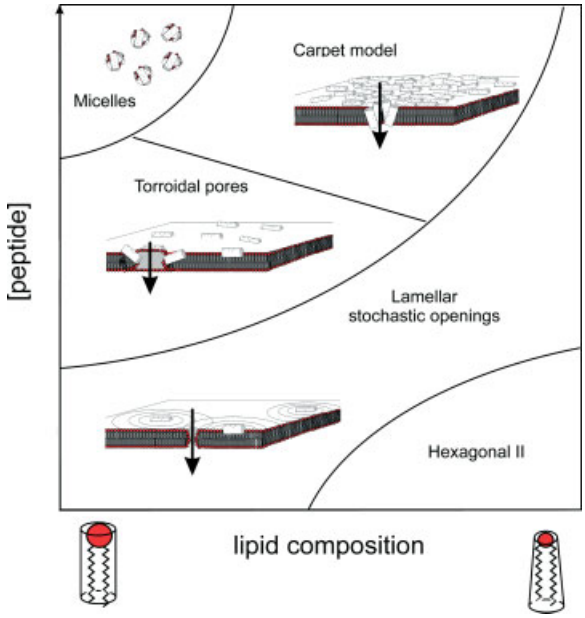

Figure 1. A schematic phase diagram is shown where the supramolecular assemblies of peptide–lipid mixtures are illustrated as a function of the concentration of membrane-associated peptide and the lipid composition. Within the diagram a selected number of models are shown that have been suggested to explain pore formation, membrane lysis and antimicrobial action (*cf* text and Refs 41,46 for additional details). Note that the diagram is intended to illustrate the concept rather than to provide detailed phase boundaries. Depending on the peptide sequence the torroidal pore and/or the carpet model may be applicable. Furthermore, torroidal pores may also be formed with peptide alignments parallel to the membrane normal.

is necessary that the peptides cross the plasma membranes of the bacterial, fungal or cancer cells. Once inside the cell they can interact with biological macromolecules and/or organelles (for reviews refer [15,27,28,47,48]). In this article biophysical investigations of the membrane interactions of magainins and related peptides are reviewed with some focus on the work performed in our own laboratory.

Table 1. Sequences of peptides presented in this review

Magainin 2	GIGKF LHSAK KFGKA FVGEI MNS-NH_2
PGLa	GMASK AGAIA GKIAK VALKA L-NH_2
Cecropin A	KWKLF KKIEK VGQNI RDGII KAGPA VAVVG QATQI AK-NH_2
LAH4	KKALL ALALH HLAHL ALHLA LALKK A-NH_2
Distinctin chain 1	E NREVP PGFTA LIKTL RKCKI I
Distinctin chain 2	NLVSG LIEAR KYLEQ LHRKL KNCKV
Alamethicin (F50/7)	Ac-Aib-Pro-Aib-Ala-Aib-Aib-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Phl
Ampullosporin A	Ac-Trp-Ala-Aib-Aib-Leu-Aib-Gln-Aib-Aib-Aib-Gln-Leu-Aib-Gln-Lol
Zervamicin IIB	Ac-Trp-Ile-Gln-Iva-Ile-Thr-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phl

The peptides composed exclusively of the 20 conventional amino acid residues are presented by the one-letter code for better visibility and the peptaibols are listed by the three letter code. The following abbreviations are used for the non-standard residues of the latter compounds: Aib, α-aminoisobutyric acid; Iva, D-isovaline; Hyp, *trans*-4-hydroxy-L-proline; Phl, L-phenylalaninol; Lol, L-leucinol. The *N*- and *C*-terminal protecting groups are Ac- for acetyl- and NH_2 for the carboxyamide, respectively. The two chains of the disticintin heterodimer are covalently connected by a cystine bond close to their *C*-terminus (the two sequences have been aligned accordingly).

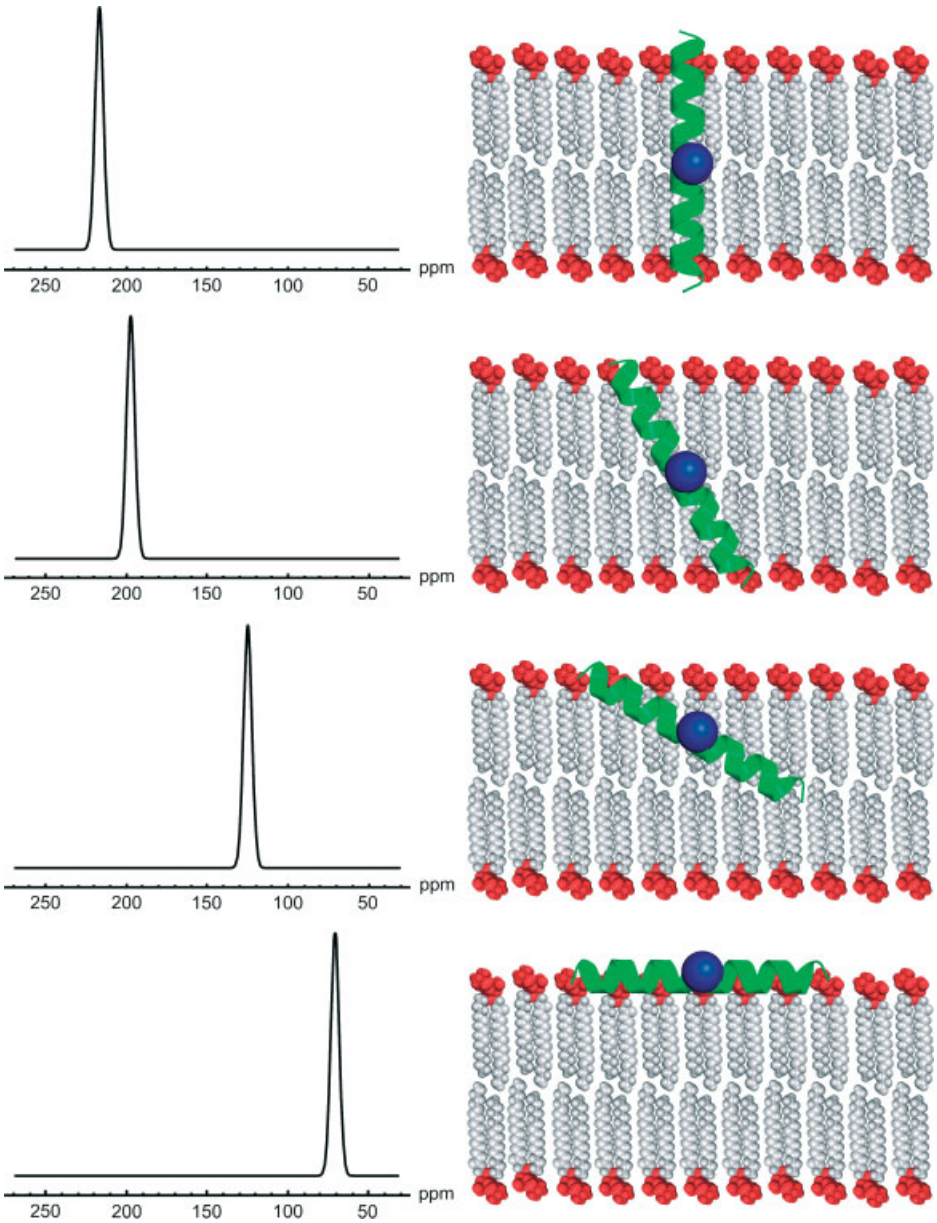

Figure 2. The correlation between the ^{15}N chemical shift and the membrane topology of labelled helices is shown. These experiments require that peptides are labelled with ^{15}N at one of their amide bonds within the helical domain, reconstituted into uniaxially oriented phospholipid bilayers [63] and the proton-decoupled ^{15}N solid-state NMR spectra recorded. Whereas transmembrane helices resonate around 200 ppm those that are oriented parallel to the surface exhibit ^{15}N chemical shifts <100 ppm [64]. The technique has early on revealed an alignment of magainins as well as of other cationic amphipathic peptides parallel to the bilayer surface (^{15}N chemical shifts <100 ppm; Table 2).

Investigations of the Membrane Topology of Host Defence Peptides

Among the linear cationic antimicrobial peptides, magainins are probably the ones that have been investigated most intensely by biophysical approaches including solid-state NMR spectroscopy (Figure 2) ([49–53]). Using a variety of techniques a number of cationic host defence peptides were found to preferentially align parallel to the surface when reconstituted into a wide variety of phospholipid bilayers (Table 2), a finding that agrees well with their amphipathic and highly charged character (reviewed in Ref. 41). This alignment of the peptides parallel to the membrane surface was early on described [50,54,55], is consistently observed also for magainin analogues [56–58] as well as for a number of related peptides that were investigated more recently [32,59–61]. Indeed, molecular modelling calculations visualize how magainin 2 can cause the formation of membrane lipidic pores without the need to adopt transmembrane orientations or peptide–peptide contacts [62]. To understand how the peptides form pores in such a configuration requires a more profound analysis and the consideration of novel mechanisms involving stochastic fluctuations, membrane phase properties as well as modulations of the bilayer shape and physical chemistry. A number of biophysical investigation that have altered our view on how these peptides interact with membranes are reviewed in Ref. 41 and a selection of them as well as some investigations performed thereafter will also be presented in this article.

Journal of PeptideScience

Table 2. Solid-state NMR and oriented circular dichroism investigations of magainin 2 and PGLa in oriented bilayers

Lipid composition	Peptide concentration (mole%)	Labelled site/s	NMR nucleus/OCD[a]	Hydration (r.h.)	Tilt angle (°)	References
Magainin 2						
POPC/POPG 3 : 1	3	A15	^{15}N	93	IP	54
POPE/POPG 3 : 1	3	I2	^{15}N	93	IP	65
POPC/POPG 3 : 1	–	–	–	–	IP	–
POPC	–	–	–	–	IP	–
POPC/POPG/chol 3 : 1:4	–	–	–	–	IP[b]	–
POPE/POPG 3 : 1	3	8 sites	^{15}N	93	90[c]	66
POPC/POPG 4 : 1	4	F16,V17	^{15}N	93	IP	67
POPC/POPG 3 : 1	–	F16	^{15}N	–	IP	68
	–	U–^{15}N	^{15}N	–	90	69
DMPC/DMPG 3 : 1	<2	–	OCD[a]	–	IP	70
	3.3	–	–	–	20% TM	–
	10	–	–	–	Mostly TM	–
PGLa						
POPE/POPG 3 : 1	2	Several	^{15}N	93	IP[d]	49
POPC/POPG 3 : 1	–	A20	^{15}N	–	IP	67
DMPC	0.5	Several[e]	^{19}F, ^{2}H	–	89–97[f]	71–73
	2	–	–	–	55–57[f]	–
PGLa in the presence of equimolar amounts of magainin2						
phosphatidylcholines[g]	2	A10, A14	15N	95,100	5 topologies[g]	–
DMPC/DMPG 3 : 1	2	Several[e]	^{2}H	–	20	74
DMPC	2	Several[e]	^{2}H	–	20–22	71

DMPG, 1,2-dimyristoyl-*sn*-glycero-3- phospho-(1′-*rac*-glycerol); IP, in-plane; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); r.h., relative humidity; TM, transmembrane.
[a] Oriented circular dichroism.
[b] In the presence of cholesterol the chemical shift increases by 16 ppm indicating less tilted alignments.
[c] Structure calculation using eight parameters from four consecutive sites indicates an α-helical conformation oriented parallel to the surface.
[d] The analysis of powder pattern line shapes indicates that residues 3 and 6 exhibit a high degree of motion, whereas the *C*-terminus is immobilized.
[e] In contrast to ^{15}N solid-state NMR spectroscopy, labelling of several sites is required to obtain topological information from ^{19}F or ^{2}H solid-state NMR spectra.
[f] The transition between the IP (tilt angle around 90°) and the more 'tilted' state (tilt angle 55°) is favoured by the addition of DMPG, a reduction of r.h. and/or by increasing the peptide/lipid ratio.
[g] When the PGLa alignment is tested as a function of membrane hydrophobic thickness (di-C20:1-PC, POPC, DMPC, di-C12:0-PC and di-C10:0-PC) five different configurations have been observed ranging from IP to TM (Salnikov & Bechinger, unpublished).

For example, the heterodimeric peptide distincin has been studied in oriented phospholipid membranes using a combination of ^{15}N and ^{2}H solid-state NMR spectroscopy [60]. As the two nuclei provide highly complementary information, it is possible to obtain accurate tilt- and rotational pitch angular information from a peptide labelled simultaneously with $^{2}H_3$-alanine and at a ^{15}N amide position [75]. The solid-state NMR measurements indicate that the distinctin dimer undergoes pronounced conformational changes when inserting into the membrane [60]. In the bilayer environment the amphipathic helix of chain 2 aligns at a tilt angle of 88° relative to the membrane normal thereby anchoring the polypeptide in a stable fashion. In contrast, chain 1 is more loosely associated with the lipid bilayer at a tilt angle of 66°. When compared to the membrane interactions of the isolated chain 1 this latter value is decreased by about 5° in the heterodimer. Functional studies in combination with this structural data suggest that the membrane interactions of distinctin are dominated by chain 2. It has been suggested that the role of chain 1 is to protect the peptide in solution from proteases through a compact dimer of dimer arrangement [76]. The studies on dimers thereby provide valuable insight into the role of peptide–peptide and peptide–lipid interactions within the membrane environment (*cf* also Ref. 13).

Histidine-Rich Models for Amphipathic Linear Peptide Antimicrobials

In order to further test if in-planar configurations explain both the pore formation and the antimicrobial activities of amphipathic peptides the so-called LAH4 sequence was designed in which the polar face consists of histidines rather than lysines and where the hydrophobic region consists of alanine and leucines (Table 1). Two lysines at each terminus assure good solubility of the peptides in aqueous environments. The histidines exhibit pK values around 5.5 and this modification therefore allows one to tune the hydrophobic moment of these sequences by changing the pH [35]. These histidine-rich sequences exhibit a high propensity to adopt α-helical conformations in membrane environments [35,77]. The regions encompassing the α-helical structures in the presence of dodecylphosphocholine micelles environments are pH-dependent and shift from a *C*-terminal (encompassing residues 9–24 at pH 4.1) to a more *N*-terminal localization (residues 4–21 at pH 7.8). At intermediate pH two shorter helices are interrupted by a hinge region formed by residues 10–13 [35], and it is believed that this flexible domain facilitates the membrane insertion during the in-plane to transmembrane transition.

When the topologies of LAH4 peptides were investigated in oriented phospholipid bilayers an alignment parallel to the bilayer surface was observed at pH <6 when the histidines are cationic, whereas transmembrane orientations are found when the histidines are discharged [78,79]. As the transition is reversible it is possible to evaluate the transfer energy of amino acid side chains from the membrane interface to the membrane interior. This has been achieved by modulating the composition of the polar face through amino acid replacements and by analysing the resulting shifts in the transition pH [80,81]. Furthermore, dynamic light scattering indicates that the peptides form suspensions of small α-helical aggregates at neutral pH, but exhibit a hydrodynamic radius which agrees with an extended monomer in acidic solutions [82].

Interestingly, members of the LAH4 family of peptides exhibit membrane pore-formation in model membranes [82] and antimicrobial action at both neutral and, to an even larger extent, at acidic pH thereby indicating that a well-defined transmembrane channel structure is not required to explain its biological actvities [77]. More recent investigations show that derivatives of LAH4 exhibit potent antimicrobial action against a number of clinical isolates, and these activities are more pronounced when the peptides occur in an in-planar orientation [83]. This has allowed for the design of a third generation of LAH4 peptides exhibiting interesting antimicrobial and antiplasmodial activities also under *in vivo* conditions [58].

Different Supramolecular Assemblies are Formed by Antimicrobial Peptide–Lipid Mixtures

The experimentally observed in-planar topology of amphipathic peptides is concomitant with an alignment of their hydrophobic moment parallel to the bilayer normal. Structure–activity studies has revealed that the pore-forming and antimicrobial activities of the peptides correlate with this topology [77]. The interaction of the peptides with the membrane interface results in a decreased order parameter of the phospholipid fatty acyl chains and considerable curvature strain on the membrane [84–86]. The peptide–lipid interactions thereby resemble in many aspects the membrane interactions of detergents or detergent-like molecules [46]. At high peptide concentrations such interactions cause the disruption of the bilayer integrity as suggested by the 'carpet model' [44]. However, it should be kept in mind that at lower detergent (peptide) concentrations their effect on the membrane supramolecular assembly may be quite different and range from channel formation to an even more stable lipid bilayer packing [41,46], the end result being much dependent on a variety of environmental factors such as lipid composition, peptide concentration, pH and temperature (Figure 1).

On the basis of the experimental findings it has been suggested that, in analogy to detergents, a full description of the peptide–lipid interactions requires extensive phase diagrams (Figure 1) where the previously suggested mechanisms such as carpet- [44], torroidal pore-, wormhole- [42,43] and lysis model are represented by distinct areas [41,46]. Notably, our improved understanding of the peptide–lipid interactions, and in particular the realization that the peptides are active in their surface-associated state, has been used to successfully design short sequences as well as peptide mimetics with potent antimicrobial properties [87–94].

The Effects of Membrane Lipid Composition on Antimicrobial Peptides

An important variable of such phase diagrams is the lipid composition of the membrane which is indeed quite different when bacterial and eukaryotic membranes, or when membranes from different species are compared to each other [46]. Differences in the macroscopic phase properties can therefore be at least a part of the explanation how antimicrobial peptides kill one species but not another or how they select bacterial over eukaryotic cells [46]. In particular, the surface charge density of the membrane has been shown to exhibit an important effect on the amount of membrane-associated peptide [95] and in view of the presence of negatively charged lipopolysaccarides and anionic lipids at the outer surface of bacterial cells, electrostatic interactions have been suggested to be an important determinant for the selectivity of the peptides for bacterial over eukaryotic and for tumour over healthy cells [95–99].

When the existing data for magainin 2 are reviewed the peptide exhibits stable in-planar membrane alignments under all conditions so far investigated (Table 2) with only subtle changes that have been detected in the presence of cholesterol [65]. In contrast, the membrane interactions of PGLa in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) membranes are dependent on the peptide-to-lipid ratio and bilayer hydration, with two distinct alignments, one in-planar and another one whose tilt angle differs by about 30° (Table 2).

Despite its stable in-planar topology some interesting differences have also been observed for magainin even when quite similar lipid systems, such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and DMPC bilayers, are compared to each other. First, neutron diffraction experiments indicate that in the presence of this peptide water penetrates the hydrophobic region of DMPC but not POPC membranes [42]. Second, subtle differences have been observed when the macroscopic phase properties of membranes composed of these lipids have been studied as a function of magainin 2 concentration by ^{31}P solid-state NMR spectroscopy [100].

In contrast to most charged amphipathic sequences that have been shown to adopt stable surface-associated states [32,50,54–61], a range of different topologies has been observed for other membrane-associated peptides, in particular the less polar sequences [101–104]. Therefore, a full description of amphipathic peptides needs to consider a series of equilibria where they move from the water phase to an interface-associated state, followed by membrane insertion and oligomerisation [105–108]. Solid-state NMR spectroscopy (Figure 2) and oriented circular dichroism have proven valuable techniques to investigate such transitions between in-plane and transmembrane alignments where the membrane topologies are a function of hydration, peptide-to-lipid ratio and lipid composition [78,104,109,110]. A prime example is the LAH4 peptide described above, which changes alignment in a pH-dependent fashion and has thereby allowed us to study the in-plane to transmembrane equilibrium in a highly controlled manner [78–81]. Other examples are found within the peptaibol family (Table 1), amphipathic helical peptides containing Aib residues and other predominatly uncharged amino acid side chains [111]. These peptides have been described to form channels after having undergone a number of topological transitions [17,106].

The uncharged dodecameric alamethicin peptide (*cf* Table 1), paradigm for the formation of pores by the association into transmembrane helical bundles, exhibits indeed transmem-

brane alignments in DMPC and POPC [112–114], but not in phosphatidylethanolamine membranes [104,115]. For other peptaibols, such as the 15 residue zervamicin II or ampullosporin A it has been shown that their alignment depends on the thickness of the phosphatidylcholine membranes and that they exhibit in-planar alignments in POPC or DMPC but transmembrane orientations in membranes composed of fatty acyl chains of 12 or 10 carbon atoms [114,116]. As it is generally believed that 1-palmitoyl-2-oleoyl-phospholipids represent well the hydrophobic thickness and the fatty acyl composition of natural membranes the question therefore arises if the antimicrobial effect of even these more hydrophobic peptides is solely related to the formation of transmembrane helical bundles, or if their spectrum of membrane-pore forming activities also encompasses a number of additional possibilities that are related to the carpet, wormhole/torroidal pore or detergent-like models established for the cationic linear peptides (Figure 1) [41,42,44,46]?

It is of interest to note that in membranes composed of dimyristoylphospholipids PGLa undergoes a transition from in-planar and 'tilted' alignments to a transmembrane orientation upon addition of equimolar amounts of magainin 2 [71] which has been correlated to the synergistic enhancements of antimicrobial activities which are observed when magainin 2 and PGLa are tested in combination with antimicrobial assays [20,117,118]. Synergistic enhancements have also been observed for other mixtures of antimicrobial compounds [119–121]. As both PGLa and magainin 2 exhibit in-planar topologies when equimolar mixtures of the two peptides are investigated in POPC bilayers (Salnikov & Bechinger, unpublished) it remains unclear how such synergies arise but one could imagine that one component of a mixture has a strong potential to weaken the membrane or the lipopolysaccaride layer thereby allowing the passage of a second component. The site of action can thus be intracellular or a membrane component which would otherwise remain inaccessible to the latter [47].

Peptide-Induced Condensation and Flocculation Phenomena

It is noteworthy that cationic peptides have been demonstrated to efficiently complex and condensate nucleic acids [122,123]. Among those, the designed antimicrobial peptide LAH4 has been investigated in some detail and it has been shown that this peptide exhibits potent transfection activities for DNA and siRNA [124,125]. Structural investigations indicate that in the DNA transfection complex about one peptide associates with two base pairs predominantly by electrostatic interactions [126], a density that is reduced by almost 50% upon acidification of the medium such as it occurs in the endosome [127]. The liberated peptide is thus available for membrane interactions and lysis of the endosomal compartment concomitant with the release of the nucleic acid into the cell interior. Such biophysical and structural insights have helped to improve the biological activities of this family of peptides [128–130].

Furthermore, it has been observed that the peptides associate with negatively charged vesicles thereby neutralizing the membrane surface charge density. When neutrality is reached the vesicles associate to form large flocculating complexes [131]. Although this association results in fusion at high ratios of acidic phospholipids, vesicle association is reversible at lower phosphatidylserine concentrations. Such observations suggest that cationic peptide antimicrobials have the capacity to cause condensation and flocculation of biological macromolecules inside the cell, to cause membrane fusion and/or the aggregation of whole cells which concomitantly affects the survival and progression of such microorganisms. These characteristics and the self-assembling properties of cationic peptide antimicrobials have so far not been investigated in much detail [70,132,133], and despite the decades of intensive investigations since their first discovery many questions about their mechanisms of action remain to be resolved.

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