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Review

Differential scanning calorimetry and X-ray diffraction studies of the specificity of the interaction of antimicrobial peptides with membrane-mimetic systems

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Abstract

Interest in biophysical studies on the interaction of antimicrobial peptides and lipids has strongly increased because of the rapid emergence of antibiotic-resistant bacterial strains. An understanding of the molecular mechanism(s) of membrane perturbation by these peptides will allow a design of novel peptide antibiotics as an alternative to conventional antibiotics. Differential scanning calorimetry and X-ray diffraction studies have yielded a wealth of quantitative information on the effects of antimicrobial peptides on membrane structure as well as on peptide location. These studies clearly demonstrated that antimicrobial peptides show preferential interaction with specific phospholipid classes. Furthermore, they revealed that in addition to charge-charge interactions, membrane curvature strain and hydrophobic mismatch between peptides and lipids are important parameters in determining the mechanism of membrane perturbation. Hence, depending on the molecular properties of both lipid and peptide, creation of bilayer defects such as phase separation or membrane thinning, pore formation, promotion of nonlamellar lipid structures or bilayer disruption by the carpet model or detergent-like action, may occur. Moreover, these studies suggest that these different processes may represent gradual steps of membrane perturbation. A better understanding of the mutual dependence of these parameters will help to elucidate the molecular mechanism of membrane damage by antimicrobial peptides and their target membrane specificity, keys for the rationale design of novel types of peptide antibiotics.

Keywords: Host defense peptide; Lipid discrimination; Membrane mimetic; Bacterial membrane; Peptide antibiotic

Contents

Abbreviations: CL, cardiolipin; DiPoPE, dipalmitoleoyl PE; DEPE, dielaidoyl PE; DLPC, dilauroyl PC; DMPC, dimyristoyl PC; DMPE, dimyristoyl PE; DMPG, dimyristoyl PG; DOPC, dioleoyl PC; DOPE, dioleoyl PE; DPPC, dipalmitoyl PC; DPPE, dipalmitoyl PE; DPPG, dipalmitoyl PG; DSC, differential scanning calorimetry; H_{II}, hexagonal type II; L_α, liquid-crystalline phase; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGLa, peptidyl-glycine-leucine-carboxyamide; PS, phosphatidylserine; Q_{II}, cubic type II; T_H, L_α-H_{II} phase transition temperature; SM, sphingomyelin

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1. Introduction

The interaction of peptides with membranes has been a subject of interest in membrane biophysics for a number of years. Such studies have been applied for peptides corresponding to segments of membrane proteins, for the purpose of understanding the mechanism of action of membrane-active peptides as well as for designing peptides which can modulate membrane properties. Interest in the latter research area has increased recently because of the dramatic increase in the number of bacteria which are resistant to conventional antibiotics [1,2]. This health issue has been one of the dominant concerns in the last few years of the World Health Organization, who published a retrospective warning on the occasion of the World Health Day in 1997. In particular, in the field of hospital infection control, there is increasing concern regarding both the rapid emergence of resistant strains with high epidemic potential and the fact that bacteria show reduced susceptibility to vancomycin, a drug of last resort, as e.g. described for a methicillin resistant clinical strain of *Staphylococcus aureus* [3]. There are reports of about a dozen new drugs that show promising antibacterial activity by interfering with protein synthesis, cell wall formation or DNA replication, but in the long run these drugs are not likely to overcome resistance [4]. Therefore, urgent action is needed to develop novel antibiotic agents [5].

One alternative approach is based on host defense peptides of around 15–40 amino acid residues which have evolved in nature to contend with invaders as an active system of defense [6]. Important stimulation in the field of innate immunity came from the pioneering work of Erspamer [7], isolating a great variety of different classes of pharmacologically active molecules in frog skin and from the discovery of magainins, peptide antibiotics from the skin of the South African clawed frog *Xenopus laevis* [8], the isolation of the first pure antibacterial factor from insects [9] as well as from the discovery of antimicrobial peptides from mammalian white blood cells [10]. The biological activity of host defense peptides mostly stems from their ability to perturb the lipid bilayer structure of membranes, i.e. their barrier function, and is not related to binding to a specific membrane receptor site. Support for this argument comes from studies on synthetic melittin, magainin and cecropins with all the amino acids in the D configuration. It has been shown that the all-D peptides exhibit antibacterial and hemolytic activity identical to that of the naturally occurring L forms [11]. The molecular mechanism underlying this membrane damage is, however, still a matter of debate [12]. Moreover, host defense peptides often exhibit a high specificity towards their target membrane, i.e. some of these peptides are toxic to humans, while others have toxicity which is restricted to microorganisms. These latter antimicrobial peptides often exhibit a higher activity against certain microorganisms than against others and in addition may also lyse mammalian cell membranes. Therefore, the elucidation of the specific target membrane damaging properties of these peptides is supposed to be a key for the rationale design of novel types of peptide-antibiotics, which should selectively lyse bacterial membranes. This is likely to be accomplished only if the molecular basis of the action of these peptides is known.

Therefore, in order to gain insight into the specificity towards particular lipid components exhibited

by lytic peptides the differences in architecture of eucaryotic and procaryotic cell membranes (e.g. [13]), which differ markedly in their lipid composition, have to be taken into account. The cell envelope of Gram-negative bacteria is more complex, consisting of an inner and an unique outer membrane, which has a distinctive highly asymmetric composition with the lipopolysaccharides being located exclusively in the outer layer and phospholipids being confined to the inner layer of the outer membrane. Studies with the antimicrobial peptides magainin [14,15] and defensin [16] showed that these peptides can permeabilize this outer membrane and are able to cross this barrier gaining access to the inner membrane. The inner (cytoplasmic) membrane is essentially a bilayer of lipids like the cytoplasmic membrane of Gram-positive bacteria. There exists well documented information concerning the phospholipid composition of individual genera and species of Gram-negative and Gram-positive bacteria [17,18]. Although changes in lipid composition may occur depending on environmental conditions, the concept of a typical lipid composition of cell membranes is well accepted. In addition, the phospholipid and glycolipid composition from one group to another may vary to a larger extent for Gram-positive bacteria than for Gram-negative ones. A common feature in any case is the presence of large amounts of negatively charged phospholipids and PE. The phospholipid composition of some selected microorganisms are given in Table 1, which indicates that in general higher amounts of PE are found in the inner membrane of Gram-negative bacteria as compared to the cytoplasmic membrane of Gram-positive bacteria. For example, PG and its derivatives are the only phospholipids of the membrane of *S. aureus*, while PE is the dominant component (82%) of the inner (cytoplasmic) membrane of *Escherichia coli*. On the other hand, in human erythrocyte membranes, which can be considered representative for mammalian cell membranes, the choline phosphatides, PC and SM, occur predominantly in the external leaflet, whereas the aminophosphatides, PE and PS, are found almost exclusively in the inner leaflet of the bilayer (Table 2). While a wealth on information has been gained on the interaction of lytic peptides with PC membranes, only recently has the number of studies utilizing other phospholipids increased. A set of selected examples for peptide interactions with such liposomes, using primarily data gained by differential scanning calorimetry and X-ray techniques, which yields information on the effects of the antimicrobial peptides on both the organization of the lipids and morphology of the model systems, will be presented.

Table 1
Phospholipid composition of Gram-positive and Gram-negative bacteria

Species	Phospholipid composition (w%)			
	PG	PE	CL+lysoPG	Others
Gram-positive[a]				
S. aureus	57	0	43	0
Staphylococcus epidermidis	90	0	1[b]	9
Bacillus megaterium	40	40	5[b]	15
Bacillus subtilis	29	10	47[b]	14
Gram-negative[a]				
E. coli	6	82	12[b]	0
Salmonella typhimurium	33	60	7[b]	0
Pseudomonas cepacia	18	82	0	0

[a]In percentage of total phospholipid of cytoplasmic membrane.
[b]Only CL (cardiolipin); data adapted from [17,18].

Table 2
Phospholipid composition (% of lipid phosphorus) of human erythrocyte membranes and their dominant localization (e.g. [117])

Phospholipid	Percentage	Membrane leaflet
Phosphatidylcholine	31.0	outer
Sphingomyelin	23.5	outer
Phosphatidylethanolamine	28.4[a]	inner
Phosphatidylserine	13.4	inner
Phosphatidylinositol	1.2	inner
Phosphatidic acid	2.2	inner

[a]About 1/3 is comprised by the alkenyl analog, ethanolamine plasmalogen, which was shown to promote the formation of non-bilayer structures [58,59].

2. Lipid discrimination by antimicrobial peptides as probed by DSC

There is evidence that some antimicrobial peptides have specificity for particular membrane lipid components. For example, the peptide cinnamycin appears to have specificity for PE [19] and sapecin exhibits a specific affinity for cardiolipin [20]. Gener-

ally, antimicrobial peptides show preferential interaction with negatively charged phospholipids as demonstrated e.g. for magainins, which effectively permeabilize PG-rich membranes and kill more effectively bacteria whose inner membranes contain higher amounts of PG ([21]; Matsuzaki, this issue). Recently, high-sensitivity differential scanning calorimetry studies were performed to gain insight on the impact of the membrane structure regarding the lytic activity of antimicrobial peptides, i.e. to understand their target cell specificity. Such thermodynamic studies allow one to investigate the influence of the peptides on the phase transition properties of membrane systems, from which the nature of lipid-peptide interactions can be assessed [22,23].

McElhaney and coworkers recently studied the effects of the antimicrobial peptide gramicidin S on the thermotropic phase behavior of large multilamellar vesicles of DMPC, DMPG and DMPE by high-sensitivity differential scanning calorimetry [24]. The results demonstrated that the effects varied markedly with the structure and the charge of the lipid polar headgroup. DMPE showed only small reduction in the temperature, enthalpy and cooperativity of the gel to liquid-crystalline phase transition after the liposomes have been exposed to high temperatures. Similarly, DMPC vesicles showed modest decreases in the temperature, enthalpy and cooperativity, although the pretransition is abolished. Exposure to high temperatures is not required to observe these effects. In contrast, gramicidin S has a much greater effect on the phase behavior of the anionic DMPG by substantially reducing the temperature, enthalpy and cooperativity of the main phase transition and abolishing the pretransition at lower peptide concentrations as compared to DMPC.

Furthermore, microcalorimetry was used to study the effects of the human neutrophil peptide, HNP-2 [25], on the thermotropic behavior of liposomes mimicking bacterial and erythrocyte membranes [26]. In this study the lipid composition of the liposomes represented the major phospholipid components of these cell membranes: PE and the negatively charged PG for bacterial membranes, and the zwitterionic PC and SM for erythrocyte membranes. In the presence of this antimicrobial peptide, which exhibits a triple stranded β-sheet configuration [27], the phase behavior of liposomes containing negatively charged phosphatidylglycerol was markedly altered. Already at very low peptide concentrations (lipid-to-peptide molar ratio of 500:1), a slight shift of the phase transition temperature and the occurrence of a shoulder was observed. Addition of HNP-2 to liposomes mimicking bacterial membranes, i.e. PE/PG mixtures, resulted in phase separation presumably into peptide-rich and -poor domains. The preference for negatively charged membrane surfaces is also in accordance with vesicle leakage experiments [28], as had been proposed by White et al. [29]. In this respect it is interesting to note that leakage experiments showed that in the case of rabbit defensin, cardiolipin seems to be essential for creating lesions as no leakage was observed for liposomes composed of PE and PG. On the other hand, HNP-2 did not affect the phase behavior of membranes mimicking erythrocyte membranes (equimolar mixtures of PC and SM) as well as of the pure lipids. Moreover, a similar behavior has been observed with the antimicrobial peptides peptidyl-glycine-leucine-carboxyamide (PGLa) from the skin secretion of the frog

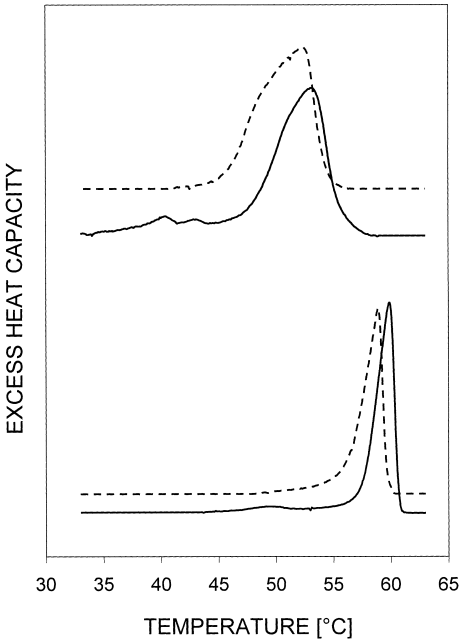

Fig. 1. Excess heat capacity functions of DPPG/DPPE liposomes (broken line) in the presence of PGLa (solid line) at a lipid-to-peptide molar ratio of 100:1 (top, DPPG/DPPE 1:1 mol/mol; bottom, DPPG/DPPE 1:3 mol/mol). Experiments were performed in 10 mM Na-phosphate buffer, pH 7.4 at a scan rate of 0.25°C/min.

X. laevis [30] and protegrin-1 [31], isolated from pork leukocytes, despite their different secondary structures. While PGLa was shown to adopt an α-helical structure in the presence of negatively charged phospholipids [30,32], a hairpin-like β-sheet structure was reported for protegrin-1 [33]. Again phase separation was observed in liposomes that contained negatively charged phospholipids. In mixtures of PGLa and DPPE/DPPG this effect was manifested by a minor transition at low temperatures and a shift of the larger one to slightly higher temperatures (Fig. 1). Concomitantly, a slight increase of cooperativity was detected [34]. These results suggest that the peptide forms specific domains with the negatively charged PG, thereby decreasing the amount of PG in the peptide-poor domains and hence inducing the temperature shift owing to the increased content of PE in these domains. This is supported by the fact that in DPPG/PGLa mixtures, a second, relatively high cooperative transition was found at elevated temperature in addition to the main transition of DPPG (Fig. 2). In contrast to the pure lipid, no pretransition was found for the peptide-affected domains, indicating an untilting of the hydrocarbon chains which is due to membrane penetration of the peptide in accordance with wide-angle X-ray measurements [30]. Moreover, analysis of the enthalpy of peptide-rich and -poor lipid domains revealed that a distinct fraction of PG is associated with PGLa. Using lipid-specific fluorescent probes it was

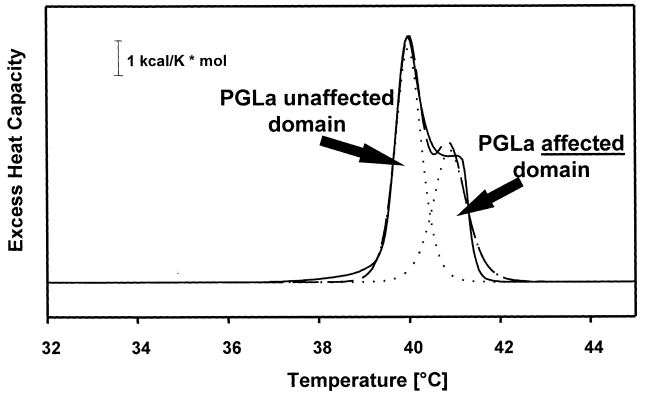

Fig. 2. Excess heat capacity functions of DPPG liposomes in the presence of PGLa at a lipid-to-peptide molar ratio of 50:1. Experiments were performed in 10 mM Na-phosphate buffer, pH 7.4 at a scan rate of 0.25°C/min. Deconvolution of data is indicated; experimental data (solid line), fitted overall (broken line) and individual components (dotted line).

shown that amphipathic helical, membrane active peptides of the A- and L-type [35] were also able to induce phase segregation in zwitterionic-acidic lipid mixtures (PC/PG), most likely owing to preferential association with the negatively charged lipid [36]. Further, it was suggested that peptide-lipid charge-charge interactions were capable of modulating existing domain composition in the course of the main phase transition in this mixture.

As discussed recently by Epand [37], the role of the lateral organization of the complex lipid mixture in membranes upon the interaction of lytic peptides should be considered. Although in a fluid membrane segregation of lipids is rarely observed, lateral domain organization of biological membranes is thought to be of physiological relevance. One example for a possible phase separation in the liquid-crystalline phase comes from DSC measurements on the L_α-H_{II} phase transition of dipalmitoleoyl phosphatidylethanolamine (DiPoPE) in the presence of magainin 2. These traces showed a minor and a major peak, which is shifted to higher temperatures with increasing amounts of peptide, suggesting the occurrence of peptide-rich and peptide-depleted domains [38]. Furthermore, very recently Uragami et al. [39] have provided evidence by use of nearest-neighbor recognition methods that in bilayers the headgroup mismatch between PG- and PE-like lipids is sufficient to induce lateral heterogeneity in the fluid phase. Immiscibility was also found by DSC experiments to exist in the lamellar gel phase in DPPG/DPPE mixtures between 5 and 40 mol% of DPPE [40]. In this concentration range a phase separation of highly enriched DPPG was observed. These effects were explained as a consequence of the molecular shape of the phospholipids differing markedly in their headgroup properties as well as the packing characteristics of the hydrocarbon side chains. The effective area of the PG headgroup is larger than predicted by its geometrical size owing to electrostatic repulsion (e.g. [41]) and therefore the cross-sectional area of the headgroup matches the hydrophobic cross-sectional area. In contrast, PE exhibits a smaller headgroup area as compared to the cross-sectional area required by the acyl chains. Moreover, adjacent PE lipids interact intermolecularly by hydrogen bonding between the amino and phosphate groups, which favors a close lipid packing [42]. Therefore, PE will

tend to adopt structures with higher intrinsic curvature, while PG will rather form flat lamellar lipid aggregates.

It can be assumed that the molar ratio of PG and PE may be important in determining microscopic differences in their lateral organization, packing and/or mobility, which can be amplified by the interaction with other membrane constituents and in particular by interaction with membrane-active solutes in the environment. Evidence for this comes from studies on the insertion of alamethicin into model membranes composed of PC and PE with identical acyl chains, which depended strongly on the molar ratio of these lipids, emphasizing again the importance of the lipid headgroup packing [43]. These authors also concluded that the specific physical characteristics of the phospholipids, i.e. the cross-sectional area of the lipid headgroup and the hydrocarbon side chains, are important factors in determining whether and how the lipid bilayer will be affected by membrane-active peptides. These observations further emphasize that the headgroup structure and composition may be important in defining the two-dimensional organization in membranes and it can be expected that further studies focusing on this aspect will help to elucidate the role of the phospholipid matrix in the interaction between membranes and antimicrobial peptides.

3. The role of membrane spontaneous curvature in peptide interaction

Both phosphatidylglycerol and phosphatidylethanolamine are major phospholipid components in bacterial plasma membranes as described earlier. As mentioned above PE is characterized by a cone-shaped molecular geometry [44,45] and possesses a large spontaneous curvature [46]. This property makes PE prone to form nonlamellar structures. Such lipids can also comprise a large fraction of other biomembranes [47]. The importance of the proper balance between lamellar and nonlamellar phase forming lipids has been widely discussed [48–50]. The presence of the latter significantly increases membrane monolayer curvature stress, thereby conferring upon cell membranes a degree of nonlamellar-forming propensity, which is believed to be essential for normal membrane function such as membrane fusion (e.g. [51–55]). It has also been suggested that the presence of nonlamellar-preferring phospholipids in bilayer membranes induces a frustrated curvature stress, which in turn may affect the conformational state and hence the activity of membrane proteins [56]. Moreover, Richard and Raquel Epand demonstrated that this curvature strain can provide energy for certain membrane processes [57]. They showed that incorporation of a few percent of lysophosphatidylcholine into large unilamellar vesicles of monomethylated PE at neutral pH is an exothermic process as detected by calorimetry corresponding to the relief of curvature strain in bilayers having the tendency to convert to the inverse hexagonal phase. These observations suggest that considerable energy may be released upon incorporation of amphipathic peptides into membranes which have a low radius of spontaneous curvature.

The effect of peptides on membrane spontaneous curvature can be evaluated on the basis of their effect on the lamellar (L_α) to inverse hexagonal (H_{II}) phase transition temperature (T_H) as detected by calorimetry. This will yield information on the bilayer (de)-stabilizing property of amphipathic peptides, which is helpful in elucidating the mechanism of membrane damage by lytic peptides. Such DSC studies are usually performed with liposomes composed of phosphatidylethanolamines which are prone to adopt the H_{II} phase (e.g. [44,58,59]). Frequently, DiPoPE has been used because it exhibits a T_H of 43°C, which allows convenient detection of temperature shifts induced by antimicrobial or cytolytic peptides. It should be noted that although lamellar gel to liquid-crystalline phase transitions are easily detected by DSC owing to their large enthalpy change, problems may arise with the detection of the less cooperative and enthalpic lamellar to nonlamellar phase transitions despite the use of high sensitivity instruments. For example, the enthalpy for the L_α-H_{II} phase transition of DOPE is around 0.3 kcal/mol [60] and even less for the L_α-Q_{II} transition of monomethylated DOPE [61]. Hence, the observation of nonlamellar phases may require additional techniques like ^{31}P-NMR and X-ray diffraction to detect such phases and the latter technique has the advantage that the supramolecular structure of the lipid aggregates can be assigned in detail [62].

Initial studies were performed with the 18L model peptide, which features the consensus sequence averaged from a number of naturally occurring lytic amphipathic α-helical peptides [35], and Ac-18A-NH_2, an 18 amino acid residue peptide that mimics α-helical segments of exchangeable human plasma apolipoproteins [63]. Based on the difference in the charged residue distribution on the polar face of the α-helix, a cross-sectional molecular shape concept, defining class A (e.g. apolipoproteins) and class L (lytic) peptides, was postulated, by which membrane stability might be controlled [64]. In fact, it was found that class L peptides and or class A peptides have opposite effects on the L_α-H_{II} phase transition temperature of DiPoPE, i.e. the former lowered and the latter raised T_H. By analogy to the reciprocal effects of the molecular shape of phospholipids on membrane structure, it was proposed that the inverted wedge shape of class L helixes destabilizes such membranes, while the wedge shape of class A helixes stabilizes the PE bilayer [65].

It was also shown that the wasp venom peptide, mastoporan [64], as well as the antimicrobial peptide magainin 2 [38], shifted T_H to higher temperatures. DSC data revealed that already very low magainin concentrations strongly raised the T_H of DiPoPE (1.8°C at a lipid-to-peptide molar ratio of 1000:1). A similar behavior was reported for magainin analogs [66]. These findings are interesting in view of a possible mechanism of membrane lysis by the antimicrobial peptide magainin (see also chapter by Matsuzaki), which binds selectively to negatively charged phospholipids. There is evidence that the peptide forms a dynamic peptide-lipid supramolecular complex pore [38,67]. Interestingly, permeabilization of lipid bilayers composed of PG occurred at a lipid-to-peptide molar ratio of about 100:1, while permeabilization of bilayers composed of other negatively charged lipids such as PS, PA, and cardiolipin (CL), occurred at much higher peptide concentrations (lipid-to-peptide molar ratios of 50:1 to 10:1). The latter three classes of phospholipids are known to be able to adopt inverse hexagonal structures under conditions of reduced interlipid electrostatic repulsions. Moreover, incorporation of PE also inhibited the magainin-induced pore formation. These results were explained by postulating that the peptide imposes a positive curvature in the bilayer, facilitating the formation of the pore of a torus type, and therefore the pore formation is inhibited by the presence of negative curvature-inducing lipids [38]. Thereby, it was found that PEs, exhibiting the largest hydrophobic cross-section, were most efficient at inhibiting pore formation [38], which can be correlated with their higher ability to promote nonlamellar structures [55].

A direct correlation between the hemolytic activity of magainin analogs and their potency to raise the lamellar to inverse hexagonal phase transition temperature was reported [66]. Also δ-lysin, a hemolytic peptide from *S. aureus*, which cannot be unambiguously classified to the scheme of Segrest of amphipathic helixes [35], was shown to increase T_H of DiPoPE to a similar extent than magainin (1.5°C at a lipid-to-peptide molar ratio of 1000:1) [68]. Moreover, similar to magainin 2 the phase transition was hardly discernible above 0.2 mol% of peptide. The high sensitivity of this phase transition to very small amounts of incorporated amphipathic peptides is shown in Fig. 3. In analogy to observations with melittin, which like δ-lysin consists of a single polypeptide chain of 26 amino acids and a cationic carboxyl terminal end, the interaction of δ-lysin with PE can be explained based on the molecular shape of phospholipids. As shown for melittin, the tendency towards the formation of discoidal structures can be counterbalanced by the H_{II} phase preference of PE [69], being consistent with a relief of the lateral pressure of the hydrocarbon chains, which in turn will

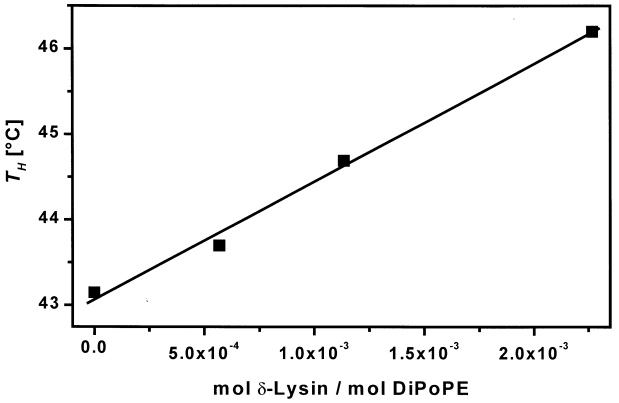

Fig. 3. Effect of staphylococcal δ-lysin on the L_α-H_{II} phase transition temperature of DiPoPE as determined by high-sensitivity DSC experiments.

result in a stabilization of the bilayer structure. A different behavior was found for melittin/dihexadecyl PC mixtures indicating the impact of the nature of the phospholipid matrix. There, above the chain melting transition, X-ray scattering data were characterized by a broad continuous transform with superimposed peaks arising from nonlamellar structures [70]. In addition, this peptide/ether lipid system was sensitive to swelling, increasing the fraction of the broad continuous transform, suggesting a sequence of lamellar-cubic-micellar structure of type I in agreement with predictions from general amphiphile/water binary phase diagrams [71].

On the other hand, the cyclic decapeptide gramicidin S, a bacterial peptide toxin from *Bacillus brevis*, was found to decrease T_H [72], i.e. owing to the presence of gramicidin S the tendency of the phosphatidylethanolamine lipid bilayer to adopt structures with a negative curvature is increased, thus destabilizing the lipid bilayer toward the formation of nonlamellar structures. For example, the probable existence of inverted cubic phases for a mixture of DMPE and gramicidin S were proposed from supplementary X-ray reflections, which could not be indexed on a lamellar phase [72]. Moreover, in the presence of the peptide, the formation of isotropic components were observed in the ^{31}P-NMR spectra of heterogeneous lipid mixtures such as occur in *Acholeplasma laidlawii* B and *E. coli* membranes [72]. The appearance of an isotropic signal in the ^{31}P-NMR spectrum of aqueous phospholipid dispersions is observed for fast tumbling lipid aggregates, like small unilamellar vesicles and micelles, or for cubic or other three-dimensionally ordered inverted nonlamellar phases. As ^{31}P-NMR spectroscopy cannot distinguish between these putative lipid-peptide aggregates due to their fast tumbling in respect to the ^{31}P-NMR time scale (e.g, [73–76]), these lipid aggregates were further investigated by a more extensive X-ray diffraction study. These latter experiments clearly showed that the interaction of gramicidin S with the microbial lipid extracts promotes the formation of a nonlamellar structure, which could be identified as a bicontinuous cubic lipid phase of the space group Pn3m [77]. As an example, Fig. 4 shows selected diffractograms obtained for the total extract of

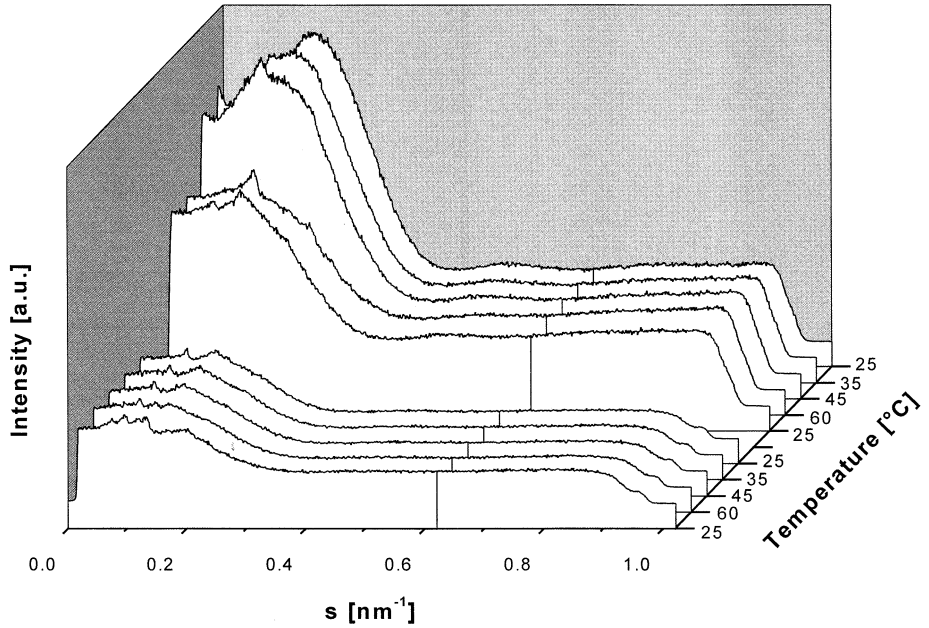

Fig. 4. Small-angle X-ray diffractograms of an aqueous dispersion of *E. coli* membrane total lipid extract (five rear frames) and a mixture of these lipids with gramicidin S at a lipid-to-peptide molar ratio of 25:1 (five front frames). Data recorded from the first heating (selected temperatures at 25, 35, 45, 60°C are shown) and cooling to 25°C are shown; $s = 1/d = 2\sin(\Theta)/\lambda$, where λ is the wavelength of the X-ray beam and 2Θ the scattering angle.

E. coli membrane lipids. The combination of X-ray diffraction and ^{31}P-NMR spectroscopic data acquired with these microbial lipid extracts is consistent with the existence of a polydomain specimen in which a lamellar phase coexists with the nonlamellar phase. These observations indicate that gramicidin S has considerable potential for disrupting the structural integrity of lipid membranes by markedly decreasing the energetic barriers against the formation of inverted (i.e. type II) nonlamellar lipid phases. It is therefore possible that interactions of these peptides with lipid bilayers result in a significant increase in monolayer curvature stress, a property that may well be key to their membrane disruptive properties ([72] and the gramicidin contribution in this issue by Prenner et al.).

A recent study has shown that alamethicin exhibits similar properties [78]. The effect of alamethicin on membrane spontaneous curvature was probed with DEPE. Thereby, it was found that addition of as low as 0.5% (w) peptide can induce the formation of nonlamellar structures over a wide temperature range. Phase assignment, based on the ratios of the Bragg peaks, revealed again the occurrence of a cubic phase of the space group Pn3m. X-ray diffraction further demonstrated that the lattice spacing of the cubic phase did not change over an alamethicin concentration range of 1–10% (w). Moreover, it was reported that the lattice spacings of the H_{II} phase, which was found to coexist with the lamellar phase for some samples and temperatures, was not affected by the presence of the peptide. This can be due to the fact that alamethicin is either expelled from the H_{II} phase or that incorporation of the peptide does not alter the membrane spontaneous curvature. It was suggested that this peptide may change the thickness and/or flexibility of the bilayer, thereby inducing the formation of the cubic phase. However, the authors argue that the formation of such phases is still not sufficiently understood that one would be able to draw solid conclusions [78].

Finally, gramicidin A and C were shown to be even able to promote H_{II} phase formation in DOPC, which is known to form only a lamellar phase at physiologically relevant temperatures [79]. Both peptides, whereby the tryptophan in position 11 of gramicidin A is replaced by tyrosine in gramicidin C, induce to a similar extent this nonlamellar phase. Distinct bands were found by sucrose density centrifugation experiments which were analyzed by X-ray diffraction and ^{31}P-NMR, demonstrating a quantitative phase separation between a lamellar phase and an H_{II} phase, the latter being enriched in peptide. Promotion of negative curvature by these peptides was further shown by the conversion of lysophosphatidylcholine micelles into bilayer structures [80]. In addition, small angle X-ray diffraction experiments revealed that upon increasing the length of gramicidin A, the peptide lost its ability to induce H_{II} phase formation indicating a requirement of hydrophobic mismatch between the peptides and the lipid acyl chains [81]. This phenomenon was further investigated systematically using gramicidin A and hydrophobic α-helical transmembrane peptides that resemble the gramicidin channel [82,83]. It was suggested that the extent to which the length of the membrane-spanning part of intrinsic membrane proteins matches the hydrophobic core of the bilayer may be an important factor in determining membrane structure and function.

4. Membrane location of peptides and its effect on membrane structure

There is ample evidence that amphipathic peptides can adopt a variety of membrane locations depending on various parameters, among which the phospholipid composition and the peptide concentration are two of the determinants. These aspects were studied intensively by Huang and coworkers within the last decade (see below). In a series of systematic studies with antimicrobial peptides, they showed that there exists a certain critical concentration for insertion, firstly described for alamethicin [84]. In general, it seems that below this concentration the peptides are aligned parallel to the membrane surface, while above this concentration they are oriented perpendicular to the membrane plane. Information on changes in the bilayer structure caused by the peptide adsorption and/or insertion can be obtained from X-ray diffraction and also by neutron scattering. Very recently White and coworkers demonstrated, using a novel X-ray diffraction method based on absolute-scale refinement [85–87], that they can obtain quantitative information about the depth of penetration

and orientation of peptides in lipid bilayers in the liquid-crystalline phase [88]. So far, mostly bilayer structures of low resolution have been obtained owing to the thermally disordered hydrocarbon chains. This has been improved by the determination of the position of specifically labeled sites of the lipids, in particular bromination of the double bonds of acyl chains, which was shown to provide information about the structure of the hydrophobic core and to sense changes in bilayer structure [87]. This approach was further extended by developing liquid crystallography for the determination of complete one-dimensional structures of liquid-crystalline bilayers [89,90], which gives information on the positions of specific structural groups such as e.g. phosphate or carbonyl groups and also on the transbilayer spatial distributions of water [91]. Based on the completely solved structure of DOPC [86], changes in the brominated double-bond distributions provided a basis for the accurate determination of the transbilayer distribution of the class A amphipathic α-helical peptide Ac-18A-NH_2 in oriented multilamellar bilayer of DOPC [88]. The diffraction results showed that this peptide was located in the bilayer interface close to the glycerol moiety. Its bilayer distribution could be described by a Gaussian function, whereby the width is a measure of the diameter of the helix. These data indicated that the amphipathic class A peptide penetrated the hydrocarbon core to about the level of the phospholipid double bond. Moreover, a slight decrease in bilayer thickness with a concomitant shift of the double-bond distribution toward the bilayer center was observed arising from a small increase in lipid-specific area caused by the peptide.

These results are in accordance with earlier observations on alamethicin and magainin which also demonstrated a decrease of the bilayer thickness upon adsorption of the peptides to a membrane surface. It was proposed that if the hydrophobic volume of the phospholipids remains constant, the decrease of bilayer thickness must be compensated by expansion of the cross-sectional area of the lipid hydrocarbon chains. For example, calculation of the area expansion of the acyl chain region of diphytanoyl PC in the presence of alamethicin revealed that it remained constant for each adsorbed alamethicin molecule (280 ± 20 Å) below a lipid-to-peptide molar ratio of 47:1 [92]. Interestingly, this area expansion correlates approximately with the cross-sectional area of one peptide molecule, which implies that the lipid headgroups will separate laterally within the membrane plane. The membrane thinning observed in this concentration range was linearly dependent on the peptide concentration. In a subsequent study, the coexistence range (adsorbed and inserted peptide) as well as the insertion phase at full lipid hydration, being relevant to the physiological conditions, were investigated by X-ray diffraction using oriented lipid bilayers [93]. These experiments, which are critical to sample disorder as discussed by the authors, showed that at high peptide concentrations the bilayer thickness increased again with the content of inserted alamethicin. This observation was explained by postulating that the hydrophobic region of the fluid diphytanoyl PC bilayer will have to match the hydrophobic portion of the alamethicin molecule. Moreover, it was found that peptide adsorption induced chain disorder over a large area [92]. A long range effect of amphipathic peptides upon bilayer organization was also proposed from earlier DSC and temperature scanning densitometry studies on DPPC/melittin and DPPC/δ-lysin mixtures, respectively [94,95]. Finally, it should be mentioned that neutron scattering in the plane of dilauroyl PC (DLPC) and diphytanoyl PC bilayers revealed a pore structure of the barrel-stave type for high alamethicin concentrations [93,96].

Further, it was demonstrated that magainin behaves in many aspects similarly to alamethicin, such as exhibiting two distinct orientations when interacting with a lipid bilayer depending on the critical concentration for peptide insertion, which roughly correlates with the concentration required for cytolytic activity [97] or membrane thinning below this concentration [98]. It is important to emphasize, however, that these experiments were performed with PC/PS bilayers, as magainin interacts more strongly with acidic phospholipids. However, in contrast to alamethicin, neutron in-plane scattering indicated a different pore structure at high magainin concentrations [99]. In agreement with suggestions of Matsuzaki (for details see chapter by Matsuzaki in this issue), a toroidal or wormhole model was proposed on the basis of the scattering data. In this model the lipid bends back on itself like the inside of a torus, inducing a lateral expansion in the head-

group region of the bilayer, the spaces of which are filled by the peptide molecules in accordance with earlier DSC data which indicated that magainin is associated with the headgroups [100].

As has been reported for transmembrane proteins and as outlined above upon membrane insertion of antimicrobial peptides, one can expect that the peptides may often display a different hydrophobic length as compared to the hydrocarbon chains of the lipid interacting with. It is now a commonly accepted idea that this hydrophobic mismatch between phospholipids and peptides is compensated by adjusting the hydrophobic core of the lipid bilayer to the hydrophobic length of the peptide [101,102]. Harroun et al. [103] emphasized that in order to understand nonspecific interactions of peptides and lipids, it is important to consider the elastic properties of these molecules. While lipid bilayers are very deformable owing to the flexibility of the hydrocarbon side chains, at least globular proteins are rather rigid, the thickness compressibility of lipid bilayers and the volume compressibility of proteins, respectively, differing by two orders of magnitude [104,105]. Although one can assume that small amphipathic peptides have a high degree of flexibility (see e.g. Blondelle et al., this issue), it is conceivable that when a peptide is inserted perpendicular to the membrane surface, the lipid acyl chains will compensate an eventual mismatch of the hydrophobic length between peptide and lipid. It was suggested that the energy of the membrane deformation for hydrophobic matching is less than the energy cost of hydrophobic mismatch.

To test this idea, gramicidin D, known to form well defined dimeric channels in lipid bilayers [106], was embedded in DLPC and DMPC bilayers at high peptide concentrations (lipid-to-peptide molar ratio of 10:1) and near full lipid hydration. In the liquid-crystalline phase the bilayer thickness, i.e. the phosphate-to-phosphate distance of the opposite membrane leaflets, as determined with refined methods from X-ray lamellar diffraction patterns [92,107, 108], was found to increase by 1.3 Å for the DLPC/gramicidin D mixture and to decrease by 2.6 Å for the DMPC/gramicidin D mixture. Hence, the peptide apparently stretches DLPC bilayers and thins DMPC bilayers toward a common thickness of 32.4 ± 0.3 Å owing to hydrophobic matching. It was suggested that upon embedding peptides in a membrane, hydrophobic matching creates a strain field in the lipid bilayer that in turn gives rise to a membrane-mediated attractive potential between peptides [103]. Therefore, membrane in-plane scattering with X-ray was applied to directly measure the gramicidin-gramicidin correlation in the liquid-crystalline phase of bilayers. The nearest-neighbor separation of the peptide was calculated to be 26.8 Å in DLPC bilayers but shortens to 23.3 Å in DMPC bilayers. These experiments clearly give evidence for hydrophobic matching and membrane-mediated peptide-peptide attractive interactions, which were just recently theoretically analyzed by the same authors [109]. In this paper they show that these processes can be described with a simple elasticity theory of membrane deformation, whereby the energetics of deformation are expressed as a function of bending rigidity and thickness compressibility of the bilayer.

5. Bilayer disruption by amphipathic peptides

Structural and thermodynamic studies as described above indicate that several steps are involved in the process of peptide-lipid interaction leading finally to membrane perturbation and permeabilization. Moreover, they present evidence that bilayer defects and pore formation do not represent distinct classes of membrane-lytic motifs, but rather that there is a continual gradation from one to another. So far, the 'carpet model' ([110]; Shai, this issue) has not been discussed in this respect, although some of the features described before by X-ray and neutron scattering are in accordance with this model. Like pore formation, this model suggests that at low peptide concentrations the peptides accumulate at the bilayer surface and that penetration of peptides occur by reaching a threshold concentration, i.e. critical concentration for peptide insertion, at which the bilayer surface is largely occupied by the peptide molecules. At this high relative peptide level the lipid bilayer is destabilized owing to massive peptide insertion, inducing disruption of the gross bilayer structure. It is even conceivable that pore formation is an intermediate step in this process (for details see Shai, this issue). The mechanism by which antimicrobial and

cytolytic peptides finally damage the membrane will be determined by the peptide and lipid structure. The carpet model also implies that the membrane integrity is strongly affected by the peptides, comparable to the action of detergents, with which amphipathic peptides share some general properties.

Data gained on melittin and δ-lysin seem to support the idea of gradual membrane perturbation. Temperature scanning densitometry [94] and DSC as well as X-ray studies [111] showed that melittin has pronounced effects on the phase behavior of DPPC already at very low peptide concentrations (lipid-to-peptide molar ratio of 1000:1), abolishing the pretransition of the lipid at a DPPC/melittin molar ratio of about 100:1. Similar results were obtained by DSC for δ-lysin/DPPC mixtures [95]. Since these effects cannot be accounted for only by local perturbations around the sites of interaction, long range effects beyond the immediate neighborhood of the incorporated peptide are supposed to be involved. This idea was supported by ultrasonic experiments on melittin/DPPC [112]. These results were discussed in terms of a local phase separation concept, where the peptide-affected domains create line defects in an ordered lipid lattice. Such a defect-like action of melittin or δ-lysin at high lipid-to-peptide molar ratios may be explained by shifting the percolation balance [113] of gel- and fluid-like states that may coexist. However, at high melittin concentrations (lipid-to-peptide molar ratio of 15:1), disk-shaped particles were found for DMPC/melittin mixture [114], suggesting a detergent-like action under these experimental conditions.

Very recently, the interaction of staphylococcal δ-lysin with multilamellar vesicles composed of dimyristoylphosphatidylcholine has been more extensively studied over the whole concentration range by spectroscopic and X-ray techniques as well as DSC [115]. Similarly to the DPPC/δ-lysin system, the addition of smaller quantities of peptide had little effect on the phase transition temperatures of DMPC bilayers, but did affect the cooperativity and enthalpy of the transitions. However, increasing the peptide concentration to molar lipid-to-peptide ratios lower than 125:1 promoted the formation of two populations of lipid particles, as deduced from the occurrence of a new phase transition, characterized by slightly increased temperature and markedly reduced cooperativity and enthalpy (Fig. 5). These two distinct fractions could be separated by centrifugation. Small-angle X-ray scattering measurements confirmed that the pelleted fraction consists of multilamellar vesicles, as can be deduced from the sharp Bragg reflections, while the form of the small-angle X-ray scattering curves obtained from the supernatant with its broad side maxima and minima was characteristic for particle scattering. It is worthwhile to note that these results demonstrate that DSC is also capable of detecting such morphological changes, albeit indirectly. The experimental scattering curves were further analyzed by indirect Fourier transformation, which can be interpreted as originating from particles with the shape of a flat inhomogeneous lamellae, i.e. single bilayer lipid aggregates. Detailed analysis of the X-ray data showed that with increasing amounts of δ-lysin, initially formed unilamellar vesicles transformed into disk-shaped lipid-peptide aggregates of a diameter of about 14 nm and a bilayer thickness of 5.2 nm. Interestingly, a hydrodynamic radius of 6.9 nm, as estimated from gel filtration experiments, was reported for the disk-shaped particles of melittin/

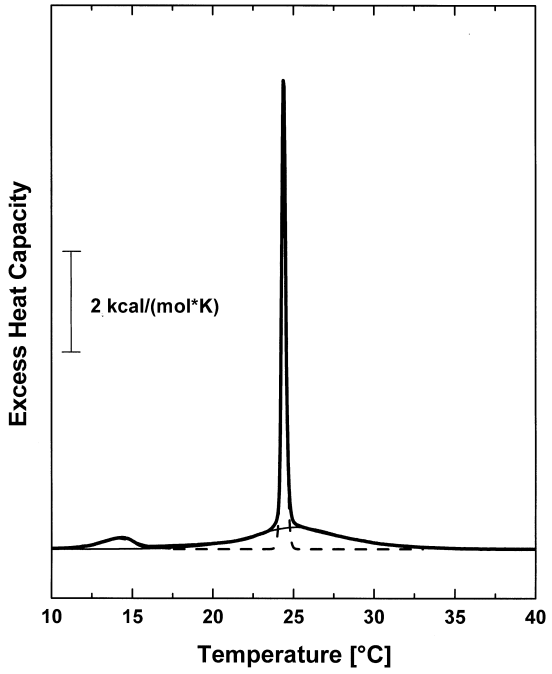

Fig. 5. High-sensitivity DSC thermograms of mixtures of DMPC and δ-lysin at a lipid-to-peptide molar ratio of 30:1 recorded with a scan rate of 0.25°C/min. Simulation of the sharp (dashed line) and broad (thin solid line) component are indicated.

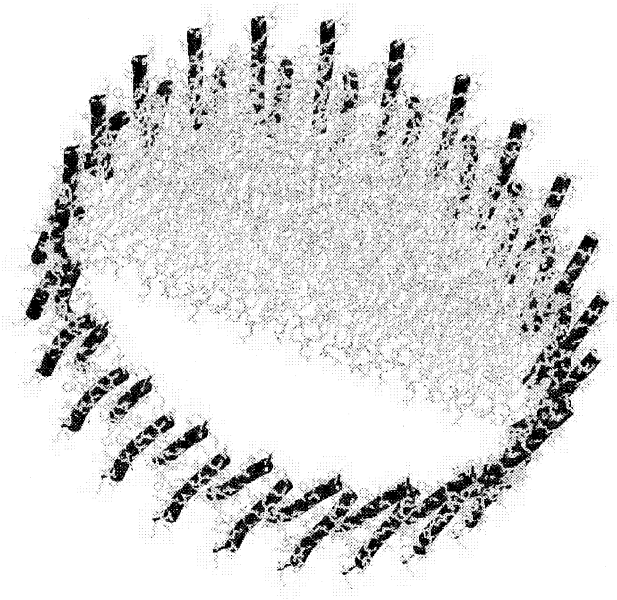

Fig. 6. Model of a lipid disk surrounded by a δ-lysin ring drawn by Rasmol 2.6. The discoidal lipid bilayer (only half of the PC molecules are shown) is circumscribed by 24 dimers of δ-lysin (α-helices are shown as rods, theoretical model of the peptide dimer was taken from the Brookhaven Protein Databank 1DHL).

DMPC [114]. Moreover, modeling of the X-ray data verified a discoidal lipid bilayer with a diameter of 12 nm, which is surrounded by a peptide ring of about 1 nm thickness. A respective model drawn by Rasmol is shown in Fig. 6, where the rim is composed of partially displaced δ-lysin dimers (data taken from the Brookhaven Protein Databank 1DHL) oriented perpendicular to the bilayer plane. However, it has to be noted that the experimental approach and analysis used did not allow to determine accurately the orientation of the peptide helix in the disk-shaped micelle, but was rather derived from the molecular properties of the peptide and lipid molecules. These data support the idea that lytic peptides may have concentration-dependent effects on the membrane structure inducing bilayer perturbations of long-range order at low peptide concentrations and exhibiting a detergent-like action at high peptide concentrations. Pore formation, as also reported for δ-lysin [116], is not necessarily in contradiction to this proposal, but may be either a transient state or may occur at distinct environmental conditions particularly as function of the lipid class.

6. Conclusion

Differential scanning calorimetry and X-ray diffraction studies have provided evidence that antimicrobial peptides discriminate between different classes of phospholipids. These peptides show preferential interaction with negatively charged phospholipids, major components of bacterial membranes, while they show only minor effects on the phase behavior of zwitterionic lipids such as PC and SM, major components of mammalian membranes. This is in contrast to cytolytic peptides like melittin or δ-lysin, which perturb PC bilayers already at very low peptide concentrations. At high peptide concentrations they exhibit detergent-like action. These observations are in accordance with results obtained by other techniques which, however, have not been the focus of this chapter but are dealt with by other authors of this special issue. Furthermore, it was shown that antimicrobial peptides can induce phase segregation in mixed phospholipid systems containing negatively charged lipids.

DSC and X-ray diffraction studies on the interaction between antimicrobial and cytolytic peptides with PE, another major component of bacterial membranes, demonstrated that the peptides perturb the PE bilayer by a different mechanism. This can be explained by the molecular geometry of the lipid and peptide molecules. While some peptides (e.g. magainin, PGLa or δ-lysin) impose a positive membrane curvature strain, others (e.g. gramicidins, alamethicin) amplify the intrinsic negative membrane curvature strain of this lipid. The former effect can lead to the formation of peptide/lipid pores, the latter will promote the formation of nonlamellar lipid structures. Both mechanism will result in massive membrane perturbation. Moreover, novel X-ray diffraction and in-plane scattering techniques have revealed quantitative information on both the location of the peptide and the related effect on lipid ordering and membrane structure. These results, like e.g. membrane thinning, will significantly contribute to a better understanding of the action of antimicrobial peptides on the molecular level.

The wealth of information gained by these complementary techniques will not only be helpful for the understanding of the molecular mechanism(s) of membrane perturbation, but also emphasize the role

of the phospholipid matrix in this process. It can be expected that the mutual knowledge accumulated in the field of membrane biophysics and peptide chemistry will strongly enhance our understanding of the membrane damage by antimicrobial peptides. This in turn will facilitate the design of novel peptide antibiotics which are urgently needed because of the dramatic increase in the number of bacterial strains being resistant to conventional antibiotics [5].

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