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Review

Lipid-induced conformation and lipid-binding properties of cytolytic and antimicrobial peptides: determination and biological specificity

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Abstract

While antimicrobial and cytolytic peptides exert their effects on cells largely by interacting with the lipid bilayers of their membranes, the influence of the cell membrane lipid composition on the specificity of these peptides towards a given organism is not yet understood. The lack of experimental model systems that mimic the complexity of natural cell membranes has hampered efforts to establish a direct correlation between the induced conformation of these peptides upon binding to cell membranes and their biological specificities. Nevertheless, studies using model membranes reconstituted from lipids and a few membrane-associated proteins, combined with spectroscopic techniques (i.e. circular dichroism, fluorescence spectroscopy, Fourier transform infra red spectroscopy, etc.), have provided information on specific structure–function relationships of peptide–membrane interactions at the molecular level. Reversed phase-high performance chromatography (RP-HPLC) and surface plasmon resonance (SPR) are emerging techniques for the study of the dynamics of the interactions between cytolytic and antimicrobial peptides and lipid surfaces. Thus, the immobilization of lipid moieties onto RP-HPLC sorbent now allows the investigation of peptide conformational transition upon interaction with membrane surfaces, while SPR allows the observation of the time course of peptide binding to membrane surfaces. Such studies have clearly demonstrated the complexity of peptide–membrane interactions in terms of the mutual changes in peptide binding, conformation, orientation, and lipid organization, and have, to a certain extent, allowed correlations to be drawn between peptide conformational properties and lytic activity.

Keywords: Antimicrobial peptide; Peptide–lipid interaction; Secondary structure; Structure–activity relationship; Membrane specificity

Abbreviations: Aib, amino-isobutyric acid; ATR-FTIR, attenuated total reflection Fourier transform infrared; CD, circular dichroism; cmc, critical micellar concentration; DPG, diphosphatidylglycerol; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; IR, infrared; lpc, lysophosphatidylcholine; lpg, lysophosphatidylglycerol; LPS, lipopolysaccharide; LUVs, large unilamellar vesicles; NMR, nuclear magnetic resonance; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGLa, peptidyl-glycine-leucine-carboxyamide; RP-HPLC, reversed phase-high performance liquid chromatography; RT, retention time; SDS, sodium dodecyl sulfate; SM, sphingomyelin; SPR, surface plasmon resonance; SUVs, small unilamellar vesicles; TFE, trifluoroethanol

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Contents

1. Introduction

The number of new antimicrobial peptides isolated and characterized from various sources nowadays exceeds 150 distinct molecules and continues to grow [1]. While many conventional antibiotics disable or kill bacteria over a period of days, antimicrobial peptides kill almost instantaneously, i.e. within minutes. These antimicrobial peptides mostly affect cells by interacting with the lipid components of their membranes. However, the molecular mechanism underlying peptide-mediated cell lysis, i.e. whether these peptides form pores, dissolve the membranes like detergents, or induce membrane defects, remains a matter of debate [2]. Furthermore, the inhibition of DNA biosynthesis resulting in cell death has also been proposed as a secondary mechanism of action for a number of antimicrobial peptides [3–5].

Another unsolved question is the influence of the cell membrane composition on peptide specificity towards a given organism. Although many defense peptides show a high specificity towards bacterial membranes, antimicrobial peptides can also exhibit toxicity, i.e. non-specific membrane lysis, of mammalian cells such as erythrocytes. There is also evidence that some antimicrobial peptides have specificity for particular membrane lipid components which influence their secondary structure [6–9]. A better understanding of the molecular basis of the specificity against different microbes would then facilitate the design of more potent and specific agents. Specifically, the structural changes of antimicrobial and cytolytic peptides upon interaction with membranes not only is a function of membrane lipid composition, but also of temperature, pH, and ionic strength. A consideration of the aqueous phase of all of these elements is therefore essential to the understanding of the mode of binding of these peptides. The main reason for the difficulties in establishing a direct correlation between the lipid-induced conformation of lytic peptides upon binding to cell membranes and their specificities toward given organisms is the lack of experimental model systems that mimic the complexity of natural cell membranes. Indeed, biomembranes are complex, two-dimensional anisotropic systems whose functions are determined by their

chemical composition, physical state and organization, all of which are interdependent, and strongly influence the induced conformation of peptides and proteins upon binding. Nevertheless, a number of spectroscopic techniques have been utilized to address the peptide–lipid interaction issues using model membranes reconstituted from lipids and a few membrane-associated proteins. While information on specific structure–function relationships of peptide–membrane interactions have been obtained at the molecular level, there is limited information on the dynamic changes associated with the energetics of such interactions. Recently, reversed phase-high performance chromatography (RP-HPLC) and surface plasmon resonance (SPR) have emerged as useful techniques for the study of the dynamics of the interactions between cytolytic peptides and lipid surfaces. This review will focus on the use of three of the existing spectroscopic techniques (i.e. circular dichroism (CD), fluorescence spectroscopy, and Fourier transform infrared resonance spectroscopy (FTIR)), as well as on the use of RP-HPLC and SPR for the study of peptide–lipid interactions. Another powerful tool, multidimensional solid-state nuclear magnetic resonance (NMR) spectroscopy, is the focus of the review by Bechinger in this issue.

2. Lipid-induced peptide conformations

2.1. Common characteristics

The induction into a specific conformation upon interaction with a lipid surface has been established as a requirement for lytic activity to occur [10,11]. The amphipathic properties of membranes provide a unique environment for the binding of peptides, which influences the peptide structure and resulting biological activity. While the secondary structures of cytolytic and antimicrobial peptides vary between peptide classes, they have the common feature of being cationic under physiological conditions [12,13] and of forming amphipathic structures [14]. The amphipathic character enables membrane permeabilization and/or perturbation. The net positive charge of these peptides is generally +2 or more, which is believed to facilitate their interactions with negatively charged membrane phospholipids. In particular, these peptides bind to the negatively charged lipopolysaccharide molecules which comprise the outer leaflet of the outer membrane of Gram-negative bacteria. Therefore, the outer membrane of Gram-negative bacteria, which serves as barrier to hydrophobic antibiotics, is relatively ineffective in protecting these cells against cationic antimicrobial peptides [15]. Similarly, the cationic character of these peptides is likely to facilitate their interactions with the negatively charged sialic acid molecules which project out of the erythrocyte surface [16]. The insertion into the lipid bilayer of the erythrocyte membrane, which is at a distance from the sugar molecules, would then depend on the affinity level of the peptides for the sugar molecules (i.e. on their cationic character and ability to cross the sugar barrier). The D-enantiomers of a number of antimicrobial and cytolytic peptides were found to exhibit similar activity to their L-counterpart [17–20]. This lack of stereospecificity suggests that the peptides interact with achiral components of the cell membrane and that an appropriate hydrophobic environment is the main requirement for peptide-mediated lysis and/or cell death to occur.

2.2. α-Helical peptides

The two main conformations induced upon binding to membranes are the α-helix and β-sheet. Cytolytic and antimicrobial α-helical peptides include melittin, magainin, alamethicin, cecropin A, as well as a number of de novo designed antimicrobial peptides (reviewed in [13]). These peptides exist as disordered structures in aqueous solution and fold into an α-helical conformation upon interaction with hydrophobic solvents or lipid surfaces. α-Helical peptides are often found to be amphipathic and can either absorb onto the membrane surface or insert into the membrane as a cluster of helical bundles. While the helical structure is well established by means of CD, FTIR, and/or NMR, the orientation of these peptides in the membrane remains controversial and conclusions largely depend on the techniques used [19,21,22]. As expected, the action of these peptides on membranes appear from several studies to be highly sensitive to the membrane lipid composition [23–30].

2.3. β-Sheet peptides

In contrast to the linear α-helical peptides, β-sheet peptides are cyclic peptides constrained either by disulfide bonds or cyclization of the backbone. They largely exist in the β-sheet conformation in aqueous solution that may be further stabilized upon interactions with lipid surfaces. Defensins are among the most characterized β-sheet-forming antimicrobial peptides. Different mechanisms involving either the perturbation of bilayers or the formation of discrete channels have been suggested for these peptides based on high resolution crystallography [31] and 2D-NMR studies [32]. Other β-sheet peptides are the protegrins and tachyplesins which adopt hairpin-like β-sheet structures [33,34].

2.4. Other cytolytic and antimicrobial peptides

Other cytolytic and antimicrobial peptides include short amino-isobutyric acid (Aib)-containing peptides which adopt 3_{10}-helices [35], gramicidin A which adopts a left-handed, antiparallel, double-stranded β-helix [36–38], and cyclic peptides, such as the lantibiotics [39], gramicidin S [40], and ternatin [41]. In contrast to other antimicrobial peptides, proline–arginine-rich peptides cannot form amphipathic structures due to the incompatibility of high concentration of proline residues in such structures and have been proposed to adopt a polyproline helical type-II structure [42,43]. Similarly, the tryptophan-rich peptide indolicidin was reported to adopt a polyproline type-II helix [44].

2.5. Antimicrobial versus cytolytic peptides

Despite the current level of the understanding of the molecular basis of antimicrobial and cytolytic peptide action, the de novo design of peptides having high antimicrobial activity and low mammalian toxicity, or a narrow spectrum of activity, still represents a major challenge. Since the sites of action toward both bacterial and mammalian cells are the membrane lipids, complete specificity represents a difficult task to achieve. However, the design of peptides having structural properties which result in an optimal therapeutic index has been the goal of a number of studies. Interestingly, subtle changes in a peptide sequence may have a significant effect on the peptide antimicrobial and/or hemolytic activity. Thus, studies using omission or substitution analogs of various α-helical peptide sequences have shown that high amphipathicity [11,45–47], high hydrophobicity [28,45–48], and/or high helicity [49,50] were favorable toward hemolytic activity. On the other hand, decreases in hydrophobicity or amphipathicity resulted in either increased [11,49–51] or decreased [45,52] antimicrobial activities, depending on the peptides studied. From these studies, it appeared that a decrease in antimicrobial activity occurs below a certain threshold of decreased amphipathicity. Low amphipathicity may result in a decreased affinity between the peptides and cell membranes. Recently, similar studies using D-amino acid substitution analogs of gramicidin S, a β-sheet cyclic peptide, have agreed with the previous results and showed that high amphipathicity and high hydrophobicity correlated with high hemolytic activity, while decreased amphipathicity resulted in increased antimicrobial activity [53,54]. In addition to an amphipathicity threshold, the latter studies indicated that a minimum size of the peptide hydrophobic domain is required for antimicrobial activity to occur.

Based on the few differences in the structural properties of the antimicrobial, non-hemolytic, peptide cecropin A and the hemolytic and antimicrobial peptide melittin (i.e. reversed polarity and different peptide length), the Boman and Merrifield groups designed cecropin A-melittin hybrid peptides with the aim of improving the antimicrobial activity of cecropin A without carrying over the toxic properties of melittin, as well as with the aim of gaining a better understanding of the key features in the specificity of these peptides [55–57]. The hybrid concept was later applied to generate cecropin A-magainin 2 hybrid peptides [58,59]. A number of these hybrid peptides were found to have enhanced antimicrobial activity and a broader spectrum of action than either parent sequences while showing low lytic effect on mammalian cells. These studies indicated that both the polarity and flexibility of the molecule are key elements for the specificity of these peptides.

A recent comparative study between the hemolytic and antifungal activities of cationic peptides emphasized the effect of assay parameters in the peptide activities [60]. Typical activity determinations are

carried out in high ionic strength buffer for hemolytic activity and in low ionic strength buffer for antifungal activity. However, significant differences in activity were found when performing the two assays in the same low ionic strength buffer, with non-hemolytic peptides in standard conditions becoming hemolytic in low ionic strength conditions. The hemolytic activity of these peptides was also found to depend on the freshness and antigen expression of the red blood cells. While these studies did not compare the peptide-induced conformations under such environments, ionic strength is expected to greatly influence the conformation that peptides adopt in aqueous solution and therefore their interactions with cell membranes. The use of standard protocols for the determination of peptide antimicrobial, antifungal, and hemolytic activities and their relevance to in vivo systems is obviously an important issue. This is not, however, the focus of this review.

3. Tools to study lipid-induced conformation and binding affinity of peptides to lipid surfaces

As mentioned earlier, the difficulties in studying the influence of cell membrane composition on the lytic specificity of peptides arise from the lack of lipid systems that mimic the complexity of natural cell membranes and which are compatible with spectroscopic measurements. Biomembranes consist mainly of lipids, proteins, and carbohydrates held together primarily by non-covalent interactions. While the common structure of the lipid bilayer forms the basis of membranes in all organisms, considerable variations in the structure and complexity of membranes exist between eukaryotic and prokaryotic cells. Furthermore, the structure and organization of the cell envelope clearly differ in Gram-positive and Gram-negative bacteria, while the phospholipid compositions vary among strains and depend on the growth conditions. Despite these major differences, but also because of the complexity of these systems, no existing technique has, to date, allowed a direct correlation to be established between the membrane-specific characteristics and their influence on the lytic specificities of peptides. Nevertheless, a great advance in the understanding of the effect that the membrane lipid-composition and structure have on the induced conformations and binding affinities of peptides has occurred in the last decade. This is mainly due to the development of novel techniques or novel applications of existing techniques as described below. The use of pure synthetic lipids also provides great advantages in understanding the influence on peptide conformational behavior of the chemical structure, polymorphic behavior, and dynamic properties of the lipid membrane, as well as the influence of environmental factors, such as temperature, pH, ionic strength, hydration level, and organic co-solvent.

3.1. Circular dichroism spectroscopy

Several approaches are used in CD spectroscopy to simulate the effect of membranes on peptide conformations. Organic co-solvents and detergents below their critical micellar concentration (cmc) have been and still are often used to mimic the hydrophobic core of the membrane, whereas micelles and lipid vesicles are applied as model membranes. Methanol, ethanol, acetonitrile, 1,1,1,3,3,3-hexafluoroisopropanol and trifluoroethanol (TFE) are the most frequently used membrane-mimetic organic co-solvents to determine the induced conformations relevant to peptide antimicrobial activity [61,62]. While these solvents are known to stabilize the secondary structure for which the sequence has propensity (i.e. α-helix or β-sheet), the induced conformation in the presence of these co-solvents does not necessarily reflect the peptide conformation induced upon interacting with lipid surfaces. For example, while the CD and FTIR spectra of peptides corresponding to the interaction site with lipids of the hemagglutinin of influenza virus clearly indicate the induction into a β-sheet structure in the presence of egg-phosphatidylcholine, the peptides fold into an α-helical conformation in 50% TFE [63]. The secondary structure of magainin, an antimicrobial peptide from the skin of the South African frog *Xenopus laevis* [64], was also found to be different in TFE–water mixtures than in lipidic milieu [26,65,66]. Another example is the significantly different conformations reported for melittin, the main component of bee venom [67], when bound to membranes and in the presence of detergent micelles or TFE [68].

Monomeric sodium dodecyl sulfate (SDS) represents another model for lipid-like environments.

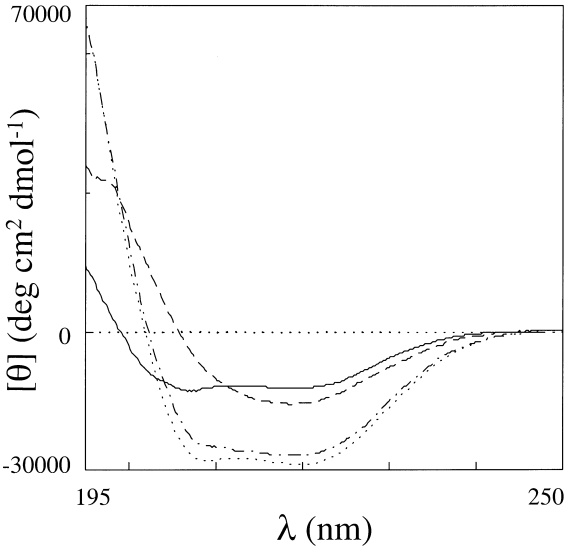

Fig. 1. Conformation of Ac-KYA$_{13}$K-NH$_2$ in (——) 5 mM MOPS, pH 7.0, (— — —) 2 mM SDS, (- - -) 10 mM SDS, and (— - —) lpc/lpg micelles [72].

SDS is a surfactant that can provide a hydrophobic environment for polypeptides [61]. At low concentrations (2–4 mM, i.e. below the cmc), SDS is in a monomeric state and has been described as a mimetic of the interior of proteins. SDS induces a β-sheet conformation in short peptide sequences by providing a hydrophobic template that promotes the stabilization of the peptide conformation through hydrophobic interactions between a monomeric peptide strand and a monomeric SDS strand [69]. Thus, peptides found to adopt an α-helical conformation in TFE were induced into a distinct β-sheet structure in the presence of low concentration of SDS [61]. At high concentrations (greater than 4 mM, i.e. above the cmc), SDS forms micelles that are used as mimetics of negatively charged bilayers and that provide an anisotropic environment similar to lipid vesicles. Micellar SDS has been reported to be a stabilizer of α-helical conformations [61,69–71]. An example of such variations in induced conformations in the presence of different SDS concentrations is shown in Fig. 1 [72]. Thus, a clear β-sheet conformation was observed in 2 mM SDS (87% β-sheet), while the peptide showed an α-helical conformation in both micellar SDS (80% α-helix) and lysophosphatidylcholine/lysophosphatidylglycerol (lpc/lpg) micelles (68% α-helix). At this point, it should be noted that lipids exhibit a different packing geometry in micelles, than in vesicles which is expected to affect their interaction with peptides. Nevertheless, aqueous micelles are still among the most commonly used lipid systems. They are generated from surfactant or lysolipid molecules [73] and have the advantage of yielding optically transparent supramolecular lipid aggregates, which are required for CD spectroscopy.

Liposomes or vesicles represent a more complex model system for mimicking biological membranes [73] and are generally classified into four groups according to the number of lamellar and size distribution: small unilamellar vesicles (SUVs diameter 25–50 nm), large unilamellar vesicles (LUVs diameter around 100 nm), multilamellar vesicles (diameter 100–800 nm), and giant unilamellar vesicles (diameter >1 μm) [74]. In a manner similar to micelles, SUVs yield optically clear solutions and exhibit strains in lipid packing owing to the high membrane curvature of these small particles. This can considerably affect the ability of amphipathic peptides to bind and, in particular, to insert into the hydrophobic core of such bilayers. To avoid such issues and to more closely relate to natural cell membranes, LUVs consisting of one or two lipid components are more commonly used. Specifically, comparative studies are commonly carried out using negatively charged lipids

Table 1
Main phospholipid components of illustrative organisms

	Zwitterionic			Negatively charged			
	PE	PC	SM	PS	PG	DPG	lpg
E. coli IM	82				6	12	
S. aureus					57	5	38
C. albicans	70	4	15	11			
C. neoformans	29	51		16		4	
Erythrocyte outer membrane leaflet		50	50				

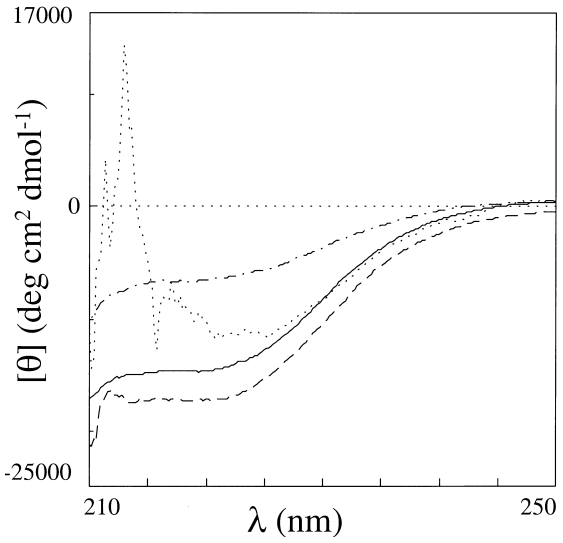

Fig. 2. CD spectra of YKLLKLLLPKLKGLLIKL-NH_2 in (———) *Staphylococcus aureus* mimetic liposomes (PG/DPG/lpg (57/5/38)), (— — —) *Cryptococcus neoformans* mimetic liposomes (PE/PC/PS/DPG (59/51/16/4)), (- - -) *Candida albicans* mimetic liposomes (PE/PC/SM/PS (70/4/15/11)), and (— - —) erythrocyte mimetic liposomes (PC/SM/cholesterol (37/37/26)). The activities of this peptide against the respective organisms are MIC = 22, 11, and 83 μg/ml, and HD_{50} = 149 μg/ml.

and zwitterionic lipids [2]. As illustrated in Table 1, general information on peptide–cell-type interaction preferences can be derived from such studies. Thus, the main lipid components of the erythrocyte plasma membrane are zwitterionic lipids, while Gram-positive and Gram-negative bacterial plasma membranes as well as fungi are characterized by a comparably large amount of negatively charged lipid [75–77]. The influence of the lipid composition on peptide induced helicity is illustrated in Fig. 2 using model peptides and liposomes composed of the different lipid ratios shown in Table 1.

A lipid discrimination by antimicrobial peptides was also demonstrated for peptidyl-glycine-leucine-carboxyamide (PGLa) [29]. This cationic, 21-amino acid residue peptide isolated from the skin of the South African clawed frog *X. laevis* [78], is practically devoid of secondary structure in aqueous environments. Partial β-structure and a minor amount of α-helix were detected at high peptide concentration, suggesting the formation of aggregates which would result in increased intermolecular hydrogen bonding between the peptide molecules. Partial β-structure was also observed at high temperatures for PGLa [29] and magainin 2 [79]. PGLa did not change its rather structureless conformation in the presence of model membranes composed of phosphatidylcholine (PC) and sphingomyelin (SM) [29] or of PC and cholesterol [80], which are the predominant components of the outer leaflet of most mammalian cell membranes, such as the erythrocyte membranes (Table 1). These observations indicate that PGLa does not interact with these lipids, which correlates with the lack of hemolytic activity of this peptide. However, PGLa exhibits a conformational change from an unordered structure in solution to a predominantly α-helical structure (around 60%) in the presence of negatively charged liposomes composed of phosphatidylglycerol (PG) and phosphatidylethanolamine (PE), lipids mimicking bacterial plasma membranes (Table 1). This occurs independently of the phase state of the lipids [29]. Magainin has a similar behavior, although it does not exhibit sequence homology to PGLa. Thus, magainin was also found to be unordered in aqueous solution and to adopt an α-helix in the presence of liposomes composed of negatively charged phospholipids [25,81,82].

Cecropin A, isolated from the immune hemolymph of the moth *Hyalophora cecropia* [83], is composed of two helices connected by a hinge region formed by Gly-Pro [84], while the mammalian cecropin P1 forms a continuous α-helix [85]. From CD measurements and sequence comparisons, it was suggested that cecropin B shares the helix-hinge-helix motif of cecropin A [86]. All of these peptides have in common an amphipathic N-terminal region with a strong cationic face and a large hydrophobic C-terminal region. To gain information on the role of these regions with respect to membrane lysis, Wang et al. designed two cecropin analogs, the first one having two amphipathic helices, termed cecropin B1, and the second one having two hydrophobic helices, termed cecropin B3 [87]. The interaction of these peptides with liposomes composed of varying ratios of phosphatidic acid (PA) and PC were studied by CD spectroscopy. Cecropin B, B1 and B3 had no secondary structure in aqueous solution, but adopted an α-helical structure upon interacting with liposomes and SDS micelles. The extent of α-helix formed depended on the amount of negatively charged lipids and was most pronounced for the hydrophobic cecropin analog. Furthermore, binding

and leakage experiments revealed very similar behavior for cecropin B and B1. While the binding of both peptides increased with increasing amounts of negatively charged lipids, as shown by surface plasmon resonance, the efficiency of liposome disruption decreased. On the other hand, binding of cecropin B3 was negligible, although cecropin B3 was more efficient in disrupting liposomes containing a large fraction of PA. Only one rate constant for each peptide was found by stopped-flow CD measurements, which depended on the charge of the peptide, being fastest for cecropin B1 (net charge 11+) and slowest for cecropin B3 (net charge 3+), mainly reflecting the stronger binding to negatively charged liposomes. This study also demonstrated that the helical structure formed early in the interaction of all of these peptides and was maintained throughout the whole binding process. In contrast, stopped-flow fluorescence data revealed a two-step process for cecropin B and B1 and a one-step process for cecropin B3, which may reflect different mechanisms of action. It was suggested that the more cationic peptides form pores, while the hydrophobic analog perturbs the bilayer structure through accumulation of the hydrophobic peptide in PC domains [87], as had been proposed earlier for cecropin P1 [88].

Covalent binding of lipids to quartz surfaces represents another route to study peptide–lipid interaction by CD spectroscopy. Such an approach was first presented with the coating of quartz plates with octadecyl groups [89,90]. To mimic an aqueous–lipid interface, the CD spectra were recorded while the plates were submerged in aqueous buffer. Using model leucine/lysine-containing amphipathic peptides, these studies clearly demonstrated the induction of α-helical or β-sheet conformations upon binding to the C_{18}-coated plates, with α-helical peptides having higher affinity for the lipid surface. The lower binding affinity observed for β-sheet peptides may be explained by the occurrence of an equilibrium between a multimeric self-aggregated peptide complex and a peptide–C_{18}-surface complex, both being stabilized through hydrophobic interactions. In a manner similar to the monomeric SDS hydrocarbon chain, the C_{18}-coated plates can then be envisioned as a hydrophobic template mimicking a hydrophobic strand necessary for monomeric β-sheet induction. Similar induced conformations were recently presented for the NC1 domain of chicken collagen upon interacting with CD quartz plates that were coated with C_4, C_8, and C_{18} groups [91]. Although this method does not allow a precise quantitation of the extent of the induced conformation, it remains a powerful technique for the evaluation of the conformational propensities of peptides upon interactions with lipid surfaces and provides insight into the binding affinity of peptides.

Another approach that yields information on the various steps involved in the interaction of antimicrobial peptides and membranes is the use of oriented lipid layers in CD spectroscopy [92]. Using this technique, a transition from the surface-bound peptide to a transmembrane state was reported for alamethicin [93] and magainin 1 [94]. Alamethicin, a hydrophobic antibiotic peptide of 19 amino acid residues, contains a large amount of the unusual Aib (i.e. eight Aib residues) and adopts an α-helical structure in organic solvents [95]. Alamethicin and other synthetic peptides rich in Aib residues were reported to adopt a partial 3_{10}-helical structure upon incorporation into bilayers [96,97]. Studies using oriented lipids in conjunction with CD spectroscopy also showed that a number of α-helical peptides initially lie parallel to the membrane plane at lower lipid-to-peptide molar ratios and insert perpendicular to the membrane plane above a certain threshold concentration (e.g. around a lipid-to-peptide molar ratio of 30 for magainin [94]). These ratios were shown to depend strongly on the lipid composition and hydration state. A similar behavior was also suggested for the β-sheet peptide, protegrin 1, although some differences appear to exist when compared to the α-helical peptides [98].

3.2. Fluorescence spectroscopy

Fluorescence spectroscopy is a highly sensitive technique extensively used to study peptide–lipid binding and peptide penetration into membranes. The wide variety of fluorescence probes makes the method suitable for studying a great range of interactions. For example, the release of fluorescence dyes from lipid vesicles provides useful information on peptide binding ability and membrane perturbation properties [7,99]. The leakage of dyes mediated by the peptides was found, however, to be greatly af-

fected by the mode of mixing the lipid and peptide solutions, and therefore such experiments can lead to erroneous conclusions on the membrane-lytic effect of peptides [99]. Due to the high sensitivity of fluorospectrophotometers and ease of use, fluorescent labeling has greatly replaced the earlier radioactive labeling approach for peptide–lipid interaction and penetration studies. Thus, peptide uptake into liposomes can be determined by quantitating the fluorescence intensity of the fluorescent label (e.g. dansyl group) of the remaining peptide in solution after separation by chromatographic techniques [100,101]. Measurements of quenching of tryptophan fluorescence by aqueous and hydrophobic quenchers are also commonly used to study peptide penetration into artificial membranes [7], as well as peptide aggregation state within a bilayer [102,103]. Recently, measurements of the fluorescence decay of tryptophan emission provided information on the distribution of tryptophan rotamers for peptides bound to phospholipid bilayers [104]. Thus, the distribution and environment of tryptophan rotamers in peptide–lipid complexes were found to depend on the peptide sequences and induced conformations. As for the other spectroscopic techniques described, conclusions drawn from fluorescence experiments on peptide orientation in the lipid bilayer, as well as their aggregational state, remain controversial and appear to be highly dependent on the systems used for the particular studies.

Depth-dependent fluorescence technology, utilizing lipids with bromine atoms or spin labels selectively attached to certain positions along the acyl chains, is an emerging technique to determine the structure of membrane proteins, as well as to obtain information on peptide penetration into membranes [49,103,105–108]. The parallax method [109] and more recent distribution analysis [110] are then used to extract such information from the quenching data. The difficulties in using this technique derive from the uncertainty of the local concentration of quenching lipids and of the fluorescence of non-quenching lipids, the existence of multiple conformations of membrane-bound peptides, and incomplete binding. Work presented by Rodionova et al. [111] and Ladhokin [105] using the outer membrane protein OmpA and a membrane spanning peptide MSP, respectively, have strengthened the suitability of this technique when combined with the distribution analysis method for the evaluation of membrane penetration of peptides and proteins. For example, a deeper insertion of OmpA, combined with subsequent structural rearrangement which resulted in a different exposure of tryptophan residues to the lipid quenchers, could be detected using the distribution analysis method, which was not detected using the parallax method alone [111].

3.3. *Attenuated total reflection Fourier transform infrared spectroscopy*

ATR-FTIR represents a powerful tool to simultaneously study lipids and peptides without introducing a probe, since vibrational modes of both molecules are present in the IR spectrum (the method with its precautions and limitations are reviewed in [112,113]). In contrast to most other spectroscopic techniques, light scattering does not affect such measurements. FTIR spectroscopy can be performed either as a transmission or an internal reflection experiment, the latter becoming widely used. A major advantage of ATR-FTIR is that the absorption of water molecules in the buffer solution is strongly reduced as compared to transmission experiments. Therefore, only small amounts of material (μg range) are necessary for recording IR spectra of membranes and peptides. When using synthetic peptides, one has to be aware that solvents commonly used in peptide synthesis and purification, in particular trifluoroacetic acid, absorb in the range of the amide I band, and therefore have to be thoroughly removed to allow a detailed analysis. Polarized ATR-FTIR spectroscopy also provides information on the orientation of different molecular parts of both the peptides and phospholipids [114]. To evaluate the molecular orientations from dichroism data, the orientation of the transition dipole moments as well as the refractive index have to be known. In this respect, it was pointed out that the determination of molecular order in supported lipid membranes, in particular, in the case of films which are much thinner than the IR wavelength, critically depends on the assumptions made about the evanescent electric field amplitudes in the membrane [115]. The difficulties in overcoming uncertainties on the orientation of the transition dipole moment and on the refractive index are dis-

cussed by Goormaghtigh et al. [113], while the complexity and influence of samples preparation on the interpretation of spectroscopic data are discussed by Tamm and Tatulian [112]. The potential of ATR-FTIR to yield information on the orientation of peptides in a membrane, an important aspect of understanding the molecular mechanism of membrane-active peptides, was demonstrated with the synthetic peptide LAH_4 [113], which exhibits a pH-dependent orientation as shown by solid state NMR spectroscopy [116]. The presence of the amide I (1657 cm^{-1}) and amide II (1546 cm^{-1}) bands indicate an α-helical structure for this amphipathic peptide. Based on the fact that the amide I dipole and amide II dipole are nearly parallel and perpendicular to the helix long axis, respectively, the orientation of LAH_4 was deduced from the dichroic difference spectra of the parallel and perpendicular polarized spectra [113]. At pH 4, LAH_4 was suggested to be oriented parallel to the membrane plane of PC model membranes, while at pH 9, it was perpendicular to the plane. In addition, information on the hydrophobic, polar and electrostatic contributions during membrane interaction could be obtained due to the presence of histidine residues, which have a pK_a value around 5.5.

The interaction of melittin with model membranes has been widely studied to gain information on the location of this hemolytic peptide. Experiments using supported PC bilayers showed that the orientation of melittin strongly depended on the state of lipid hydration, adopting a transmembrane orientation at low hydration but aligning preferentially parallel to the membrane surface in fully hydrated bilayers [117]. Furthermore, this orientation is also affected by the lipid composition. Thus, CD and FTIR spectroscopy studies performed at low hydration revealed that melittin is oriented perpendicular to the membrane plane in the presence of saturated dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG). In contrast, melittin lies parallel to the membrane plane in the presence of mono-unsaturated dioleoylphosphatidylcholine, dioleoylphosphatidylserine, and dioleoylphosphatidylglycerol [118]. On the other hand, a transmembrane orientation of melittin was found in oriented 1-palmitoyl-2-oleoylphosphatidylcholine bilayers at ambient humidity [102,119]. Although no clear general conclusions could be made regarding the orientation of melittin when bound to membranes, the observed significant effects of the acyl chains indicated the insertion of melittin into the membrane hydrophobic core. Similar effects were reported for the bacteriotoxin δ-lysin from *S. aureus* [119] and for transmembrane segments of the channel-forming peptide phospholamban [120]. Thus, FTIR analysis of oriented dipalmitoylphosphatidylcholine bilayers showed that δ-lysin lies randomly within the membrane [121]. On the other hand, data from fluorescence experiments indicated that the toxin lies parallel to the surface, only weakly penetrating the hydrophobic core with its apolar face [122]. Furthermore, ^{2}H- and ^{31}P-NMR experiments showed that the location of the peptide in the membrane depends on the experimental temperature relative to the gel-fluid phase transition temperature of dipalmitoylphosphatidylcholine [123]. In contrast to these hemolytic peptides, polarized ATR-FTIR studies showed that cecropin P1, isolated from mammals, is oriented nearly parallel to the membrane surface in model membranes consisting of PE and PG (liposomes mimicking the phospholipid composition of the inner membrane of *Escherichia coli* [124]). This is consistent with observations that the predominantly α-helical cecropin P1 did not change significantly the order parameters of the acyl chains of the same PE/PG liposomes [124].

In addition to the membrane location of peptides, the conformational polymorphisms of small amphipathic peptides can be deduced from FTIR studies, which is of general interest in elucidating the mechanism(s) of action of these peptides. For example, ATR-FTIR studies performed with peptide hormones showed a conformational change from random structure to β-sheet upon binding to lipid model membranes [125,126]. A change in secondary structure was also found for human calcitonin upon binding to supported lipid bilayers. Initially, a small fraction of α-helical structure was formed, while the formation of a larger fraction of β-sheet structure was kinetically delayed relative to the formation of α-helix [127]. While signal peptides are expected to form amphipathic helices [128], the secondary structure of the signal peptide of the *E. coli* λ phage receptor LamB was shown to depend on the packing properties of lipid monolayers. At low surface pressure, an α-helix was detected, whereas a β-sheet was

formed at high surface pressure [129]. The influence of the lipid-to-peptide molar ratio and lipid composition on the induced conformation of a synthetic peptide corresponding to the N-terminal 23 amino acid residues of the HIV envelope glycoprotein gp41, which exhibits membrane fusion activity has also been described [130,131]. In the presence of negatively charged lipids, the peptide transformed from an α-helical structure at low peptide concentrations to a β-sheet at high peptide concentrations. The surface-bound form was suggested to induce the aggregation of lipid vesicles, while the inserted α-helical form causes bilayer disruption [130]. Furthermore, IR spectroscopy revealed that, in the absence of cations, the peptide which associated with the vesicles was predominantly a pore-forming α-helix, whereas in the presence of Ca^{2+} the conformation switched to a fusogenic, predominantly extended β-type structure. Liposomes composed of zwitterionic phospholipids (i.e. PC and PE) and cholesterol also induced a β-type structure that became fusogenic in a dose-dependent fashion [132]. From these studies, it was concluded that it is not an α-helical, but an extended structure adopted by the HIV-1 fusion peptide that destabilizes cholesterol-containing electrically neutral membranes.

Detailed studies also exist on synthetic hydrophobic transmembrane helices. Systematic FTIR studies revealed that the peptide $K_2GL_{24}K_2A\text{-}NH_2$ forms a very stable transmembrane α-helix, although small distortions of its α-helical conformation are induced in response to any mismatch between peptide hydrophobic length and the hydrophobic thickness of the PC bilayer [133]. Furthermore, the peptide alters the conformational disposition of the acyl chains in contact with it, minimizing the extent of hydrophobic mismatch. This effect was less pronounced using PE model membranes [134]. In contrast to the polyleucine-based transmembrane helix, FTIR spectroscopic data indicated that the conformation of Ac-K_2-$(LA)_{12}$-K_2-NH_2 is sensitive to the composition of the surrounding medium, with the occurrence of conformational transitions from an α-helical to a 3_{10}-helical structure [135]. Furthermore, Ac-K_2-$(LA)_{12}$-K_2-NH_2 retained a predominantly α-helical conformation in both the gel and liquid–crystalline phases of short to medium chain PC (hydrocarbon chain length <18), but undergoes a reversible conformational change at the gel/liquid–crystalline phase transition, when incorporated into bilayers composed of long chain PC (hydrocarbon chain length ≥ 18) [136].

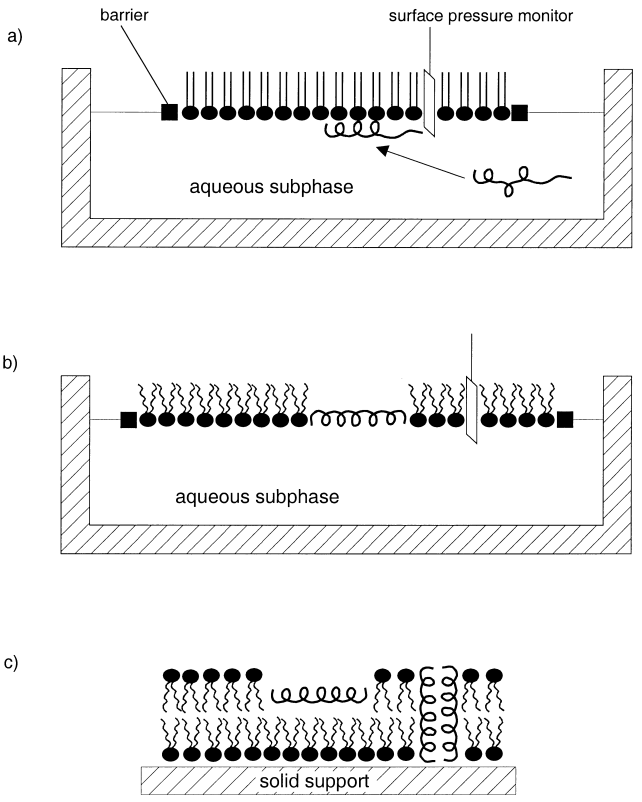

Fig. 3. Schematic representation of membrane model systems that allow the study of: (a) binding of peptides to the membrane surface (monolayer at high surface pressure); (b) penetration of peptides into the membrane interface (monolayer at low surface pressure); and (c) membrane insertion of peptides (supported bilayer) by polarized ATR-FTIR spectroscopy. Supported monolayers can be also used instead of monolayers formed at the air–water interface which are shown in the scheme [137].

Less is known about the conformational changes of antimicrobial peptides that may be associated with the individual steps of binding to the membrane surface, penetration of the membrane interface, and insertion into the bilayer hydrocarbon core. Polarized ATR-FTIR spectroscopy of supported membrane preparations (mono-, bi- and multilayers), combined with the use of monomolecular lipid films formed at the air–water interface of a Langmuir trough, provide useful models to investigate these different steps of lipid–peptide interactions (Fig. 3) [112,137]. Early FTIR studies on the interactions of melittin with

phospholipid monolayers at different surface pressures, and with small unilamellar vesicles above or below their phase transition temperature, showed that melittin, bound to the lipid surface, contains less α-helical structure than when inserted into the lipid bilayer [138]. Most interestingly, as the penetration depth of melittin increases, more ordered structures appear. This is in accordance with recent data on cecropin A, which demonstrated that the peptide inserts in both monolayer and bilayer model membranes, but inserting deeper in bilayers, which results in a more highly ordered peptide structure [137]. In addition, the phospholipid bilayer was more significantly perturbed as compared to the lipid monolayer. Minor changes in lipid absorption were attributed to a reorientation of the lipid ester group out of the membrane plane. Conformational changes were also reported for nisin, a peptide widely used as an antimicrobial food preservative. Although nisin is stabilized by five thioether bonds providing some rigidity to the peptide, its secondary structure is sensitive to the solution environment [139]. The amide I band indicated that the peptide adopts mainly unordered and β-turn structures in water, while the addition of TFE or binding to membranes promoted the formation of β-turns [140]. Nisin showed different affinity to negatively charged and zwitterionic lipids, though the amide I band of the peptide bound to these lipids was very similar. Furthermore, the phase state of these lipids had no influence on the binding affinity of nisin, suggesting that the nature of the membrane interface is less important for the action of this peptide. Finally, amphipathic peptides consisting of leucine and lysine residues of various lengths (5–22 mer), designed to yield ideal α-helices with a single lysine residue per putative turn [141], were shown to exhibit length-dependent lytic activities, which was accompanied by a change of secondary structure [142]. Thus, in both the bulk solid state and at the air–water interface, the shorter peptides (nine residues and less) folded predominantly into antiparallel β-sheets, whereas the longer peptides adopted α-helical structures. Furthermore, in the case of the shorter peptides, the random conformation present in the bulk state was not detected at the air–water interface. This result suggested that the interface induces a more strictly defined peptide structure. On the other hand, the structure and orientation of the peptides did not depend on the nature of the interface, i.e. air–water or DMPC monolayers at various surface pressures. In summary, these findings demonstrate that the flexibility of small amphipathic peptides may play a role in their biological activity.

3.4. *Reversed phase-high performance liquid chromatography*

RP-HPLC has been shown to be a sensitive analytical tool for the study of peptide conformation at aqueous–lipid interfaces [89,143–150]. The hydrocarbon groups of RP-HPLC columns have been shown to induce alterations in secondary structure, which results in variations in the retention behavior of sequence related peptides [143,146,147]. Thus, the dynamics of hydrophobic–hydrophobic interactions between peptides and the *n*-alkyl groups of the RP-HPLC stationary phase lead to the induction of secondary structures specific to each peptide. The deviation between experimental retention times (RTs) and the theoretical RTs calculated based on amino acid retention coefficients [151–153] is clearly due in part to induced secondary structures. Therefore, the sensitivity of the RP-HPLC systems lies in the fact that peptides and proteins interact with these surfaces in an orientation-specific manner via a specific hydrophobic contact area. Changes in this hydrophobic contact area can occur as a result of conformational or orientational changes, which, in turn, alter the binding properties of the peptides. Thus, RTs of peptides are a physical parameter which is highly sensitive to the conformational status of the peptides upon interaction with the hydrophobic surface. For example, peptides that were able to be induced into amphipathic α-helices were found to elute significantly later than predicted, while those designed to be inducible into β-sheet conformation eluted earlier than predicted [143]. Furthermore, perturbation of the amphipathicity and/or helicity of peptides by single or double sequence mutations was found to correlate with variation in the peptides retention behavior relative to the parent sequences [11,89,147,148,154].

Comparative RP-HPLC and CD studies using C_{18}-coated plates [90] provided evidence for the occurrence of a continuum of induced conformations for

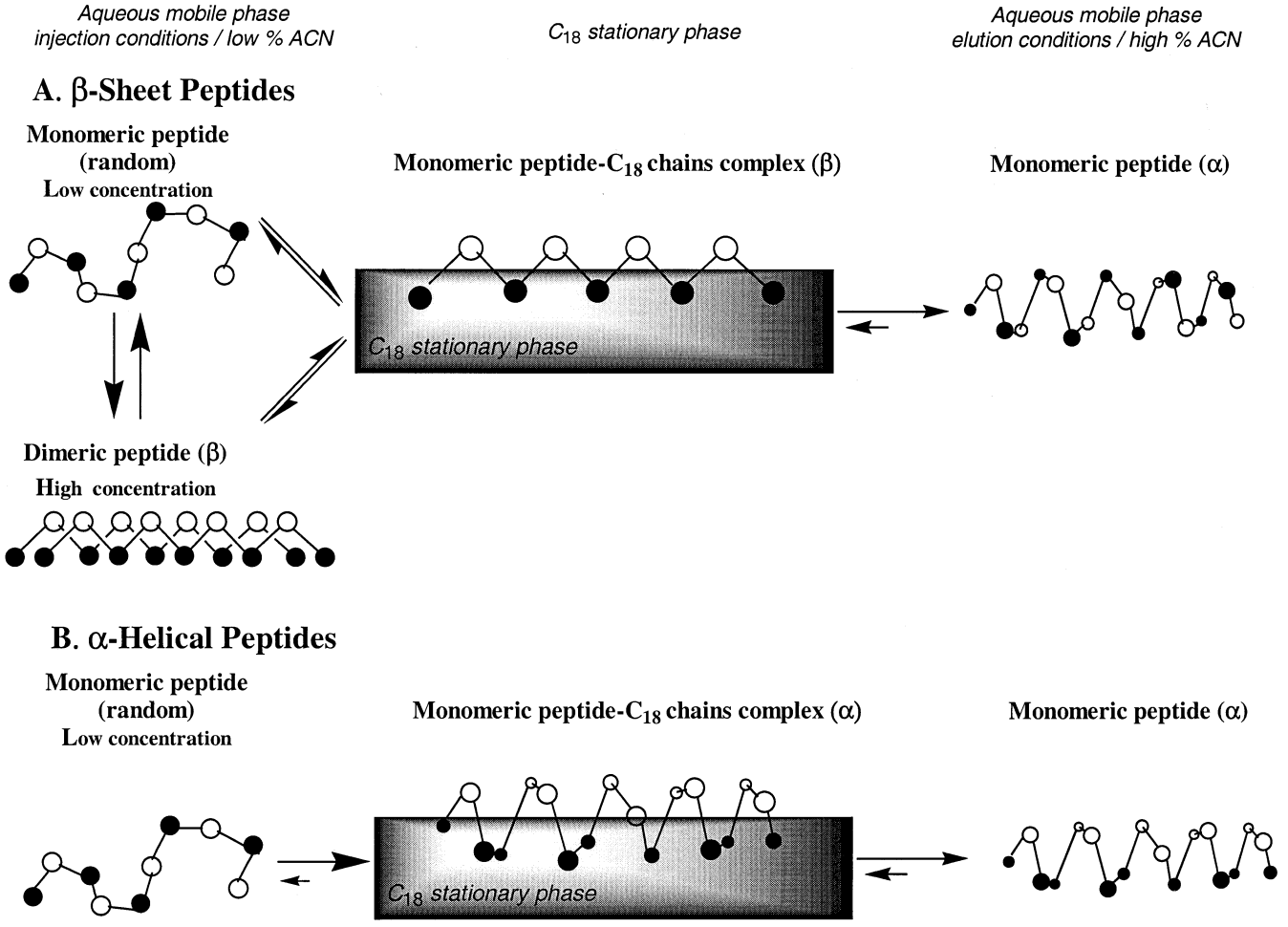

Fig. 4. Schematic of the conformational equilibria occurring during the RP-HPLC elution process of amphipathic β-sheet and α-helical peptides.

β-sheet forming peptides at different equilibrium stages during the RP-HPLC elution process (Fig. 4). In a first stage, the β-sheet conformations are induced resulting from the interaction of the peptides to the C_{18}-groups of the stationary phase which competes with intermolecular hydrophobic interactions. This would explain the lower binding affinity of such peptides to lipid surfaces. In contrast, no competition exists for hydrophobic interactions in the case of α-helical peptides. The final stage of the RP-HPLC elution process involves an equilibrium between the peptide-bound β-sheet or α-helical conformation and free peptide α-helical conformation in organic solvent (RP-HPLC mobile phase). The extent of the equilibrium shift depends on the specific binding affinity of each peptide which then affects the peptide RT. Thus, the shift toward the free peptide conformation occurs at a low percent of organic solvent for β-sheet peptides having low binding affinities, which explains their observed early RTs. Based on the distances between the hydrophobic and hydrophilic faces of amphipathic α-helical and β-sheet peptides, the differences in binding affinity, and, in turn, in RT between α-helical and β-sheet peptides, can be rationalized by a moderate insertion of the hydrophobic side into the lipid layer for β-sheet peptides and a deeper penetration of the α-helical peptides within the lipid layer (Fig. 4).

Temperature-dependent gradient elution RP-HPLC has also been used to investigate the binding properties of model amphipathic peptides with lipid-like surfaces [154]. The interaction of the peptides with the C_{18} ligands were characterized in terms of the binding affinity and hydrophobic binding domain

as a function of temperature. These studies revealed that peptides adopting either an α-helix or β-sheet upon adsorption to the surface undergo a significant conformational transition. However, the rate of this transition was much slower for α-helical peptides relative to β-sheet-forming peptides, indicating the stabilizing effect of the hydrophobic surface on peptide secondary structure. This phenomenon of differential stabilization of peptide secondary structure upon binding to a hydrophobic surface cannot be readily characterized by other techniques.

To understand the forces that control the specific binding and conformational changes of peptide–membrane interactions, the analysis of the energetics of binding and insertion of peptides into the membrane, particularly as a function of peptide conformation and lipid conformation and composition, is an essential step towards the characterization of the action of antimicrobial peptides. However, there have been very few studies which have addressed the binding characteristics of membrane-active peptides in terms of their relative affinity for different phospholipids. This is due to the nature of the existing techniques which cannot be easily used to analyze large numbers of peptides. Membrane-binding assays with SUVs and LUVs generally require a physical separation step to prepare distinct phases for analysis by dialysis or centrifugation, which can be tedious [155,156]. Alternatively, peptide partitioning can be directly measured by changes in fluorescence quenching or enhancement of tryptophan. However, this approach relies on the presence of a fluorescent probe, which if not an intrinsic fluorophore, must be incorporated by covalent modification of the peptide and/or lipid [157,158]. The RP-HPLC studies described above clearly demonstrated the potential of this technique for the quantitative determination of the changes in the conformation and binding affinity of peptides as they interact with a lipid-like surface. However, while the RP-HPLC sorbents provide a hydrophobic surface that may partially mimic the physicochemical properties of lipid surfaces, there are significant differences between the conformational and interactive properties of lipid membranes and the *n*-alkylsilicas used in RP-HPLC. The *n*-alkylsilicas do not contain a polar phosphate head and do not exhibit the cooperative phase transition behavior characteristic of lipid bilayers. Thus,

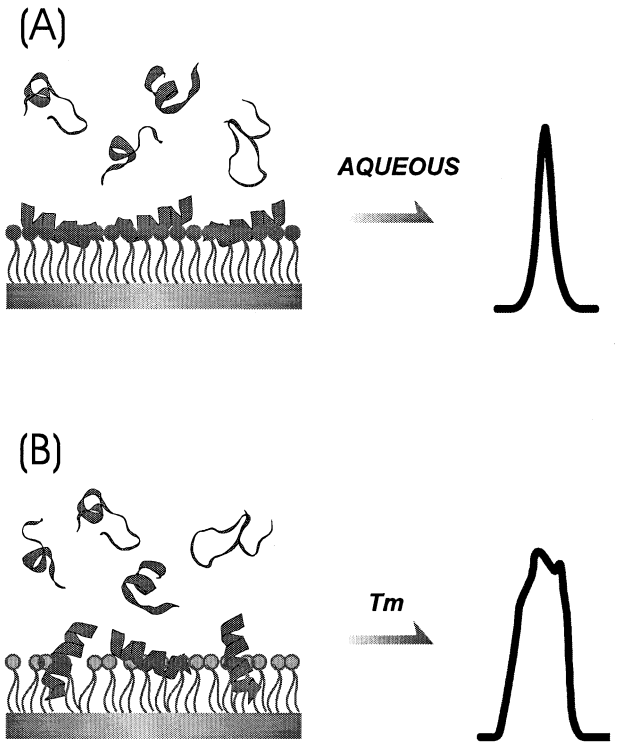

Fig. 5. Schematic illustration of the interaction of melittin with an immobilized phosphatidylcholine monolayer in aqueous conditions at temperatures where (A) the lipid and/or melittin do not undergo conformational interconversions, and (B) where interconversions are present. The corresponding elution profiles observed for melittin are also shown.

in order to capitalize on the potential of RP-HPLC techniques and incorporate the binding properties of phospholipids, a model membrane which consists of ω-amino-phosphatidylcholine derivatives covalently immobilized onto the activated surface of porous silica-based particles has been synthesized and characterized [159–161]. The interactive behavior of the cytolytic peptide melittin with this model membrane surface was examined as a function of methanol concentration and temperature using dynamic elution analysis, analogous to procedures used in RP-HPLC [154]. Melittin was found to exhibit complex binding behavior upon adsorption to and elution from the immobilized monolayer. In particular, a transition was observed in the log k' values at 20% methanol, while broad asymmetric and split peaks were observed between 0 and 20% methanol. The binding behavior observed for melittin with the monolayer compared to the linear retention plots

and Gaussian peak shapes observed for the control molecules suggests that melittin undergoes conformational changes upon binding to the immobilized PC ligands as illustrated schematically in Fig. 5. This methodology provides a novel approach to study the different conformational and orientational effects which peptides undergo while interacting with membranes surfaces and therefore offers great potential in the investigation of conformational transitions of membrane-associating peptides. As outlined earlier, the interaction of peptides with membrane surfaces involves a number of steps which include initial binding to the phospholipids through a mixture of hydrophobic and electrostatic interactions, induction of secondary structure upon binding, reorientation and insertion of the peptide into the lipid membrane, and further partitioning of the peptide in the membrane [162]. The design and preparation of more complex systems, which allow deeper penetration of the peptide into the lipid layer, will expand the range of biophysical tools available to investigate the molecular basis of peptide–lipid interactions.

3.5. Surface plasmon resonance

SPR is an expanding technique based on biosensor technology which allows the characterization of biospecific interactions of label-free compounds [163]. One of the strengths of SPR is that it allows the real time observation of the binding of ligands to a target immobilized on the sensor surface [163]. Most reports using SPR include the studies of protein–protein, protein–peptide, DNA–protein, DNA–DNA, antibody–antigen, receptor–ligand, and lipid–protein interactions [164–167]. Recently, lipid monolayers were formed on an alkane-thiol self-assembled monolayer mounted on a gold surface for SPR measurements of the binding affinity of amphipathic molecules for PC lipid monolayers [168,169]. The spontaneous fusion of the vesicles results in the formation of a hybrid bilayer that resembles a membrane surface as shown in Fig. 6. The rate of the formation of these bilayers was found to be dependent on the lipid concentration as well as on the diffusion constant of the lipid vesicles [168]. Thus, SUVs containing analogs of bacterial mucopeptides were adsorbed onto the surface of the hydrophobic self-assembled monolayer to mimic the surface of a bacterial membrane

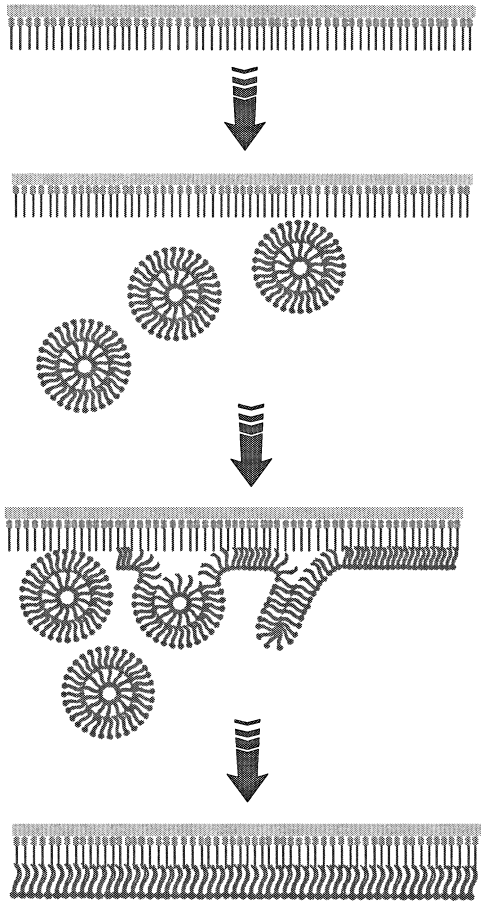

Fig. 6. Schematic illustration of the preparation of the hybrid bilayer system for SPR studies. The surface is composed of self-assembled octadecyl alkyl chains covalently attached to a gold surface. Upon application to the surface, SUVs absorb spontaneously to form lipid multilayers which are then treated to create a monolayer, thereby forming a hybrid bilayer.

[169]. A good correlation was then found between the binding affinity of different glycopeptide antibiotics to the inserted mucopeptide analogs and the in vitro antimicrobial activity of these glycopeptides. In contrast, no such correlation was obtained when measuring the binding affinities of the glycopeptides to mucopeptide analogs free in solution. Using a similar technique, the direct binding of peptides to this hybrid bilayer surface can also be studied. The resulting data then allow the thermodynamic analysis of peptide–lipid interactions in terms of relative rate constants and binding constants. Comparison of the binding of melittin to a DMPC and a DMPG bilayer revealed a much higher affinity for DMPG (Fig. 7). These results are in agreement with previous studies

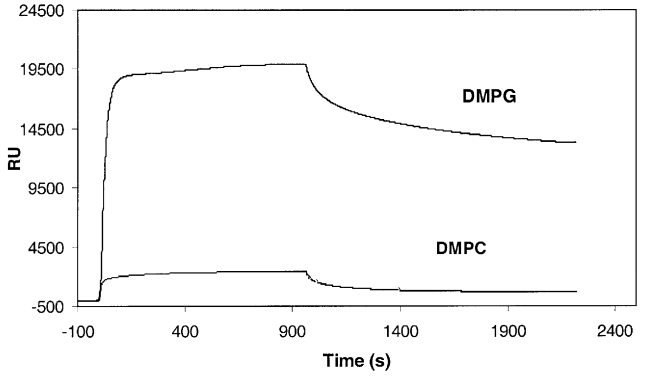

Fig. 7. Sensorgrams obtained using SPR detection of the interaction of melittin (at 90 μM) with a hybrid bilayer (illustrated in Fig. 6) composed of DMPC or DMPG. The response is proportional to the amount of peptide absorbed to the hybrid bilayer and hence to the affinity of melittin for the lipid surface.

[21] that have shown that melittin has a higher affinity for negatively charged phospholipids than for zwitterionic lipids. Similarly, it was shown that the binding capacity of the cationic antimicrobial peptides cecropin B and its analog B1 increased as the content of PA in the PC monolayer increased, but no binding was found for the hydrophobic analog B3 [87]. Although cecropin B and B1 with a higher positive net charge bound more effectively to liposomes with an increasing content of negatively charged lipids, their efficiency in lysing these liposomes decreased while B3 became more potent in lysing such liposomes. These results demonstrate that a high binding affinity of peptides to lipids does not necessarily imply high ability to lyse membranes.

Using SPR, kinetic analyses of the interaction of lysenin, a hemolytic protein isolated from the earthworm *Eisenia foetida* [170], with membrane surfaces composed of SM were also recently reported [171]. These studies showed a high association of lysenin with SM-containing membranes with a low dissociation constant. Furthermore, higher binding with no change in the kinetic parameters of lysenin–membrane interaction was obtained with membranes containing both SM and cholesterol, indicating a high specificity to SM relative to cholesterol. This is due to changes in the distribution of SM in the membranes caused by the insertion of cholesterol, which increases the accessibility of SM to lysenin. Similarly, the kinetics of the interaction of the peptide antibiotic polymyxin B and its analogs with lipopolysaccharide (LPS) were determined by means of SPR [172]. These studies showed that polymyxin B and its analogs interact with LPS in a monoexponential manner, and that the binding is dominated by hydrophobic and van der Waals forces. These results are in agreement with earlier studies using isothermal titration calorimetry [173] and stop-flow fluorescence techniques [174]. Furthermore, the poor binding affinity to LPS observed for analogs lacking amphipathic properties agreed with the premise that amphipathicity is necessary and sufficient for peptides to bind to LPS [173,174]. Kinetics of the interaction between LPS and lactoferrin-related antimicrobial peptides were similarly carried out [175]. Thus, an amphipathic α-helical region exposed on the outer surface of lactoferrin was found by SPR to bind specifically to LPS of *E. coli* O111. These results showed the importance of this region to the binding and antimicrobial pathogenesis of lactoferrin.

Overall, SPR is a more rapid and convenient technique than the existing separation-based and fluorescence-based techniques and has the potential to provide a new complementary experimental approach to the study of the action of antimicrobial peptides.

4. Correlation between lipid-induced conformation and/or binding affinity and lytic activity of peptides

Attempts to correlate the antimicrobial, antifungal, and/or hemolytic activity of peptides with their induced conformation in various lipid environments are part of most of the studies directed toward understanding the mechanism of action of these peptides, as well as toward the design of novel therapeutically useful peptides. As anticipated, great variations in activity are often obtained when comparing antimicrobial or antifungal activities to hemolytic activities, and a number of naturally occurring and engineered peptides exhibit significantly greater antimicrobial activities than hemolytic activities. In contrast, subtle variations in activities often exist when comparing activity against different bacterial and/or fungal species. Due to the difficulties in completely mimicking the changes in membrane compositions between microorganisms and/or bacterial strains, the relationship between the peptide antimicrobial and antifungal specificities and the pep-

tide–lipid binding preferences is not totally understood.

As shown for several antimicrobial peptides, binding preferences to certain lipid types can be somehow related to the peptide specificity toward given microorganisms. In particular, cationic peptides disrupt membranes composed of acidic phospholipids better than those composed of zwitterionic phospholipids. For example, magainin was found to effectively permeabilize PG-rich membranes and to more effectively kill bacteria whose inner membranes contain higher amounts of PG [6]. Similarly, the antimicrobial peptide semiplasmin exhibits higher affinity to negatively charged dioleoylphosphatidylglycerol vesicles than to zwitterionic lipids (the extracytosplasmic region of erythrocyte cells is composed of zwitterionic lipids), which could reflect the lack of hemolytic activity at the concentration where it exerts its antimicrobial activity [7]. Sapecin also exhibits a specific affinity for diphosphatidylglycerol [8]. On the other hand, the hemolytic peptide cinnamycin appears to have specificity for the zwitterionic lipid PE [9]. Preincubation of cinnamycin with PE significantly reduces the extent of hemolysis induced by this peptide. In addition, the permeability of PE-containing liposomes is increased by cinnamycin, but this does not occur with liposomes composed of other phospholipids.

Peptide–C_{18} interactions were also found to provide insight into biomolecular recognition events at aqueous–phospholipid membrane interfaces. Using different series of sequence-related amphipathic α-helical peptides, excellent correlations were observed between the antimicrobial activities and the RTs during RP-HPLC [11,147]. Excellent correlations were also reported between the RP-HPLC behavior of the β-sheet peptide analogs of cyclic gramicidin S and their binding affinity to bacterial LPS, as well as between the RP-HPLC retention times and hemolytic activities of these peptides [54].

5. Conclusions

The interaction between cytolytic peptides and lipid bilayers plays a crucial role in their membrane perturbation activity and/or disruption of cellular function, and subsequent cell death. During these interactions, peptides generally exhibit a conformational transition from extended random coil in aqueous environment to a stabilized unique secondary structure upon interaction with the membrane, which is either preceded by or followed by enhanced binding through electrostatic interactions. The dynamic interactions between the peptides and membrane lipids are therefore key elements in the biological activities of the peptides as well as in the specificity of their activity. Furthermore, the determination of the relative contribution of each of these interactions to the overall binding and function clearly depend on the specific sequence and membrane system used. Studies using the techniques described in this review have clearly demonstrated the complexity of peptide–membrane interactions in terms of the mutual changes in peptide binding, conformation, orientation, and lipid organization, and have to a certain extent allowed correlations to be drawn between peptide conformation properties and lytic activity. While great progress has been made in the last decade thanks to such techniques, development of stable membrane systems, that incorporate all of the elements making up the complexity of natural membranes and of the dynamic of peptide–membrane interactions, combined with sensitive analytical and spectroscopic techniques, will provide greater insight into the molecular basis of antimicrobial peptide action.

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