Peptide-Lipid Interactions: Insights and Perspectives

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As the number of membrane proteins in the Protein Data Bank increases, efforts to understand how they interact with their natural environment are increasing in importance. A number of membrane proteins crystallise with lipid molecules implicitly bound at discrete locations that are consistent with the transmembrane regions of the protein. Bioinformatics studies also point to the specific interactions of some amino acids with membrane lipids. The results of experiments using model systems are revealing how these interactions contribute to the stability of both the protein and the membrane in which it is embedded. From a different perspective, the processes involved in the binding of peptides to membrane surfaces to produce a variety of effects are being understood in ever-increasing detail. This review describes current research efforts and thinking in this area.

Introduction

It is now some 42 years since the first structure was proposed for the biological membrane by Singer and Nicolson[1] (Figure 1).

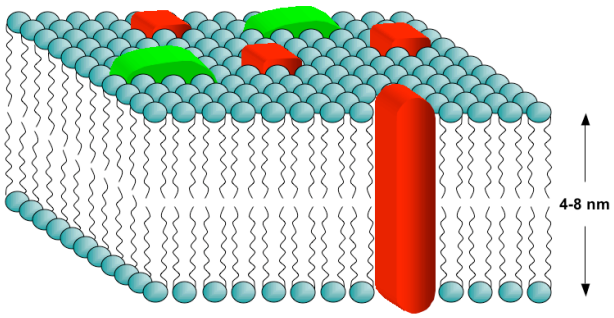


Fig. 1 The Singer/Nicolson fluid mosaic model. Peripheral proteins are indicated in green and integral proteins in red.

Their fluid-mosaic model comprised a tail-to-tail arrangment of amphipathic lipid molecules, forming a lipid bilayer. The 'tails' of the lipid molecules, the hydrophobic fatty acyl chains, associated together in the interior of the bilayer in a favourable like-with-like arrangement that kept them segregated from the hydrophilic aqueous medium on either side of the membrane. The 'heads' of the lipid molecules, hydrophilic groups such as choline, were presented on the outside of the membrane in favourable disposition for solvation by water. Proteins were present in this model as integral (membrane-spanning) structures floating in a sea of lipid, or peripheral (extrinsic), bound to the membrane surface. The model also contained proteins that traversed one of the membrane lamellae, *ie* half of the diameter of the membrane, although proteins of this type may be relatively rare, as it inevitably implies that hydrophilic groups, most notably amide bonds in the region of β-turns and random coil motifs, will be exposed in the region of the hydrophobic interior of the membrane and therefore poorly solvated. Work in recent years has demonstrated that natural membranes are much more complex than this simple model, both in terms of the lipid distributions found, and the interactions of lipids with other molecules such as proteins. This review is concerned with the chemistry of protein-lipid interactions; in particular, the factors that contribute to the stability of the membrane and the activity of those peptides and proteins that function in the membrane environment.

Membrane proteins

When Singer and Nicolson first proposed their model, little was known about the precise structures of membrane proteins beyond the fact that many were helical. It was not until Henderson and Unwin's model of bacteriophodopsin was published in 1975,[5]

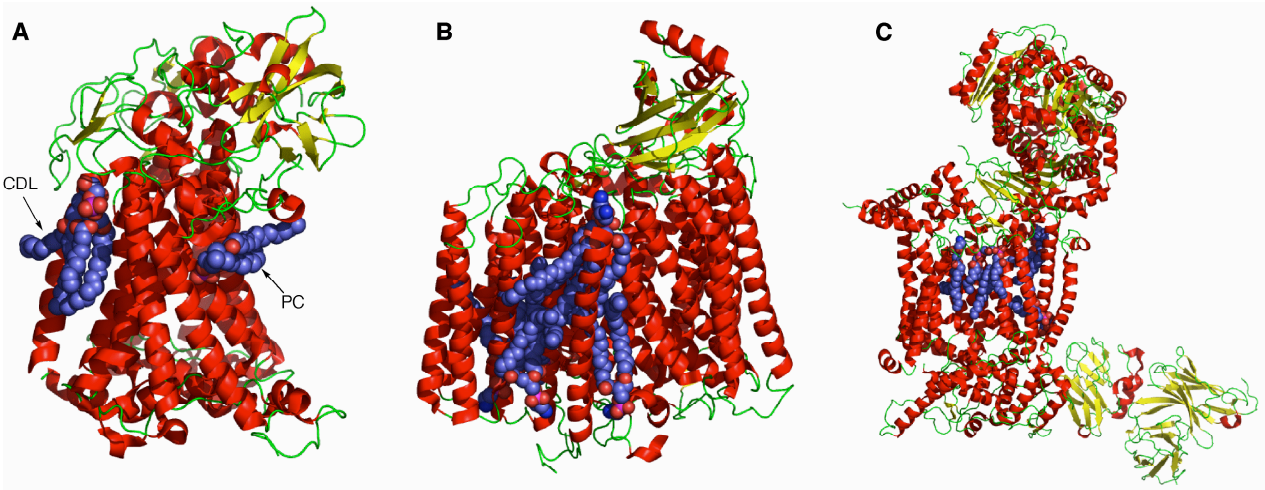


Fig. 2 Examples of membrane protein structures in the PDB. Key: α-helices are shown in red, β-sheets in yellow; CDL, cardiolipin; PC, phosphocholine. A, Photosynthetic reaction centre from *R. sphaeroides* (1m3x);[2] B, Cytochrome C oxidase from *R. sphaeroides* (1m56).[3] All lipids in this model are PE; C, Yeast cytochrome bc_1 complex (1kb9).[4] For more details on the lipids bound to this protein, see Figure 3.§

following years of analytical work by protein chemistry, that the transmembrane α-helix was described. It is still the case that membrane proteins are massively under-represented in the protein databank (PDB),[6] but nevertheless the number for which atomic coordinates are available is increasing steadily. Two fundamental types of membrane protein may be described: peripheral and integral.

Peripheral membrane proteins will frequently crystallise in the absence of lipids, but usually reveal little about the nature of their contacts with the membrane surface, which may involve direct contact with the lipids themselves, or with other cell surface-bound groups such as carbohydrates or integral membrane proteins. The most well studied surface binding proteins are phospholipases, including phospholipase A_2 C2 domain, which binds to zwitterionic lipids,[7] and phospholipase C, which binds specifically to membrane surfaces that contain phosphatidylinositol-4,5-bisphosphate through an N-terminal pleckstrin homology domain.[8] A number of proteins involved in the formation and recycling of vesicular structures, such as those involved in neurotransmitter signalling, have been recognised and display interesting activities that are dependent on membrane curvature (see below). G proteins have also received much attention as examples of proteins that bind transiently to membrane surfaces in order to reach their active sites on G protein receptors.[9]

A further type of peripheral protein needs to be recognised. A large number of antimicrobial peptides from a range of prokaryotic and eukaryotic sources have been isolated, and a significant number of these exert their activity through modification of membrane properties. The mechanisms by which these peptides bind to the membrane surface and subsequently form membrane lytic structures have been the subject of intense scrutiny, and will be discussed later in this review.

Integral membrane proteins currently number about 150 in the PDB. As methods for the preparation of crystals of these proteins improve, the number is likely to increase more rapidly.[11] Of these, a large number crystallise associated with lipid molecules (Figure 2). There are currently around 70 entries in the PDB that contain one or more bound lipids, including examples of, in decreasing order of occurrence, cardiolipin (CDL), phosphatidylethanolamine (PE), sphingosine, phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylserine (PS). This not only reflects improved methods for their crystallisation using detergent depletion methods, which are sufficiently mild to allow lipid molecules to remain bound to the protein, but also the fact that membrane proteins do have specific interactions with lipids in the membrane around them.[2,4] There are some striking examples in the PDB of multiple entries for a single protein that has been crystallised in different laboratories. A cursory glance at these reveals that some lipids are invariably bound at the same site on the protein, regardless of its origin, whereas other lipids are more variant (Figure 3). Bioinformatics approaches take the evidence for specific sites of interaction between membrane proteins and lipids a step further. By examining the distributions of amino acids across membrane-spanning segments of proteins, it has been noted that some display a non-random distribution, with a significant tendency to occur in regions of the protein that correspond to specific locations with respect to the bilayer. In one study,[12] it was observed that in transmembrane α-helices, isoleucine was located preferentially in the hydrophobic region of the extracellular lamella, phenylalanine and tyrosine were located preferentially in the region of the lipid headgroups of the intracellular lamella, tryptophan was located preferentially in the region of the headgroups of both lamellae, and arginine and lysine were located preferentially in the cytoplasmic residues flanking the intracellular end of the helix. In a more extensive study, significant differences were observed between the distributions of amino acids in α-helical and β-barrel membrane proteins, although a tendency for aromatic amino acids to occur in the region of the membrane interface was noted.[13]

These bioinformatics approaches highlight one of the essential problems with the study of peptide-lipid interactions: systematic structural biology approaches are rendered difficult by the chemical nature of lipids, and the fluid nature of the membrane environment. Protein-lipid interactions *in vivo* are intrinsically dynamic, as lipid molecules are free to move and redistribute within the bilayer in response to changes in its chemical composition. For this reason, combined studies using model lipid systems and molecular modelling approaches provide the best methods for understanding the fundamental properties of these interactions, as well as determining their biological significance.

From the above discussion, it will be apparent that that there are two types of interaction of proteins with membranes that need to be considered: binding to the membrane surface, involving a large area of contact between the two, and the interactions of integral proteins with the lipids that surround them. Both of these require a firm understanding of the chemical nature of the lipid bilayer.

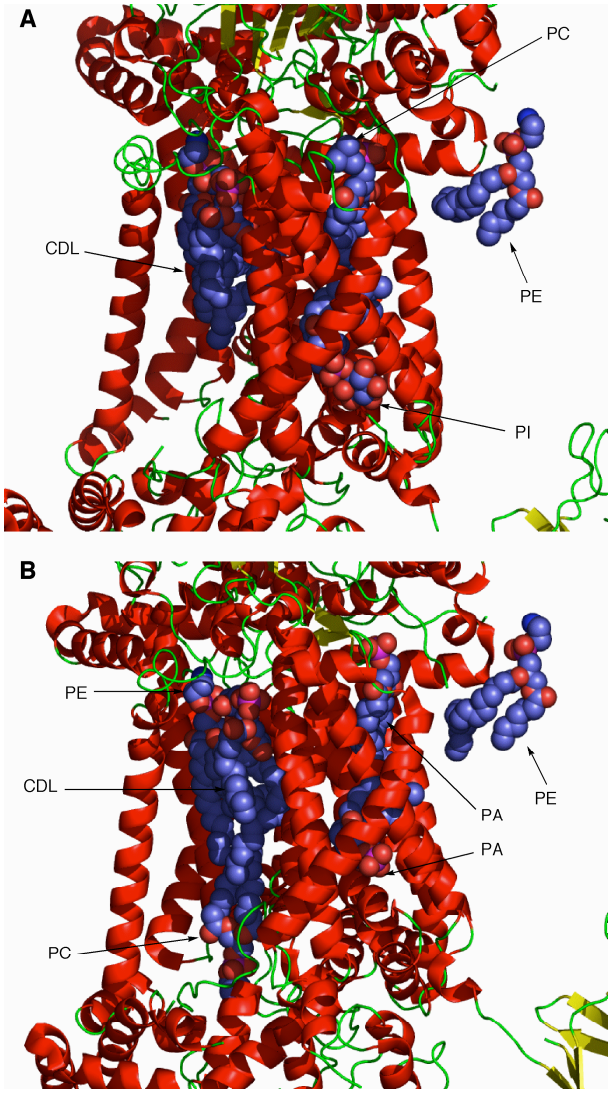


Fig. 3 A more detailed examination of the lipids bound to yeast cytochrome bc_1 complex (Figure 1C, rotated by 90 °) from alternative structures in the PDB: A, PDB entry 1kb9;[4] B, PDB entry 1p84.[10] Key: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; CDL, cardiolipin; PA, phosphatidic acid

Membrane Properties

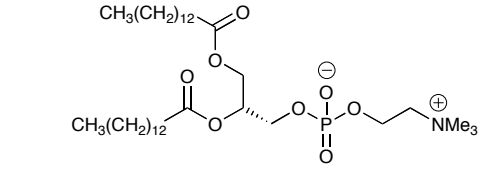
The Singer model, although simple, is still a useful starting point for understanding the structure of membranes. It is beyond the scope of this review to cover extensively the chemical nature of lipids, as this is described in many textbooks, however, it will be necessary to describe the nature of the bilayer in more detail.‡

The liquid crystalline properties of membranes

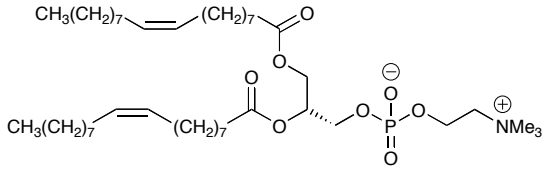
The lipid bilayer exhibits lyotropic liquid crystalline properties.[14] Simple lipids with saturated acyl chains, such as 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC, Figure 4), exhibit clear transitions between gel ($L_{\beta'}$), rippled gel ($P_{\beta'}$) and liquid crystalline (L_α) phases in water when the lipid sample is pure. These transitions are most easily revealed by calorimetry; the $L_{\beta'}$-$P_{\beta'}$ change is generally termed the pre-transition (*Tp*), and the $P_{\beta'}$-L_α change the main transition (*Tm*). Both of these phase changes are reversible and occur at characteristic temperatures, with enthalpies of the order of 3 kJ mol^{-1} for the pre-transition, and 25 kJ mol^{-1} for the main transition in DMPC (Figure 5). Langmuir-Blodgett studies reveal that lipid-lipid interactions in these membranes are dominated in the $L_{\beta'}$ phase by close contact of lipid headgroups, with the acyl chains tilting to maximise Van der Waals contact.

For unsaturated lipids, with one or more double bonds in one or both of the acyl chains, such as 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, Figure 4), phase transition temperatures are generally much lower in enthalpy, and frequently occur below 0 °C.[14] Membranes composed of these lipids tend to be much more fluid and expanded than their saturated counterparts, with lipid-lipid interactions in the L_α phase dominated by close contact of lipid acyl chains. The effects of other non-lipid constituents, such as cholesterol in eukaryotic membranes for example, are described later.

The thermal properties of phase transitions within membranes



1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)



1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)

Fig. 4 Examples of phosphatidylcholines.

are extremely useful indicators of membrane structure, as they are sensitive both to binding of solutes to the lipid headgroups, and to partitioning of solutes further into the membrane interior. The disruptions of lipid packing promoted by peptides and proteins typically produce changes in *Tm* of ~3 °C for peptide:lipid ratios of 1:10, with a concurrent decrease in the enthalpy of the transition and loss of the pre-transition.[15] Additional transitions may also be observed in these cases, which may reflect phase separation effects. Transition temperatures are also useful indicators of the degree of mixing of mixtures of lipids. Lipids that mix ideally will have a main transition temperature that is a statistical average of the values for the pure lipids, whereas those that do not mix will show independent transitions for the lipids. This is becoming an increasingly important issue, as the cases described above for DMPC and DOPC are rather simple when compared with biological membranes *in vivo*. Biological membranes are complex mixtures of lipids, proteins and carbohydrates, and it is becoming increasingly clear that they are far from ideal fluid structures.

The nature of the membrane interface. The surface of the membrane is a complex environment of intermediate polarity. Ordered (bound) water in the headgroup region influences a number of bilayer properties, including diameter, fluidity and *Tm* temperature. Extensive studies have been performed with lipids, covering a large range of hydration states, in order to determine the number of water molecules that are closely associated with them. FTIR and dipole potential measurements made on DMPC/water and DMPC/water/carbohydrate mixtures in

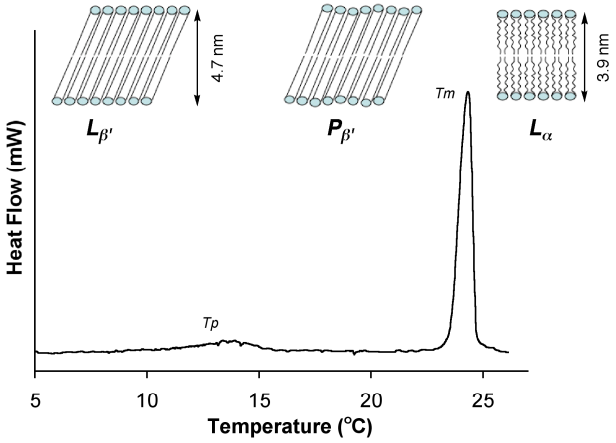


Fig. 5 DSC enthalpogram for DMPC showing phase changes between the gel ($L_{\beta'}$), rippled-gel ($P_{\beta'}$) and liquid crystalline (L_α) phases. The Temperatures of the pre- and main transitions are labelled *Tp* and *Tm* respectively.

chloroform indicate that of a total of 18 water molecules in the coordination sphere, 7 are tightly bound, 4 are loosely bound, with the remaining 7 of intermediate binding.[16] Other studies have found that the free energy of binding water molecules is zero above molar water:lipid ratios of about 5.[17] The inner sphere waters are likely to be tightly bound to the headgroup and the carbonyl oxygens and therefore the most difficult to displace. Nevertheless, bound water can be displaced by solutes, most strikingly demonstrated by the use of polyhydric alcohols and carbohydrates as cryoprotectants in liposome preparations.[18] In addition to interfacial water, some experiments provide evidence that a small amount of water localises in the hydrocarbon region of the membrane in the L_α state.[19]

Metal ions are associated with the membrane surface; divalent cations have been known to bind especially favourably to membranes for some time,[20] but recent studies suggest that monovalent cations also associate with membranes.[21] These ions may be modelled as an electrical double layer (Helmholtz model) and a diffuse layer (Gouy-Chapman model), although penetration of ions into the hydrocarbon interior of the membrane under the influence of an electrochemical potential gradient violates the latter model.[22] This does however serve to remind us of an often neglected aspect of biological membranes – the membrane potential. Most eukaryotic and many prokaryotic cells have an electrical potential gradient across the cell membrane, typically of the order of –90 to -110 mV in the case of eukaryotes, giving rise to a substantial electric field (~10^7 V m^{-1}) that has been implicated in the functioning of some proteins. For example, rotation of a transmembrane helix has been suggested as a mechanism for the gating of voltage-sensitive channels.[23] However, given that the α-helix has a modestly strong dipole moment resulting from the alignment of backbone amides, the role of membrane potential in influencing the structure and activity of membrane proteins may be worth more rigorous examination.

Lipid rafts

Most natural membranes are rather complex mixtures of lipids that display a level of microinhomogeneity when examined by microscopic, calorimetric and spectroscopic methods. This may be attributed to the formation of phase-separated domains, with condensed phase lipids termed 'rafts' coexisting with lipids in more fluid phases.[24] Rafts may be isolated as a result of their insolubility in non-ionic detergents, and as a result their molecular content has been the subject of intense scrutiny and debate.[25] Certain lipids, most notably those with high *Tm* temperatures such as glycosphingolipids (*Tm* > 40 °C), are in the gel phase in physiological conditions, and are consequently major constituents of rafts. Cholesterol also contributes to raft formation as a result of its favourable interactions with glycosphingolipids. When added to pure DMPC bilayers, cholesterol intercalates between the lipid molecules to form a mixed phase that is intermediate in nature between the gel and fluid liquid crystalline phases – a so-called liquid-ordered state.[26] In natural membranes, cholesterol has a high affinity for gel-phase glycosphingolipids, leading to the formation of rafts with a liquid-ordered state. The likely physiological consequence of cholesterol incorporation is a reduction in bilayer defects in the interface between the rafts and liquid crystalline phase lipids, thus increasing membrane stability.

Proteins are the other major constituent of a subset of rafts, termed caveolae. These rafts have a distinctive invaginated morphology that is produced by the binding of the protein caveolin-1 to cholesterol on the cytoplasmic surface of the membrane.[24,27] Interestingly, a number of cell signalling receptors are found to be localised in caveolae, including receptor tyrosine kinases, G protein receptors and proteins with glycosylphosphatidylinositol anchors,[24] fundamentally challenging the notion that protein receptors are distributed evenly over the membrane surface. Recent studies have also shown that the specific activity of phosphatidylinositol transfer proteins increases in the presence of cholesterol-rich liquid-ordered domains,[28] and the pore-forming peptide equinatoxin II interacts preferentially with membranes containing sphingomyelin.[29] Caveolae have also been implicated as the point of entry of pathogenic bacteria such as *Chlamydia* into cells.[30] As the significance of lipid rafts increases, it should be anticipated that the reasons why certain proteins are able to interact selectively with them will be examined in greater detail.

The lamellar distribution of lipids

In addition to the lateral inhomogeneities described above, most natural membranes are asymmetric from the point of view of the lipid composition of the lamellae. For example, in the plasma membrane of eukaryotes, PI, PE and PS have a higher prevalence in the inner (cytoplasmic) leaflet, whereas PC and sphingomyelin have a higher prevalence in the outer leaflet.[31] Some of these distributions may have clinical significance. For example, the early stages of apoptosis are marked by the presence of PS on the external surface of the cell, which renders the cell more amenable to a number of physiological events, such as coagulation and recognition by phagocytic cells.[31]

A number of proteins are involved in the transport of lipids across the cell membrane,[32] including some that are active (flippases, floppases and aminophospholipid translocase) and some that are passive (scramblases). Scramblases are non-specific, and act to equilibrate lipid distribution across a bilayer. Flippases and floppases are non-specific and require ATP for their activity, transporting lipids in an inward and outward direction respectively. Aminophospholipid translocases require ATP for the transfer of PS and PE molecules in an inward direction.

There is a great deal of scope for research concerning the mechanisms by which these proteins function, particularly as their activity may be of clinical significance. Furthermore, it should be apparent that synthetic molecules designed to bind specifically to membrane-bound lipids should be able to dramatically influence the rate of lipid transfer across the membrane.

Membrane curvature

Molecular volume and shape are crucial factors in determining the properties of amphipathic molecules.[33] The PCs described above (DMPC and DOPC) are common constituents of eukaryotic membranes and may be thought of as having a rectangular cross-section. When molecules of this shape are packed into a mesophase, they prefer to form structures of low intrinsic curvature, *ie* closed spherical structures of large radius. Molecules with large polar headgroups preferentially form structures with high positive intrinsic curvature, *ie* micelles, and those with small polar headgroups will form reverse micelles of negative intrinsic curvature (Figure 6). In fact, most molecules with lipidic properties will form lyotropic phases other than the L_α phase described above (such as micellar, inverse-micellar, hexagonal or cubic) under exceptional conditions. Lipids such as PE however, form non-bilayer hexagonal (H_{II}) and cubic (Q_{II}) phases much more readily, due to an inherent preference for the formation of structures of negative curvature.[34] This has important implications for a number of membrane processes *in vivo* that require localised areas of negative membrane curvature, such as membrane fusion (*eg* during exocytosis) and budding (*eg* during endocytosis).[35] A number of proteins have been characterised that are involved in membrane fusion processes, including viral fusion proteins such as influenza haemagglutinin,[36] and complex protein assemblies such as those formed by soluble NSF attachment protein receptors (SNARE) proteins,[37] which are involved in trafficking of synaptic vesicles. Mutagenesis studies on the above proteins have allowed intermediates in the fusion process to be isolated, and these hemifusion products confirm their role in the fusion process. Perhaps the best example of the effects of membrane curvature on protein activity is provided by the amphiphysins. These proteins are involved in the recycling of neurotransmitter vesicles, and feature a conserved domain, termed the BAR domain, which is found in a number of similar proteins of eukaryotic origin. These include Arf-GTP, which is involved in the formation of clathrin-coated vesicles,[38] and centaurins, which

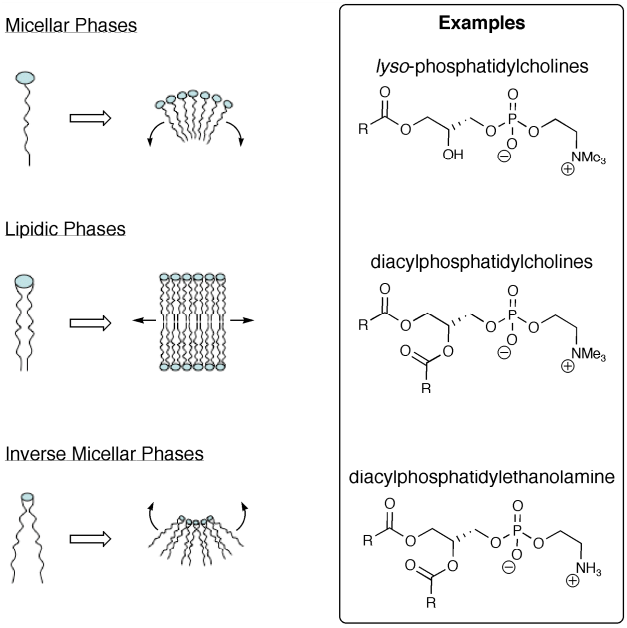


Fig. 6 The formation of aggregates of different specific curvature according to amphiphile shape.

are involved in vesicular trafficking. The BAR domain is a homodimer, produced by a head-to-head arrangement of two kinked coiled-coil motifs (Figure 7), and binds to membranes in

a fashion that is remarkably sensitive to membrane curvature, producing effects that are visible both in cell preparations and synthetic liposomes.[39]

Highly curved membrane structures are also implicated in a number of the pore formation mechanisms of antimicrobial peptides that are discussed later. In all of these cases, protein-membrane binding promotes the formation of energetically unfavourable structures characterised by a high degree of curvature strain. The factors that produce tight protein-lipid binding, and the design of new molecules to alter membrane curvature in a controllable manner are likely to be highly active research areas in the future.

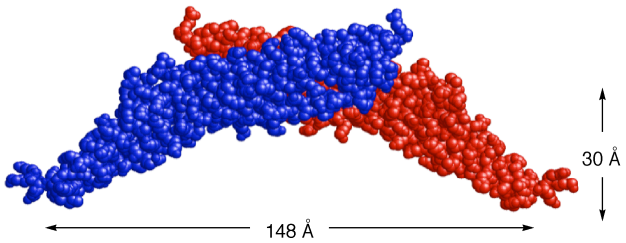


Fig. 7 Spacefilled representation of he assumed biological molecule of the BAR domain from amphiphysin (PDB entry 1uru).[39]

Peptide-Lipid Interactions

Methods for studying peptide-lipid interactions

In recent years, there have been some tremendous improvements in the methods available for studying the interactions of peptides with membranes. It is beyond the scope of this review to cover these in detail, but an awareness of the nature and potential benefits of these methods should facilitate the design of new experiments.

Calorimetry has been used for some time for the analysis of lipid phase changes. Recently however, significant improvements in the instrumentation available have enabled far more sensitive measurements to be made, giving access to the full thermodynamic parameters (ΔG, ΔH and ΔS) for a given system.[40] Isothermal titration calorimetry (ITC) allows binding isotherms to be measured, which should enable more difficult problems of relevance to peptide-lipid interactions to be addressed, such as the role of interfacial water in binding. Water sorption calorimetry has proved useful in measuring the energy associated with water binding to membranes, and should also prove useful for determining the fate of water during peptide binding.

Scanning probe microscopies have matured considerably and offer an excellent method for visualising the structures that are formed by peptides and proteins in membranes.[41] A particular advantage with these approaches is the ability to perform the experiments directly on aqueous membrane preparations. This enables the visualisation of 2D arrays of membrane proteins, (which are easier to form than 3D crystals), and the types of lipid phases that are formed by peptide-lipid mixtures. Atomic force microscopy (AFM) can provide resolutions as high as 5 Å, and has allowed the direct visualisation of the pores formed by a number of channel proteins such as OmpF[42] and α-Hemolysin in membranes. In the latter case, the 2D crystals formed with the protein assembled as a homohexamer,[43] contrasting with the structure obtained by conventional crystallographic methods, in which the protein assembled as a homoheptamer.[44] This demonstrates one advantage of studying membrane proteins in their natural environment. AFM also allows the measurement of forces between single biomolecules, potentially allowing a direct measurement of peptide-lipid interactions.[45] This may provide useful information on the various interactions that drive stable protein assembly in the membrane.

Methods for quantifying peptide-lipid binding, including surface plasmon resonance[46] and the quartz crystal microbalance,[47] now enable the association constants of peptides with supported membranes to be measured extremely accurately. Together with single molecule force measurements by AFM, it should be possible to use these methodologies to systematically probe the energetic characteristics of peptide-lipid interactions as a function of the chemical structure of the lipid and the peptide, in order to understand the relative importance of the non-covalent interactions that are involved.

In order to probe the structure of peptide-lipid adducts, a number of spectroscopic techniques have found application, including NMR,[48] linear and circular dichroism,[49] and attenuated total reflection FTIR.[50] All of these methods may be performed on oriented bilayers, providing information on the orientation of peptides with respect to the bilayer normal,[51] as well as information concerning the 2° structure of the peptide. NMR spectroscopy has proved particularly useful where information is already available for the structure of a peptide in the absence of membranes, as coupling constants and chemical shift anisotropies may be monitored to assess changes in conformation following binding.[52] Raman spectroscopy has also been applied to liposomes,[53] and should prove useful for the study of peptide-lipid interactions in the future.

Helical peptide models for integral membrane proteins

Properties such as bilayer thickness are able to influence the activity of proteins. For example, when reconstituted into synthetic membranes of differing thickness, Ca^{2+}-ATPase and (Na^+,K^+)-ATPase display maximum activity when the lipid acyl chains are 18C in length.[54] The effects of peptide-lipid interactions, both on the properties of bilayers and the activity of membrane proteins, have therefore received much scrutiny.

Hydrophobic mismatch,[55] in which the length of the hydrophobic region of the peptide is different to the diameter of the hydrophobic interior of the membrane, has been examined intensively using helical peptides.[56] These studies address some of the fundamental issues around the effects of membrane lipids on protein structure and *vice versa*. In response to hydrophobic mismatch, a number of effects are imaginable, which may be broadly considered as changes in the structure of the peptide or modification of bilayer properties (Figure 8). These effects are dependent on the nature of the mismatch, *ie* whether the hydrophobic part of the peptide is shorter or longer than that of the bilayer, and the composition of the membrane. Regardless of the changes that occur, the net effect is to reduce the extent of exposed hydrophobic residues.

Aggregation of peptides in response to changes in lipid diameter has been demonstrated for model transmembrane helical peptides, of sequence Ac-KKGL_mXL_nKKA-NH_2, where X is 3,5-dibromotyrosine or tryptophan, and 12 and 10 are typical values for m and n respectively.[57] Following incorporation of mixtures of these peptides into synthetic membranes, quenching of tryptophan fluorescence by 3,5-dibromotyrosine was used to quantify their dimerisation. Association constants for dimerisation increased markedly as the length of the lipid chains was increased, with the free energy of dimerisation increasing by 0.5 kJ mol^{-1} per acyl chain carbon. Furthermore, association constants were phase-dependent, being much higher in the liquid-ordered phase of a cholesterol:DOPC mixture than the L_α phase of pure DOPC. However, this increase in binding was much more than would be expected as a result of the change in bilayer diameter produced by the inclusion of cholesterol alone, and may therefore reflect the higher packing density in liquid-ordered phases. A change in helix tilt with respect to the membrane was a further consideration in these experiments, as a packing angle of 20° between helices is optimal for inter-helix interactions.[58] In this regard, studies on model peptides of sequence KK(A)$_m$(LA)$_n$KK, in which m = 0 or 1 and n = 5-15, demonstrated that at low peptide:lipid ratios, hydrophobic

mismatches in which the peptide was 3 Å too short or 14 Å too long did not alter the peptide orientation with respect to the membrane,[59] similar to other results using lysine as membrane-anchoring residues.[60]

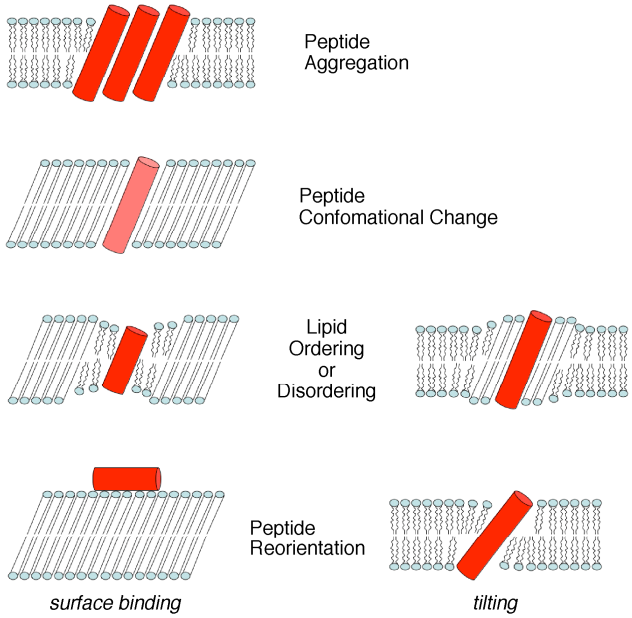


Fig. 8 Mechanisms by which peptide-lipid systems may adapt to hydrophobic mismatch. Adapted from reference 55.

Although some studies have been able to detect evidence for peptide conformational changes in response to lipid phase changes,[61] these effects have generally been very small, and it seems that most peptides adopt the same conformation regardless of lipid phase.

Lipid disordering has been demonstrated using a number of natural and designed peptides. The most striking are the so-called WALP peptides studied by Killian and co-workers.[62] These peptides were designed to incorporate differing lengths of hydrophobic stretch between anchoring tryptophan or lysine residues (Table 1).

Interestingly, the shorter peptides such as WALP16 were able to induce the formation of non-lamellar H_{II} phases, and it was noted that the inter-tryptophan distance was the crucial factor for determining the activity of W_2^{in}ALP16 and W_2^{out}ALP16, rather

Table 1 WALP and KALP peptides studied by Killian and co-workers[62]

peptide	sequence [a]	hydrophobic stretch (Å)
WALP16	Ac-GWW(LA)$_5$WWA-Etn	15.0
W_2^{in}ALP16	Ac-GAW(LA)$_5$WAA-Etn	15.0
W_2^{out}ALP16	Ac-GW(LA)$_5$LAWA-Etn	18.0
WALP17	Ac-GWW(LA)$_5$LWWA-Etn	16.5
WALP23	Ac-GWW(LA)$_8$LWWA-Etn	25.5
KALP23	Ac-GKK(LA)$_8$LKKA-Etn	25.5
KALP31	Ac-GKK(LA)$_{12}$LKKA-Etn	37.5

[a]Etn = ethanolamine

than the length of the hydrophobic segment. ^{2}H NMR experiments also provided evidence that WALP16 produced an increase in acyl chain order, with concomitant increase in bilayer diameter of 0.4 Å. Solid-state ^{1}H NMR analysis of WALP peptide/DMPC mixtures produced upfield chemical shift changes (relative to pure DMPC) for several of the key lipid signals, with the magnitude of the chemical shifts increasing with increasing peptide length. In particular, the changes observed for the choline group of the lipid were attributed to ring current effects of tryptophan. On the basis of this, the authors propose that the indole ring of tryptophan locates preferentially in the region of the acyl carbonyl groups, consistent with other reports.[63] Whether there are specific dipolar interactions between tryptophan residues and lipids in these helical peptides is open to question, although experiments using tryptophan analogues did not change the location of the tryptophan with respect to the lipid bilayer, suggesting that, at least in this case, dipolar interactions may be small.[63]

Parallel experiments with the equivalent peptides containing lysine in place of tryptophan produced different results. For example the peptide KALP23 produced an increase in bilayer thickness of 0.2 Å, compared with 1.0 Å observed with WALP23, and the longer peptide KALP31 only produced an increase in bilayer diameter of 0.3 Å, pointing to the importance of the membrane anchoring role played by tryptophan in the WALP peptides.

Taken together, these results are consistent with the earlier findings of bioinformatics studies that point to specific roles for aromatic amino acids, especially tryptophan, at the membrane interface in the region of the polar headgroups.[12,13]

Gramicidin A (gA) is a naturally occurring peptide antibiotic of sequence Formyl-V-G-A-D-L-A-D-V-V-D-V-W-D-L-W-D-L-W-D-L-W-ethanolamine. It has been one of the most well studied membrane-active peptides, principally because it has a well characterised structure and it displays interesting biophysical properties. In organic solvents, the peptide adopts helical dimer conformations. In membranes however, as a result of the unusual alternating sequence of D- and L-amino acids, the peptide adopts a $\beta^{6.3}$ right-handed helical conformation.[64] The monomer is approximately 10.5 Å in length, less than the diameter of a single lamella in most membranes. gA monomers diffuse laterally in the membrane, and monomers in different lamellae are able to form head-to-head (N-terminal-to-N-terminal) dimers (Figure 9). These dimers function as ion channels that are selective for cations, and this is the basis for their antibiotic activity.

A number of studies have examined the effects of gA incorporation on the properties of lipid bilayers. gA insertion into bilayers is accompanied by dehydration of the lipid headgroups surrounding the peptide (the so-called boundary lipids).[65] ESR studies[66] have shown that these boundary lipids become more ordered, adopting a more gel-like state than the

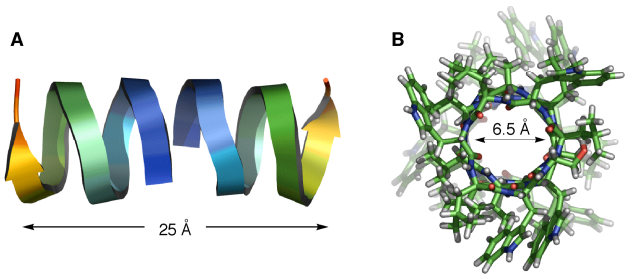


Fig. 9 The structure of the gramicidin A helical dimer in DMPC determined by solid-state NMR methods (from the PDB entries 1mag)[64]

surrounding lipids, which remain in the liquid-crystalline phase. As a result, the boundary lipids become extended in a direction normal to the plane of the bilayer, increasing the extent of the hydrophobic mismatch between the peptide and the membrane. This in turn leads to curvature strain on the membrane that, it has been suggested, contributes to the formation of H_{II} phases, which are known to form at high gA:lipid ratios.[67] Using small-angle x-ray techniques, the intrinsic radius of curvature of gA in DOPC bilayers has been estimated as –7.4 Å.[68] Other ESR studies with DMPC have concluded that the number of boundary lipid molecules with restricted motion is three to four per gA monomer.[69] The same study found that gA has little effect on the tilt angle of the lipid acyl chains in either the gel or liquid-crystalline phase of DMPC, with the gA aligned along the same axis as the acyl chains in the latter case. In the gel phase however, the gA monomer exhibited a greater tilt angle than the lipid chains. With other lipids (PE, PS and PG), gA was less well oriented. In common with many of the peptides encountered in

this review, the termini of the helical dimer in the region closest to the membrane interface are marked by the presence of tryptophan residues, which are aligned with the indole-NH groups facing the extracellular medium and the indole dipole aligned along the channel axis. These tryptophan residues serve the twin purposes of aligning the peptide in the bilayer, and enhancing channel conductance.[70]

β-Sheet models for integral membrane proteins

A significant number of membrane proteins in the PDB have a β-barrel structure. These proteins are mostly prokaryotic outer membrane proteins, isolated from Gram negative bacteria, where they serve as transmembrane pores for a range of functions, such as drug efflux, nutrient influx and protein transport. Whilst some of these are important therapeutic targets, others have potential biotechnology applications. For example, a number of autotransporters have been characterised that are able to catalyse the transfer of parts of their own polypeptide chain across the membrane through a β-barrel pore.[71] Cleavage of the transported protein fragment releases a soluble protein on the *trans* side of the membrane, leaving the pore behind. Peptides that are designed to mimic these pores may have potential applications as agents for macromolecule delivery into cells. Finally, a number of peptide toxins insert into membranes to form β-structures that are responsible for lytic activity.

An understanding of the factors that promote stable assembly in the membrane is therefore important. The group of White and co-workers have developed a peptide model for studying the formation of β-structures in membranes.[72] Their peptide, of sequence Ac-WL_5, exists in solution as a monomer with a random coil conformation. Following insertion into the membrane as a random coil, the peptide aggregates and adopts a β-sheet conformation with the inter-strand hydrogen bonds aligned parallel to the plane of the membrane, as determined by CD and IR experiments. Interestingly, peptide insertion is cooperative and coupled to β-sheet formation. Thermal denaturing experiments indicate that the midpoint temperature for unfolding of the aggregate is ~60 °C, and establishes that the process is an equilibrium, with the β-sheet aggregate in equilibrium with a membrane inserted random coil monomer, which is in turn in equilibrium with the random coil monomer in aqueous solution (Figure 10). Although the formation of β-structures by this and related peptides[73] correlates poorly with the sheet-forming propensities of the constituent amino acids in globular proteins, the observation of β-sheets in the bilayer environment points to the importance of polar interactions, in this case hydrogen bonds, within this environment. Studies using coiled-coil proteins have found that polar residues, such as asparagine, are preferentially located on the sides of α-helices that are involved in protein-protein contacts when located deep in the bilayer, but may be found on the lipid exposed surface of helices near the membrane interface,[74] which is also consistent with the increased importance of polar residues in controlling protein conformations in the bilayer interior. White estimates that the free energy gain for the formation of a hydrogen bond in the membrane environment is of the order of between 0.46 and 0.61 kcal mol^{-1} per residue when compared with a solvated peptide bond in water. Even if this value is an overestimate, it still points to the reason why some membrane proteins have extremely thermostable structures. It also explains why the Ac-WL_5 peptide forms sheet rather than helix structures in membranes, as the number of exposed peptide bonds is minimised in sheets. The adoption of helical conformations would expose a significant number of peptide bonds at the helix ends. Consistent with this, a number of longer leucine-containing peptides that we have already discussed are able to adopt helical transmembrane conformations, as the proportion of peptide bonds exposed is much smaller in these peptides.

The effects of hydrophobicity were also addressed by White and co-workers. Longer, more hydrophobic peptides such as Ac-WL_6 were found to have higher partition constants with lipid membranes and were stable towards thermal degradation.[72] This

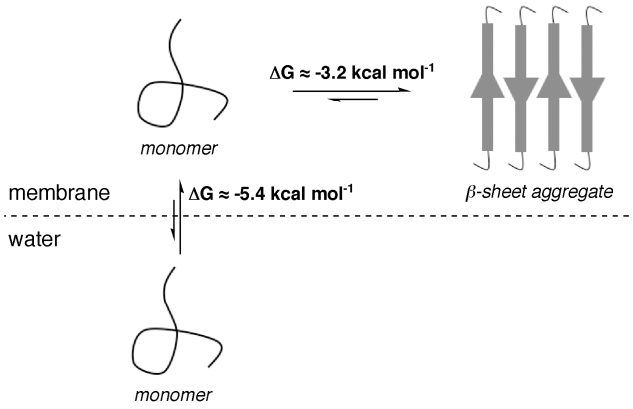


Fig. 10 Formation of β-sheet structures by the peptide Ac-WL_5. Adapted from reference 72.

was attributed in part to the cooperativity of β-sheet formation, coupled with the considerably more favourable partitioning of the monomer into the membrane. Shorter peptides, such as Ac-WL_4 had only poor interactions with membranes. A more systematic study using analogues of the antimicrobial peptide indolicidin produced a linear relationship between the free energy of partitioning from water to neutral PC membranes and peptide hydrophobicity.[75] These studies demonstrate that hydrophobicity alone is an indication of the tendency of peptides to insert into neutral membranes in some cases.

An important consideration concerning peptide aggregation within membranes is the role of Van der Waals interactions: protein-protein interactions compete with protein-lipid and lipid-lipid interactions. As a consequence, Van der Waals interactions on their own may not be sufficient to drive aggregation in the low dielectric close-packed environment of the bilayer. The association of transmembrane helices for example, requires their surfaces to have highly complementary shapes, permitting tight 'knob-into-hole' packing that favours peptide-peptide interactions over peptide-lipid interactions.[76]

Antimicrobial peptides: surface interactions

There is now a considerable body of work on the structural diversity and mechanism of action of a vast array of peptides with antimicrobial activities. It is beyond the scope of this review to describe all of these, and they have been amply described elsewhere.[77] This discussion will be restricted to those for which peptide-lipid interactions have been characterised.

Pore forming mechanisms. There are currently three proposed mechanisms by which membrane-lytic peptides function (Figure 11).[78] A common feature of all of these is initial binding of the peptide to the membrane surface, which is mediated by a combination of hydrophobic and electrostatic effects. Many antimicrobial peptides are cationic, and bind to the surface of negatively-charged membranes through electrostatic interactions. A key factor here in determining peptide selectivity is the composition of the membrane.‡ Many eukaryotic cell membranes are composed mainly of neutral (zwitterionic) lipids. This includes red blood cells, the poration of which is easily monitored by haemolysis assays, providing a measure of peptide activity. Prokaryotic cells, on the other hand, tend to contain more negatively charged lipids, such as PG and CDL. Whilst this provides a mechanism for the selectivity of cationic peptides, it is not the whole story. Peptides have also been characterised that bind selectively to membranes containing cholesterol (which is not found in prokaryotic membranes), such as streptolysin O and perfringolysin.

Whilst initial binding to the membrane may be driven by electrostatic effects, hydrophobic effects are also important. The

work to study the effects of hydrophobicity on the membrane partitioning of model peptides described earlier,[75] also found that electrostatic and hydrophobic effects were not additive, with the electrostatic effect often being overestimated. One of the reasons why the relative importance of these effects is difficult to assess is the change in conformation that many peptides undergo upon binding to the membrane surface. For example, the magainins are cationic peptides that adopt an amphipathic α-helical conformation following surface binding.[79] Protegrin-1, a peptide stabilised by two disulphide bridges, functions in the porcine immune system as a broad-spectrum antimicrobial agent. Both the parent peptide and analogues containing more or fewer disulphide bonds retain their activity, and are predicted to form amphipathic β-structures when bound to the membrane, suggesting that interaction with the membrane is sufficient to induce folding.[80] Many other peptides adopt amphipathic conformations following surface binding, as this places hydrophilic and hydrophobic residues in favourable dispositions for solvation or interaction with the lipid interior respectively. In at least one case, evidence is provided for peptide aggregation prior to membrane interaction, but it remains to be seen how general this is.[81] Cases have also been described of pH-dependent changes in the conformation of proteins leading to the exposure of hydrophobic residues that are then able to drive membrane insertion.[82]

A number of studies have addressed the nature of the interactions between peptides and the surface region of the bilayer. The human antimicrobial peptide LL-37 is an amphipathic α-helix that embeds in the bilayer in the region of the headgroups and aligns parallel to the membrane surface.[83] ^{2}H NMR studies indicate that LL-37 produces disordering of gel phase DMPC bilayers and conversely, ordering of unsaturated bilayers. Mellitin, a well-studied peptide from bee venom, inserts at low concentrations into bilayers in a similar fashion to LL-37, aligning parallel to the membrane surface whilst penetrating to the depth of the glycerol region of the lipid.[84] One novel approach to determining the orientation of the fusogenic peptide B18 in membranes has been to use solid-state ^{19}F NMR measurements on fluorine-labelled B18 analogues.[85] Incorporation of L-4-fluorophenylglycine at distinct locations in the peptide did not perturb the kinked α-helical structure of the peptide, and the measurement of ^{19}F chemical shift anisotropies in oriented membranes enabled the respective orientations of the peptide fragments N- and C-terminal to the kink to be estimated as 54° and 91° with respect to the bilayer normal, with the N-terminus of the peptide located at the membrane interface.

All of the mechanisms of membrane poration that follow binding to the surface occur once the bound peptide:lipid ratio has reached a critical value (P:L*).

The carpet mechanism (Figure 11) involves peptides binding parallel to the membrane surface to form clusters that cover the surface with a 'carpet' of peptide. In some cases, the distinction between surface binding and slight penetration into the bilayer may be difficult to determine. No generalisations can be made concerning peptide structure in these carpets, but it is generally accepted that peptide activity is not detergent-like, occurring instead by the exertion of disruptive forces on the membrane. The redistribution of lipids in one of the membrane lamellae in response to peptide binding is likely to produce significant curvature strain on the membrane, leading to disruption.

The toroidal mechanism involves initial peptide binding to the membrane surface to form an α-helix that is aligned in the plane of the membrane. Studies on magainin, a peptide from the skin of the African clawed frog,[79] demonstrate that, as with LL-37, slight penetration into the headgroup region of the bilayer occurs. This disrupts lipid-lipid interactions, leading to membrane thinning, placing the membrane under positive curvature strain. At the critical P:L ratio (P:L*), breakdown of the bilayer occurs and the peptides realign to adopt a transmembrane orientation. Self-association of the helices leads to the formation of a protein-lined pore. It should be apparent that many of the steps resemble those of the carpet mechanism, and indeed, both require peptides that are able to promote curvature strain in membranes. It should also be apparent that

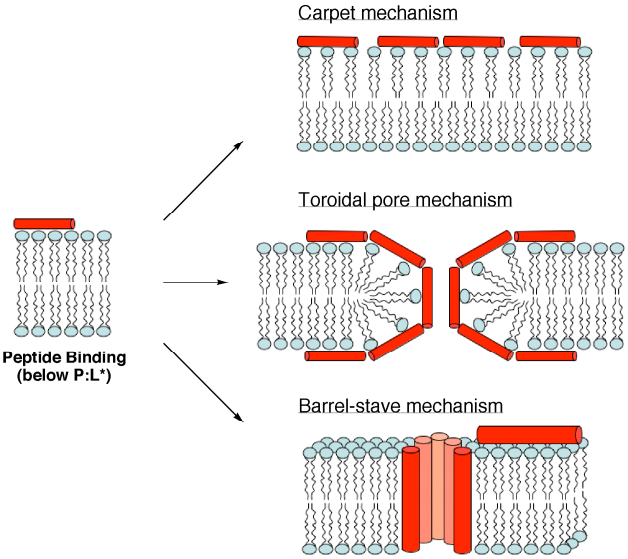


Fig. 11 Pore-forming mechanisms of antimicrobial peptides.

peptides that are able to produce curvature strain may potentially form inverted hexagonal phases if present in sufficient concentration. This has been demonstrated for the designed antimicrobial peptide MSI-78.[86] One striking example of the propensity for antimicrobial peptides to produce curvature strain is provided by the interaction of equinatoxin II with DMPC/SM membranes.[29] Freeze-fracture electron micrographs of mixtures of the toxin with DMPC/SM liposomes show the presence of small unilamellar vesicles of diameter 20-40 nm, presumably reflecting the preference of the protein-lipid mixture to form highly curved structures.

The barrel-stave mechanism is proposed for a select group of peptides, the most studied of which is alamethicin. Peptide monomers assemble on the membrane surface, oriented with respect to the membrane according to their amphipathicity. Upon reaching the threshold concentration, monomers aggregate and insert into the membrane to form a pore lined with a mixture of peptides and lipid. The pore is a dynamic molecular assembly that is able to recruit or lose peptides according to local concentration gradients. As a consequence, the sizes of the pores formed by these peptides are relatively polydisperse. The channels formed by alamethicin have a diameter of 2-3 Å, corresponding to the recruitment of 4-6 monomers. The unusual sequence of the peptide, which contains α-aminoisobutyric acid (Aib) and phenylalaninol, points to the mechanism by which the peptide forms pores. In the crystal structure of the peptide (Figure 12), the backbone is predominantly helical, with a kink at residue 14 (proline). The Aib residues enable strong interhelical packing interactions, whilst the kink increases the flexibility of the helix, an effect that studies on model peptides indicate should minimise peptide aggregation in solution.[87] A similar kink is found in the membrane-penetrating peptide buforin II. Removal of this kink by substitution of leucine for proline produced a peptide without antimicrobial activity that bound to the membrane surface.[88]

Some work has pointed to mellitin forming pores by a barrel-stave mechanism,[90] although other researchers suggest that this peptide forms pores through a toroidal pathway.[91] There is clearly some room for speculation as to the precise mechanisms by which these peptides function, and it is entirely feasible that different mechanisms may be observed in the presence of alternative lipid mixtures.

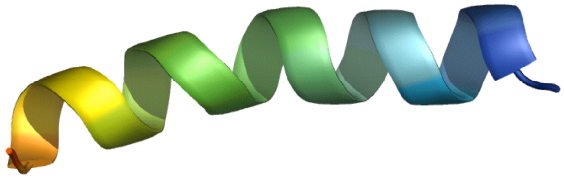
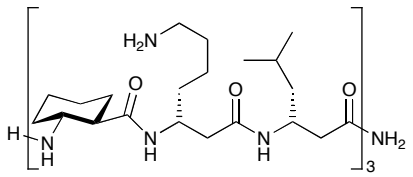


Fig. 12 X-ray crystal structure of Alamethicin (PDB entry 1amt).[89]

Designer antimicrobial peptides. Some natural peptides, such as the gramicidins, have been used in topical applications for some time. In general however, the design of new peptides with specific activity is not trivial. An understanding of the mechanisms by which antimicrobial peptides function selectively on different cell types is essential, which in turn requires that the detailed molecular basis of peptide-lipid interactions are understood. The need for this arises because the diversity of antimicrobial peptides is considerably greater than the mechanisms by which they function. Peptides that are vastly different in sequence and structure may share the same mechanism for pore formation, and this presents a serious challenge to understanding the basics for designing a new peptide. The most successful approaches have tended to commence by modification of peptide of known activity, with some useful outcomes. For example, modification of platelet antimicrobial proteins has lead to the production of novel peptides that are non-haemolytic, and therefore suitable for use in blood-based media.[92]

In order to be of more general use, such as for oral applications, the problem of digestion by proteases needs to be overcome. Approaches to achieving this have included the preparation of peptides with reduced amide bonds,[93] and the design of peptides based around β-amino acids, which are not natural substrates for proteases.[94] The groups of Gellman, DeGrado and Seebach have extensively investigated the structure and lytic activity of a series of β-peptides. These peptides tend to form helical structures that are more stable than the α-helix. Peptides **1** and **2**, for example, adopt 14-helical conformations in membranes, with hydrogen bonds between the C=O of residue *i* and the NH of residue *i*-2 forming a 14-membered ring.[95]

Peptides **1** and **2** have potent antimicrobial activity and low toxicity to mammalian cells. Furthermore, these peptides have been shown to induce lipid flip-flop in membranes containing anionic lipids and promote membrane fusion. The latter activity was attributed to the induction of negative curvature strain following the binding of these peptides to membranes at low



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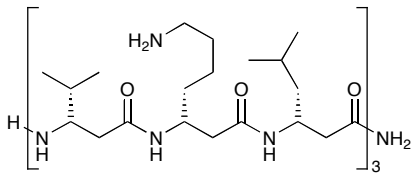

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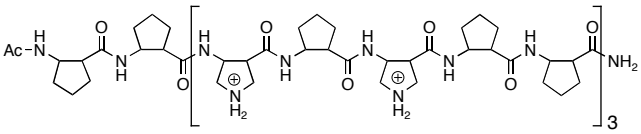
concentrations.[95] The more conformationally restricted peptide β-17, designed around analogues of *trans*-2-aminocyclopentanecarboxylic acid, forms an amphipathic 12-helix that also displays promising antimicrobial activity and low haemolytic activity.[96]

Studies on the binding of this peptide to lipid membranes have reached similar conclusions to those arrived at for peptides **1** and **2**. These peptides therefore share the common property of being able to induce negative curvature strain, and in this respect are



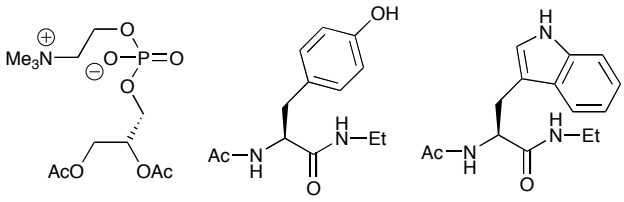
β-17

different from other antimicrobial peptides, such as magainin II, that act by inducing positive curvature strain.[97]

Fundamental lipid binding studies

This last section describes some of the current approaches that are being explored to develop small-molecule systems that reveal fundamental aspects of the interactions between amino acids and lipids. From the above discussion it will be apparent that the membrane interface is a complex environment, and it is therefore of interest to examine specific details of the interactions that occur between amino acids and lipids in the absence of complicating factors.

Work in our group has developed a model system for probing the preferred interactions between amino acids and lipids in non-competing solvents. Our system comprises a PC such as diacetyl PC (**3**) or DMPC as a host in titrimetric experiments with N-acetyl amino acid N-ethyl amides (**4, 5**) as hosts. The system maintains all of the functionality present in natural systems, and



3 **4** **5**

has allowed us to measure the association constants of each amino acid with **3** by ^{1}H NMR titration.[98] From these studies, we were able to show that tryptophan and tyrosine binding to PC headgroups was significantly more favourable than the binding of either phenylalanine or valine, a finding consistent with the bioinformatics studies described earlier.[12,13] Furthermore, both tryptophan and tyrosine formed 2:1 complexes with the lipid and in the case of tryptophan, we were able to observe intermolecular contacts by 2D ROESY spectroscopy. These were used to

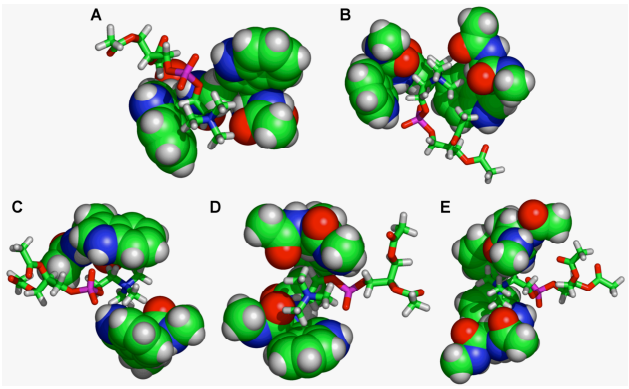
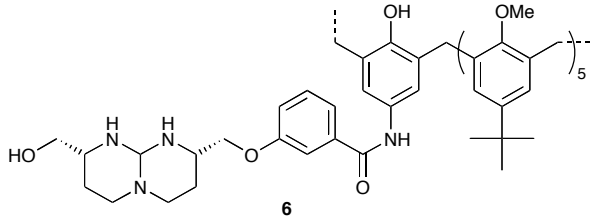


Fig. 13 Models for the interaction of tryptophan with PC headgroups. Each model is a representative structure from groups of structures generated by dynamics calculations.[98]

generate distance restraints that could be used to examine the interactions by molecular modelling (Figure 13). Adduct formation was characterised by indole-NH---phosphate hydrogen bonds, indole---choline cation-π interactions, amide C=O---choline ammonium coordination, and amide-NH---phosphate

hydrogen bonds. In more recent work, we have found that the observed association constants are markedly dependent on the water content of the sample, and titrations with DMPC in place of **3** (which is highly hygroscopic) produce significantly higher association constants.[99] During the course of this work, we also observed that water bound to the choline headgroup of DMPC was displaced, suggesting that this may be a consequence of tryptophan coordination. Although our results differ from those reported earlier for the coordination of tryptophan in transmembrane helices, our system is not restrained by the requirement of residing in the bilayer, and therefore is probably of more relevance to the surface-bound conformations described earlier for antimicrobial peptides. It is interesting to speculate that dehydration of PC headgroups by tryptophan may be responsible for some of the curvature strain effects that are seen with these peptides.

A number of synthetic receptors for PC headgroups have been designed, which shed some light on the interactions that favour lipid binding.[100] Calixarene **6** has an association constant in chloroform of 7.3 ± 0.5 x 10^4 M^{-1} with DOPE. Complex formation in this case is enthalpic, and requires both the guanidinium functionality and the calixarene cavity.[101] NMR



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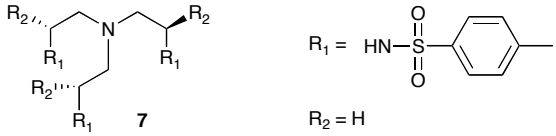
studies on the 1:1 complex are consistent with inclusion of the choline headgroup in the cavity of the calixarene. Synthetic receptors based on TREN (tris(2-aminoethyl)amine) exhibit selective binding of PC over PE and PS. These compounds, such as sulphonamide **7**, are able to mimic scramblase activity by altering the distribution of lipids within the membrane.[102] In this series of compounds, complexation with the phosphate seems to be the major determinant of binding, as derivatives with



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increased amide acidity were better at PC complexation. This echoes the important role of hydrogen bonding to the phosphate that we observed in our studies.[98]

Prospects

We have discussed a number of features of peptide-lipid interactions in this review. Some general conclusions concerning these interactions may be drawn: (1) electrostatic and hydrophobic effects contribute to peptide binding to membrane surfaces; (2) hydrophobicity is an important controlling factor in peptide partitioning into membranes. Peptides of low hydrophobicity tend to reside outside the bilayer, whereas those that are highly hydrophobic partition into all membranes, and usually do not discriminate between mammalian and prokaryotic cells; (3) tryptophan has an important role in membrane anchoring. Other aromatic amino acids may also have special roles in peptide binding; (4) most surface-active peptides are amphipathic, or can adopt an amphipathic conformation following membrane binding; (5) transmembrane peptides and proteins need complementary shapes in order to aggregate, due to the finely balanced nature of the competing peptide-lipid and lipid-lipid interactions; (6) dipolar (electrostatic) interactions are more favourable in free energy terms in the hydrophobic part of the bilayer than in water; (7) peptide binding influences the phase properties of the membrane and the distribution of boundary lipids. Lytic and fusogenic peptides mediate their activity through the curvature strain that results from these changes; (8) whilst there is some evidence that membrane-bound peptides are able to displace interfacial water, the role of water in peptide-lipid interactions is not currently well defined.

Whilst, through a great deal of effort, the factors that contribute to peptide-lipid interactions are beginning to be understood, many opportunities remain for the organic chemist to design new systems that address some of these issues.

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† Abbreviations used: AFM, atomic force microscopy; CDL, cardiolipin; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; P:L, peptide:lipid ratio; PA, phosphatidic acid; PC, phospatidylcholine; PDB, protein data bank; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin. Standard single letter are abbreviations are used for amino acids: A, alanine; G, glycine; K, lysine; L, leucine; V, valine; W, tryptophan.

‡ Supplementary information available: chemical structures of common lipids; lipid composition of typical eukaryotic and prokaryotic membranes.

§ Ray-traced images in this paper were prepared using the program PyMol (DeLano Scientific, San Carlos, CA, USA).

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