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Review

AMPs and OMPs: Is the folding and bilayer insertion of β-stranded outer membrane proteins governed by the same biophysical principles as for α-helical antimicrobial peptides?☆

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ABSTRACT

The folding and function of membrane proteins is controlled not only by specific but also by unspecific interactions with the constituent lipids. In this review, we focus on the influence of the spontaneous lipid curvature on the folding and insertion of peptides and proteins in membranes. Amphiphilic α-helical peptides, as represented by various antimicrobial sequences, are compared with β-barrel proteins, which are found in the outer membrane of Gram-negative bacteria. It has been shown that cationic amphiphilic peptides are always surface-bound in lipids with a negative spontaneous curvature like POPC, i.e. they are oriented parallel to the membrane plane. On the other hand, in lipids like DMPC with a positive curvature, these peptides can get tilted or completely inserted in a transmembrane state. Remarkably, the folding and spontaneous membrane insertion of β-barrel outer membrane proteins also proceeds more easily in lipids with a positive intrinsic curvature, while it is hampered by negative curvature. We therefore propose that a positive spontaneous curvature of the lipids promotes the ability of a surface-bound molecule to insert more deeply into the bilayer core, irrespective of the conformation, size, or shape of the peptide, protein, or folding intermediate. This article is part of a Special Issue entitled: Lipid-protein interactions.

Contents

1. Introduction

Biological membranes are complex systems involved in numerous biological functions, and consist of lipid bilayers with many different associated proteins. To get a better understanding of such systems, many groups have studied membrane-bound α-helical peptides of typically 10–30 amino acids. These peptides can have a biological function of their own (like antimicrobial [1–3], cell-penetrating [4–6] and fusogenic peptides [7–9]), represent a functional segment of a larger protein (for example the transmembrane helix of an integral membrane protein) [10,11], or serve as model peptides that are designed to investigate a specific aspect of peptide-membrane interactions (like hydrophobic mismatch) [12,13]. In all these cases, it is of interest to obtain information on the structure, orientation and dynamics of the peptide in a

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lipid bilayer with a certain composition, and how the orientation changes under different conditions. Solid-state NMR structure analysis in macroscopically oriented membranes is an excellent method for such studies under quasi-natural conditions [14–19]. Such NMR samples can be prepared using a wide range of peptides and lipid systems, making them ideal for systematic studies of lipid–peptide interactions.

We have recently gained comprehensive insight into the role of the spontaneous curvature of lipids—which is related to the shape of the lipid molecule—on the membrane insertion of amphiphilic antimicrobial peptides (AMPs) with α-helical and cyclic structures. These molecules initially bind to the membrane surface, but they can get tilted and inserted deeply into the bilayer core, provided that the spontaneous curvature allows this. By analogy, we speculate that the same physicochemical principles may also be relevant for the un-assisted folding and insertion of outer membrane proteins (OMPs), because these intermediates also consist of *amphiphilic* β-strands that are initially stretched out on the membrane surface. The membrane insertion of OMPs has been comprehensively described in the literature, but not yet in the context of lipid curvature. In this review, we will first describe our own systematic data on short α-helical and cyclic peptides and show that they agree with the results from other groups. Then we will compare the behavior of these amphiphilic peptides with experimental studies of β-barrel proteins. The overview clearly shows that the spontaneous lipid curvature has indeed the same fundamental influence on the membrane interactions of these much larger proteins.

2. Studies of α-helical amphiphilic peptides

For the last quarter of a century there has been much interest in antimicrobial peptides (AMPs), whose mechanism of action is attributed to the permeabilization of bacterial membranes [1,20–23]. AMPs are a particularly promising class of compounds, due to the fact that they directly attack the membrane of the microorganism and do not seem to induce resistance, which is often the case for traditional antibiotics. The structural behavior of such peptides in the membrane-bound state has been studied using several biophysical techniques, to gain more insight into their mechanism of action. If the peptides form oligomeric pores in the lipid bilayer, this should be connected to a transmembrane orientation [24]. This characteristic molecular alignment can be detected using, e.g., oriented circular dichroism (OCD) [23,25,26] or solid state NMR (SSNMR) methods [14,16,19,27]. Other mechanisms like the "carpet model" have been proposed [24], where the peptides cause membrane thinning and increase the permeability of membranes. In this case, the molecules remain bound to the bilayer surface without changing their orientation.

Even though the peptide orientation can be measured reliably using OCD or SSNMR, there are several caveats with these methods to distinguish pore formation from a carpet mechanism. One problem is that pores do not have to be stable under equilibrium conditions, as transient pores—driven by a concentration gradient—can induce membrane leakage on a time scale that is enough to kill bacteria. Likewise, only a small proportion of peptides could be involved in pore formation, yet be sufficient to induce irreversible damage. Methods like OCD and SSNMR are not very sensitive and can only reveal the orientation of the majority of peptides in the membrane, so it is hard to rule out pore formation by these methods. Kinetic measurements, such as fluorescence leakage from lipid vesicles, provide a complementary time-resolved approach [28,29], without providing any structural information, however.

Another problem in all these studies is the fact that they are performed in model membranes, where only one or two (in rare cases more) lipid species are present. It would be more relevant to study the peptides in native membranes from the organism of interest (i.e. for AMPs the bacteria to be killed), but this has only been done in a few cases [17,30,31]. Supported by those results, but mainly for practical reasons, simplified model membranes tend to be used, in the hope that they mimic the relevant biological membranes reasonably well. There is no consensus, however, which lipid systems should be employed as a model, and different groups and different studies have used widely varying lipid compositions. This diversity can be seen in Table 4 of reference [14], which contains a list of SSNMR studies of AMPs. Most often phosphatidylcholine (PC) lipids have been used, sometimes combined with charged phosphatidylglycerol (PG). In some cases also phosphatidylethanolamine (PE) has been included, which is a prominent component of bacterial membranes, whereas cholesterol is added to mimic eukaryotic membranes. Not only the lipid head groups have been varied, but also the acyl chains, which can have different lengths and degrees of saturation.

When comparing different studies, it is hard to know whether any reported difference between the orientations of two peptides must be attributed to a genuinely different mechanism, or rather to a coincidental difference in the lipid model membranes used. An SSNMR structure analysis requires considerable effort, as peptides have to be synthesized with isotope labels, oriented samples need to be prepared, and NMR measurements performed sometimes over several days [14]. Therefore, most studies tend to focus on a single peptide and include only few lipid systems. It is therefore hard to compare these data and draw any general conclusions.

We have recently performed a systematic study of an α-helical amphiphilic AMP, MSI-103 [32], which opened up a generalized view of how the lipids can influence the orientation of peptides, based on the old concept of spontaneous lipid curvature [33,34]. This led us to do additional studies [3,35], and altogether these results have consolidated a new picture of the importance of lipid–peptide interactions on the orientation of peptides in membranes, which will be described in the following section.

2.1. Orientation of MSI-103 in different lipid systems

MSI-103 is a cationic α-helical amphiphilic AMP, designed as a simple heptameric repeat with high charge and amphiphilicity [36]. We labeled this peptide with Ala-d_3 and studied its orientation in membranes using ^{2}H-NMR as previously described, using a wide variety of lipid systems [32,37]. (A list of lipids is given in Table 1 for convenience, where the full names and common abbreviations of lipids are listed next to the acyl chain compositions.) Upon varying the head groups and acyl chains, we found that the peptide can have two characteristic orientations: a flat orientation on the membrane surface (which we call "S-state"), and a tilted orientation ("T-state") that penetrates the bilayer core more deeply. Which of these orientations prevailed was clearly not dependent on the hydrophobic thickness of the membrane. Namely, the S-state was found both in very thin DMoPC as well as in thick DOPC bilayers, while the T-state was found in other thin and thick systems. However, there was a distinct correlation between the spontaneous curvature of the lipids and the peptide orientation: In systems with a negative spontaneous curvature, like POPE/POPG or DOPC, only the S-state was found, even at high peptide-to-lipid (P/L) ratios. On the other hand, in systems with a positive spontaneous curvature, like DLPC or lyso-lipid containing membranes, the T-state was found at high P/L, while at low P/L the peptides preferred the S-state also in these systems. We concluded that at very low concentrations, when the peptides are monomeric, they lie flat on the membrane surface with an orientation dictated by the amphiphilic character of the molecule, such that the polar and charged residues point into the aqueous phase. At higher concentration, peptides will start to interact with each other (directly by molecular contacts, or indirectly via lateral pressure [38,39]) and will be driven to insert more deeply into membranes. However, this is thermodynamically favorable only if the bilayer is composed of lipids with a positive spontaneous curvature, whereas the threshold for insertion is much higher in lipids having a negative spontaneous curvature [32]. This phenomenon can be readily understood in terms of lipid shapes, as will be described below more in detail.

Table 1
List of lipids and their corresponding hydrophobic thickness.

Lipid	Full name	Acyl chains	L_h[a] (Å)
	1,2-didecanoyl-*sn*-glycero-3-phosphocholine	di-10:0-PC	16.6
	1,2-diundecanoyl-*sn*-glycero-3-phosphocholine	di-11:0-PC	18.8
DLPC	1,2-dilauroyl-*sn*-glycero-3-phosphatidylcholine	di-12:0-PC	21.0
DLPE	1,2-dilauroyl-*sn*-glycero-3-phosphatidylethanolamine	di-12:0-PE	21.0
DLPG	1,2-dilauroyl-*sn*-glycero-3-phosphatidylglycerol	di-12:0-PG	21.0
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine	di-14:0-PC	25.4
DMPG	1,2-dilauroyl-*sn*-glycero-3-phosphatidylglycerol	di-14:0-PG	25.4
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine	di-16:0-PC	29.8
DTPC	1,2-di-O-tetradecyl-*sn*-glycero-3-phosphocholine	di-ether-14:0-PC	25.4
DPhPC	1,2-diphytanoyl-*sn*-glycero-3-phosphocholine	di-4Me-16:0-PC	n.a.[b]
DPhPE	1,2-diphytanoyl-*sn*-glycero-3-phosphatidylethanolamine	di-4Me-16:0-PE	n.a.[b]
DMoPC	1,2-dimyristoleoyl-*sn*-glycero-3-phosphatidylcholine	di-14:1c9-PC	19.2
DPoPC	1,2-dipalmitoleoyl-*sn*-glycero-3-phosphatidylcholine	di-16:1c9-PC	23.0
DOPA	1,2-dioleoyl-*sn*-glycero-3-phosphate	di-18:1c9-PA	26.8
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine	di-18:1c9-PC	26.8
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine	di-18:1c9-PE	26.8
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine	16:0-18:1c9-PC	28.3
POPE	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine	16:0-18:1c9-PE	28.3
POPG	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol	16:0-18:1c9-PG	28.3
Lyso-MPC	1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphatidylglycerol	14:0-PC	n.a.[b]
Lyso-OPC	1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphatidylglycerol	18:1c9-PC	n.a.[b]
TOCL	1,1',2,2'-tetraoleoyl-cardiolipin	tetra-18:1c9-CL	n.a.[b]

[a] Lipid hydrophobic thickness, according to Ref. [83].
[b] No data available.

2.2. Spontaneous lipid curvature and peptide orientation

The morphology of a lipid assembly in water is determined by the shape of the lipid molecules, when the head groups are exposed to the aqueous solution and the lipid chains are buried inside the assembly. The lipid shape can be described by the number $S = V/A_oL_c$, where V is the volume of the hydrocarbon chains of the lipid, A_o is the optimal area per lipid, and L_c the critical chain length [33].

Lipids with a small head group and larger acyl chain cross-sectional area ($S > 1$) can be described by a cone (Fig. 1A) and prefer to form "inverted" structures like an inverted hexagonal phase (Fig. 1B); lipids with a large head group and smaller acyl chain volume ($S < 1$) can be described by an inverted cone (Fig. 1A) and prefer to form micelles (Fig. 1C); while lipids with similar cross-sectional areas for head group and chains ($S \approx 1$) are described by cylinders (Fig. 1A) and prefer to form lamellar bilayers (Fig. 1D) [33,34]. In the hypothetical situation of being present as a monolayer, each type of lipid would thus prefer to assume a characteristic "spontaneous" curvature, which can be quantitatively described by the corresponding radius of curvature that can be measured experimentally. Provided that the radius of curvature is not too small, lipids with a moderate cone shape will nonetheless assemble as bilayers, though with a slight thermodynamic penalty. Namely, lipids with a small head group like phosphatidylethanolamine (PE) would prefer to keep their intrinsic cone shape, but this would result in some "void space" in the head group region of a planar bilayer membrane (Fig. 1E). On the other hand, lyso-lipids with only one acyl chain would prefer to keep their inverted cone shape, but this would give rise to some "void space" in the acyl chain region (Fig. 1F). This concept should be regarded as the hypothetical empty space that would be present in a flat bilayer if the lipids were to pack with their preferred shape. In reality of course, the lipids will adapt their shape to become cylindrical on average, and they simply experience some lateral pressure. Another way of describing a lipid system is in fact based on so-called lateral pressure profiles [38,40]. The lateral pressure of the membrane is not constant across the bilayer, but can be higher or lower at different cross-sections along the membrane normal. Roughly speaking, a negative spontaneous curvature corresponds to a higher lateral pressure in the acyl chain region compared to the head group region, while a positive spontaneous curvature corresponds to a higher pressure in the head group region compared to the acyl chains.

Based on this concept, we can illustrate the binding of peptides to these different types of membranes, as is illustrated in Fig. 1E and 1F. In lipids with negative spontaneous curvature, there is "void space" or low pressure in the head group region, so an amphiphilic peptide can readily bind and be accommodated here, compensating the cone shape of the lipids. However, it is hard for the peptide to insert more deeply, because of the high lateral pressure in the acyl chain region, which would put stress on the system. On the other hand, in lipids with positive spontaneous curvature, it will be less favourable for a peptide to bind to the membrane surface, because of the higher lateral pressure in the head group region. However, once the peptide is bound, it will be easier to insert more deeply into the bilayer, taking up much volume in the acyl chain region and reducing the overall stress on the lipids.

Values of spontaneous curvatures for lipids in the fluid liquid crystalline phase have been reported in the literature, but are not available for all lipids; especially for PC lipids there is not much data available. Here, we will describe lipids in terms of their radius of spontaneous curvature (R_0). It should be noted that small values of R_0 correspond to high curvature, while a completely relaxed and flat monolayer has an infinite R_0. PE lipids have a pronounced negative spontaneous curvature, with several studies reporting R_0 to be around −30 Å for DOPE [41–45]. A recent study showed R_0 to be −25 Å for DOPE and −32 Å for POPE [46]. Charged phosphatidic acid (PA) lipids also have a small head group, and one study found R_0 to be −46 Å for DOPA [42]. It is also obvious that lyso-PC lipids have a large positive spontaneous curvature, with an R_0 value of around +38 Å for lyso-OPC [47].

PC lipids, with a large head group and two acyl chains, are generally assumed to be quite close to cylindrical and assemble into bilayers. However, their actual spontaneous curvature depends critically on the length and saturation of the acyl chains, covering both positive and negative values of R_0. Since most orientational studies have been performed in PC lipids, it would be particularly interesting to compare the spontaneous curvatures of the different saturated and unsaturated PC lipids that are typically used in biophysics. Unfortunately, there is not much experimental data available. Nevertheless, it is known that PC lipids with short saturated acyl chains, like DHPC (di-6:0-PC), form micelles, and must therefore have a small radius of positive spontaneous curvature. Longer-chain PCs do not form micelles, so they must have a larger radius of positive curvature. For DPPC (di-C16:0) a value of +147 Å has

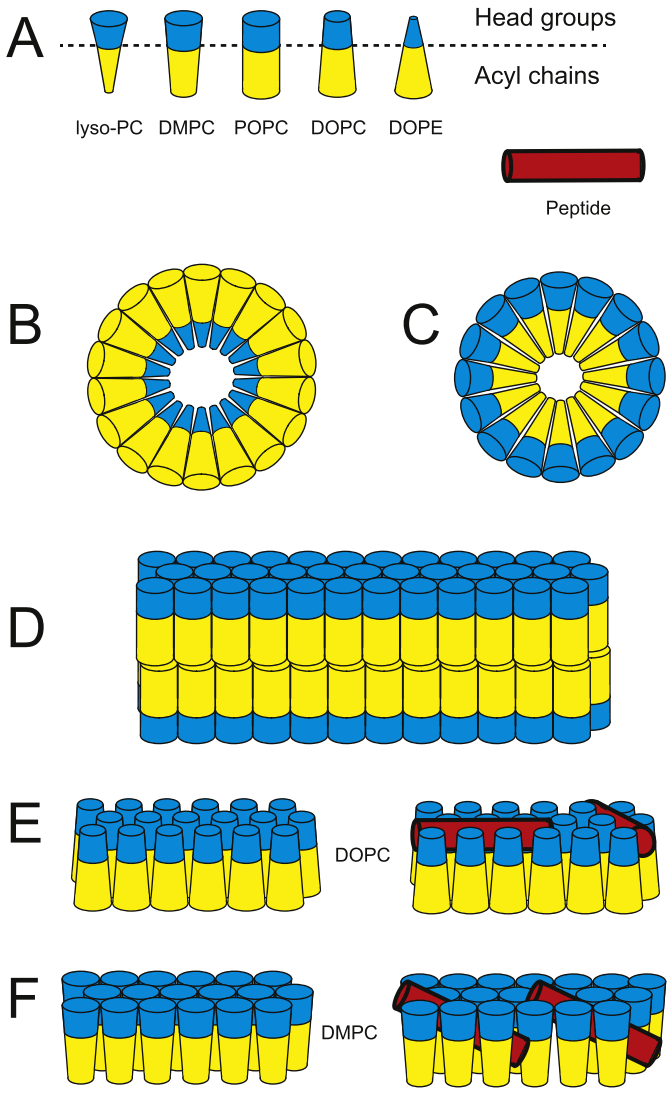

Fig. 1. Lipid shape concept: (A) Lipids with a small head group and larger acyl chain cross-sectional area can be described by a cone, lipids with a large head group and smaller acyl chain area can be described by an inverted cone, and lipids with similar cross-sectional areas for head group and chains are described by cylinders. (B) Cone-shaped lipids prefer to form "inverted" structures with a negative curvature. (C) Inverted cone-shaped lipids prefer to form micelles with a positive curvature. (D) Lipids with cylinder shape prefer to form lamellar bilayer phases. (E) Lipids with a moderate cone shape would hypothetically prefer to keep their shape, but this would result in some "void space" in the head group region of a flat bilayer. Therefore, peptides can easily bind in this head group region but cannot readily insert more deeply into the hydrophobic core. (F) Lipids with a moderate inverted cone shape would hypothetically prefer to keep their shape, but this would result in some "void space" in the acyl chain region of a flat bilayer. Therefore, in this case the peptides can more readily insert into the hydrophobic core of the membrane.

been reported [46], and according to another estimate R_0 should be around +50 Å for DMPC (di-C14:0) [48]. This might be an unrealistically strong curvature for DMPC, but we can surely take it to be positive, and may therefore also assume a somewhat smaller positive R_0 value for DLPC (di-C12:0). Unsaturated DOPC (di-C18:1), on the other hand, has a negative spontaneous curvature, with an R_0 value of about −140 Å [48,49]. No data are available for the spontaneous curvature of DMoPC (di-C14:1), but the unsaturated chains must make it negative as well. POPC (C16:0/C18:1) has a very large negative spontaneous curvature with R_0 of −450 Å [46,50], so it has almost a balanced lateral pressure profile. In general, the commonly used systems with unsaturated lipids have a negative spontaneous curvature.

When considering the simple lipid shape concept illustrated in Fig. 1, it is plausible that there is a distinct correlation between the spontaneous curvature of the lipid system and the ability of the peptides to attain the tilted T-state or transmembrane I-state orientation. In systems with a negative spontaneous curvature only the S-state can be observed (e.g. for MSI-103), while in systems with positive spontaneous curvature a more deeply inserted state is accessible at sufficiently high peptide concentration.

2.3. Orientation of other helical AMPs in different lipid systems

MSI-103 is a designer-made amphiphilic model peptide, and the results from this special peptide might not be generally valid. We therefore performed another systematic study of two natural antimicrobial peptides, PGLa and magainin 2 (MAG2), found in the skin of the African frog *Xenopus laevis* [3]. Both peptides form amphiphilic α-helices in the presence of membranes. In this case, we labeled the peptides with ^{15}N at the backbone amide of a residue in the middle of the helix to obtain an approximate tilt angle from the chemical shift of this ^{15}N-label. For PGLa, we observed a concentration dependent reorientation from an S-state at low P/L to a T-state at higher P/L in DMPC or DMPC/DMPG [51–53], just as for MSI-103 [32,37]. MAG2 had been studied previously in other model membranes, where only an S-state was found in POPC, POPE/POPG, or POPC/POPG/cholesterol [54–57].

PGLa and MAG2 exhibit a strong synergistic activity at a 1:1 molar ratio, which shows up structurally in a transmembrane orientation of PGLa (we call this the "I-state"). We had found this behavior by ^2H-NMR, using Ala-d_3 labeled PGLa in the presence of MAG2 in DMPC/DMPG (3:1) lipid bilayers [58]. Such an I-state was never observed for PGLa on its own, not even at high peptide concentration [53]. Using ^{15}N-labeled peptides, it was later shown that in the 1:1 mixture, MAG2 is not inserted, but it remains in an S-state in DMPC/DMPG (3:1) bilayers, both in the presence and absence of PGLa [3,59].

We then continued to study PGLa and MAG2 systematically, alone and in 1:1 mixtures, using many different lipid systems with positive or negative spontaneous curvature [3]. We found that PGLa, like MSI-103, will always stay in the S-state in lipids with negative spontaneous curvature, but it can attain the T-state at higher concentration in lipids with positive spontaneous curvature. MAG2 was usually in the S-state in both types of lipids, but sometimes also the T-state was found in lipids with positive spontaneous curvature (for example in DPPC). When the peptides are mixed, PGLa will flip into the I-state in lipids with positive spontaneous curvature, but it will always stay in the S-state in lipids with negative spontaneous curvature, while MAG2 has a slight tendency to reach the T-state in lipids with a positive spontaneous curvature but will also remain in the S-state in lipids with a negative spontaneous curvature [3].

In short, for the three tested amphiphilic α-helical peptides, MSI-103, PGLa and MAG2, in lipids with negative spontaneous curvature (like PE, or PC with unsaturated acyl chains) we always found an S-state under all conditions. On the other hand, in lipids with positive spontaneous curvature (like lyso-lipids, or PC with saturated acyl chains) the peptides could also reach a more inserted T-state or even a transmembrane I-state. The results on these three peptides are summarized in Fig. 2. By comparing different types of acyl chains, it was clearly demonstrated that this behavior is not correlated with the bilayer thickness [3,32].

In light of these results on three related peptides, it would now be interesting to compare the literature results on other amphiphilic α-helices that have been studied in various lipid systems, to see whether they also fit into this pattern. In several cases, one and the same peptide has been studied in both positive and negative spontaneous curvature lipids, and indeed different orientations were found. Examples are pardaxin [60], where an S-state was reported in POPC and an I-state in DMPC, or zervamicin II [61], where an S-state was found in unsaturated and an I-state in saturated PC lipids. However, in most studies usually only a single peptide and a single lipid system were used, so it is most

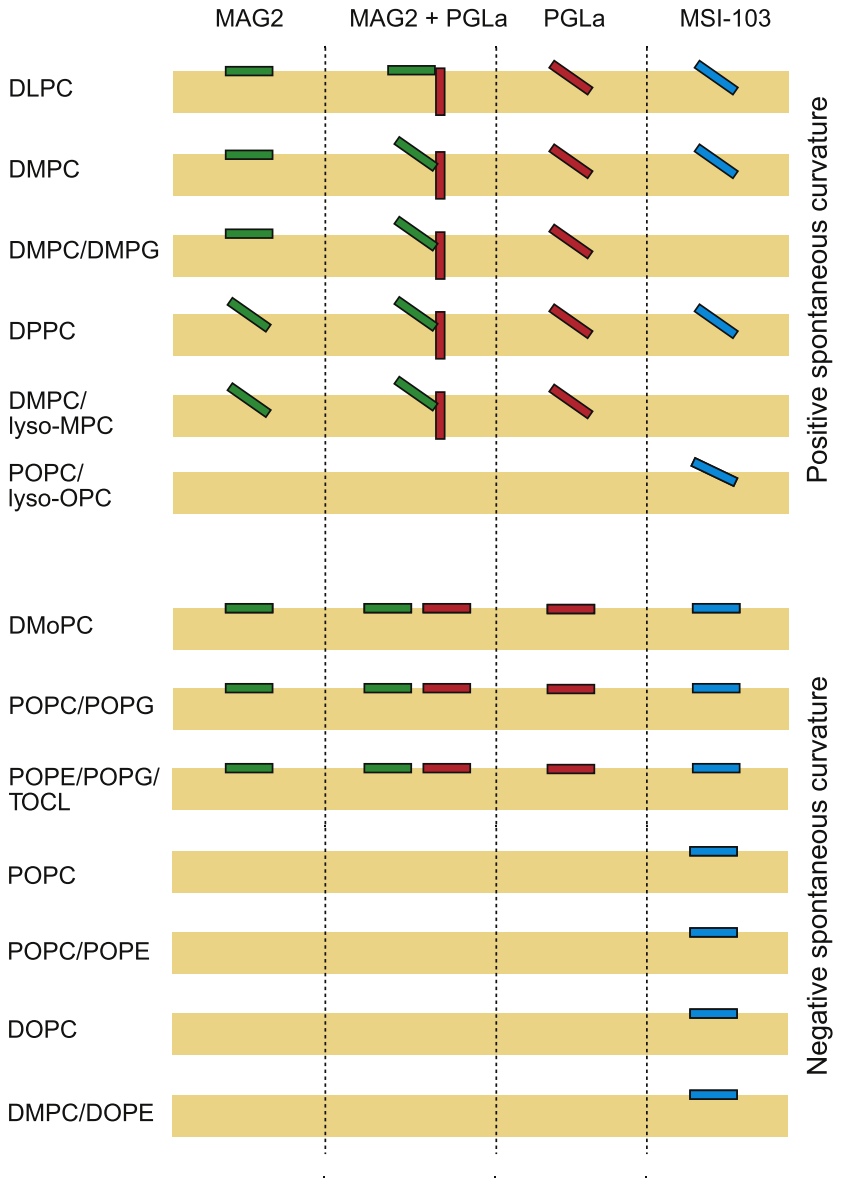

Fig. 2. Peptide orientation in lipid membranes: Overview of results from the α-helical peptides MAG2 (green, sequence GIGKFLHSAKKFGKAFVGEIMNS), PGLa (red, sequence GMASKAGAIAGKIAKVALKAL-NH_2) and MSI-103 (blue, sequence $[KIAGKIA]_3$-NH_2). The approximate orientation (S-state, flat on the surface; T-state, tilted; I-state, inserted in a transmembrane orientation) is depicted. The helix orientations were determined using solid state ^{15}N- and ^{2}H-NMR [3,32].

instructive to survey a large number of studies to search for a pattern in peptide behavior.

In Tables 2 and 3, data are collected from numerous studies of cationic, amphiphilic, α-helical peptides in oriented lipid systems using solid-state NMR methods. (Many further experiments have been performed in lipid bicelles, but they are not included here, because the aggregate shape and lateral pressure are different from a planar lipid bilayer.) In Table 2, lipid systems with a positive spontaneous curvature are shown (mainly DMPC, DLPC and DPPC), while in Table 3, lipid systems with a negative spontaneous curvature are shown (mainly POPC, POPC/POPE and POPC/POPG). It is clear from Table 2 that different orientations are found in lipids with positive spontaneous curvature—sometimes S-state, sometimes T-state and sometimes I-state—depending on the peptide studied and also on sample properties like the P/L ratio. On the other hand, as seen in Table 3, in lipids with negative spontaneous curvature *always* an S-state is found, independent of the peptide, P/L ratio, or details in lipid composition. We can clearly conclude that the spontaneous curvature of the lipids is very important for the orientation of amphiphilic peptides in membranes, as the ease of insertion correlates with the degree of positive curvature. This relationship has not always been obvious, and in fact, in some studies the lipids have not even been specified properly, only as "PC" or "PC/PG", and those studies have not been included in Tables 2 and 3.

Obviously, very hydrophobic α-helical peptides like WALP19 and WALP23 prefer to bind in a transmembrane orientation in every kind of lipid, including POPC and DOPC, as shown in Table 4 for a few cases from the literature. These peptides are not amphiphilic (with the exception of alamethicin, which is only weakly amphiphilic), so it would be energetically unfavorable for them to lie flat on the membrane surface, where hydrophobic residues would get into contact with water. On the other hand, for cationic amphiphilic peptides, an S-state is always energetically favorable, and a T- or I-state must compete with this S-state. Lipids with a negative spontaneous curvature do not support the inserted state, which makes it very hard for any peptide (probably also for a completely hydrophobic one) to insert into the bilayer, and for amphiphilic peptides this additional energy cost makes it very unlikely that they will ever insert in a stable way. On the other hand, if the lipids have a strong positive spontaneous curvature, this tends to make it more

Table 2
SSNMR studies of the orientation of cationic amphiphilic α-helical peptides in lipid systems with a positive spontaneous curvature.

Ref.	Peptide	Nuclei	Labeled	Lipids	Orientation
[84]	Aurein 1.2	^{15}N	Specific NH	DMPC	T-state [a]
[66]	BP100	^{19}F	Spec. CF_3-Bpg	DMPC/DMPG (3:1)	S-state
[85]	Bombolitin II	^{13}C	Specific	DPPC	T-state
[84]	Caerin 1.1	^{15}N	Specific NH	DMPC	T-state [a]
[86]	Cecropin A	^{15}N	Specific NH	DMPC/DMPG (4:1) (P/L = 1:40)	S-state
[84]	Citropin 1.1	^{15}N	Specific NH	DMPC	T-state
[87]	LL-37	^{15}N, ^{13}C	Spec. NH	DMPC, DMPC/DMPG (4:1)	S-state
[88]	M2δ	^{15}N	Specific NH	DMPC/DMPG (4:1)	I-state
[89]	Melittin	^{13}C	Specific CO	DTPC (ether lipid)	I-state
[90]	Melittin	^{13}C	Specific C^{α}	DMPC	I-state
[91]	Melittin	^{13}C	Specific C^{α}	DLPC, DPPC	I-state
[37]	MSI-103	^{2}H, ^{19}F	Specific	DMPC, DMPC/DMPG (3:1)	T-state
[32]	MSI-103	^{2}H	Specific Ala-d_3	DMPC, DLPC	T-state
[60]	Pardaxin	^{15}N	Specific NH	DMPC	I-state
[92]	Pardaxin	^{15}N	Specific NH	DMPC/DMPC (3:1)	I-state
[93]	PGLa	^{19}F	Specific	DMPC	T-state
[94]	PGLa	^{19}F; ^{15}N	4CF_3-Phg; spec. NH	DMPC	T-state
[52]	PGLa	^{2}H; ^{15}N	Ala-d_3; spec. NH	DMPC	T-state
[95]	Piscidin 1	^{15}N	Specific NH	DMPC/DMPG (3:1)	S-state
[96]	Piscidin 3	^{15}N	Specific NH	DMPC/DMPG (3:1)	S-state
[95]	Piscidin 3	^{15}N	Specific NH	DMPC/DMPG (3:1)	S-state
[61]	Zervamicin II	^{15}N	Uniformly	DLPC	I-state
[61]	Zervamicin II	^{15}N	Uniformly	di-10:0-PC	I-state

[a] Also shown by OCD in the same study.

favorable for the peptides to insert, provided that a threshold concentration is met.

2.4. Oriented circular dichroism studies of α-helical AMPs

Oriented circular dichroism (OCD) is another method that can be used to study the orientation of α-helical peptides in membranes [25,26,62–64]. The spectral line shape is characteristically different for peptides lying flat on the membrane surface and for peptides with a transmembrane orientation. While NMR provides inherently local information, OCD will report on the global peptide orientation (but not on local details). If, for example, one segment of a kinked peptide is surface-bound and the other segment is inserted at right angle, the signal will show an average spectrum of an obliquely tilted helix. Likewise, if half the population of straight peptides are surface-bound and the other half are inserted, the signal cannot be distinguished from the case where all peptides are tilted obliquely in the membrane. Compared with NMR, however, the OCD method is faster and less peptide is required, without the need for any labeling. OCD can thus be readily used to monitor the reorientation of α-helices as a function of peptide concentration, hydration, temperature and other factors, and it has been applied to numerous α-helical peptides [5,23,25,28,37,65–72].

OCD studies have been performed in membranes with different spontaneous curvature [23,68], where the peptide orientation was usually monitored as a function of P/L. At low peptide concentrations, the peptides were always found to lie flat on the membrane in an S-state,

Table 3
SSNMR studies of the orientation of cationic amphiphilic α-helical peptides in lipid systems with a negative spontaneous curvature.

Ref.	Peptide	Nuclei	Labeled	Lipids	Orientation
[97]	Distinctin	^{15}N	Specific NH	POPC/DOPE (4:1), POPC/DOPA (1:1)	S-state
[98]	Htt-17	^{15}N	Specific NH	POPC, POPC/POPS (3:1), POPE/POPG (3:1)	S-state
[99]	Interleukin-8α	^{15}N	Specific NH	POPC/POPG (3:1)	S-state
[87]	LL-37	^{15}N, ^{13}C	Spec. NH	POPE, POPC, POPC/POPG (4:1)	S-state
[100]	LL7-27	^{15}N	Specific NH	POPC/POPG (3:1)	S-state
[88]	Magainin	^{15}N	Specific NH	POPC/POPG (3:1)	S-state
[54]	Magainin	^{15}N	Specific NH	POPC, POPC/POPG (3:1), POPE/POPG (3:1), POPC/POPG/chol (3:1:4)	S-state
[55]	Magainin	^{15}N	Specific NH	POPE/POPG (3:1)	S-state
[56]	Magainin	^{1}H,^{15}N	Specific NH	POPC/POPG 4:1	S-state
[57]	Magainin	^{15}N	Specific NH	POPC/POPG (4:1)	S-state
[101]	MSI-78	^{15}N	Specific NH	POPC, POPC/POPG (3:1)	S-state
[32]	MSI-103	^{2}H	Specific Ala-d_3	DOPC, DMoPC, POPC, POPC/POPE, POPE/POPG, DMPC/DOPE	S-state
[101]	MSI-594	^{15}N	Specific NH	POPC, POPC/POPG (3:1)	S-state
[102]	Ovispirin	^{15}N	Specific NH	POPC/POPG 3:1	S-state
[60]	Pardaxin	^{15}N	Specific NH	POPC	S-state
[103]	Pardaxin	^{15}N	Specific NH	POPC	S-state
[104]	PGLa	^{15}N	Specific NH	POPE/POPG (3:1)	S-state
[105]	Phylloseptin-1	^{15}N	Specific NH	POPC	S-state
[105]	Phylloseptin-2	^{15}N	Specific NH	POPC, POPC/POPS (3:1)	S-state
[105]	Phylloseptin-3	^{15}N	Specific NH	POPC	S-state
[95]	Piscidin 1	^{15}N	Specific NH	POPE/POPG (3:1)	S-state
[95]	Piscidin 3	^{15}N	Specific NH	POPE/POPG (3:1)	S-state
[61]	Zervamicin II	^{15}N	Uniformly	DOPC	S-state
[61]	Zervamicin II	^{15}N	Uniformly	DPoPC	S-state
[61]	Zervamicin II	^{15}N	Uniformly	DMoPC	S-state

Table 4
SSNMR studies of the orientation of predominantly hydrophobic α-helical peptides in lipid systems with a positive or negative spontaneous curvature.

Ref.	Peptide	Nuclei	Labeled	Lipids	Orientation
[106]	Alamethicin	^{15}N	Specific NH	DMPC (P/L = 1:8)	I-state
[107]	Alamethicin	^{15}N	Specific NH	DMPC (P/L = 1:8)	I-state
[108]	Alamethicin	^{15}N	Specific NH	DMPC (P/L = 1:15)	I-state
[61]	Alamethicin	^{15}N	Uniform NH	POPC (P/L = 1:237)	S-state
[61]	Alamethicin	^{15}N	Uniform NH	POPC (P/L = 1:15)	I-state
[13]	WALP19	^{2}H	Specific Ala-d_3	DMPC	Transmembrane
[13]	WALP19	^{2}H	Specific Ala-d_3	DOPC	Transmembrane
[109]	WALP21	^{2}H	Specific Ala-d_3	DMPC	Transmembrane
[109]	WALP21	^{2}H	Specific Ala-d_3	DOPC	Transmembrane

but above a certain critical peptide concentration P/L* they started to tilt into the membrane and in some cases eventually reached an I-state. In the case of melittin in DOPC, P/L* was found to be around 1:100, and with the addition of lyso-OPC, P/L* was reduced proportionally to the amount of lyso-OPC. On the other hand, upon the addition of DOPE, P/L* was shifted to higher concentrations [68]. It can be noted that for melittin in DOPC the I-state was never reached, not even at high P/L and high amounts of lyso-OPC, which fits perfectly to the curvature concept above. Likewise, when alamethicin was embedded in DPhPC bilayers, P/L* was found to be around 1:60. With the addition of 25% lyso-OPC, P/L* was reduced to around 1:200. On the other hand, upon the addition of DPhPE, P/L* was shifted to higher concentrations [68].

On the basis of these OCD studies, our own NMR studies, and other NMR studies as summarized in Tables 2 and 3, it seems that the spontaneous curvature hypothesis holds for all peptide systems studied so far. Fig. 3A illustrates schematically how amphiphilic α-helical peptides are engaged in the process of (i) binding to the membrane surface, (ii) possibly dimerizing in-plane, and (iii) cooperatively inserting into the bilayer to form an oligomeric transmembrane pore. The position of this multi-stage equilibrium is governed by the spontaneous curvature of the lipid bilayer (all other factors being equal). We propose that the same will also hold for other cationic amphiphilic α-helical peptides. It would be interesting now to find out whether this is also true for other types of membrane-active peptides and amphiphilic proteins. In the next sections we will thus look at others classes with completely different structures.

3. Studies of β-stranded peptides

There are only few studies of the alignment behavior of β-structured peptides in membranes. The orientation of such peptides cannot be determined in a straightforward manner as for α-helices, because the ^{15}N-NMR tensor is not aligned along the peptide long axis, and there is no simple theory to link the OCD spectral shapes directly to the molecular orientation. Nevertheless, for a couple of systems there is some data available. The cyclic β-stranded antimicrobial peptide gramicidin S was shown with solid-state ^{19}F-NMR to insert into DLPC and DMPC bilayers, which have a positive spontaneous curvature, but not into

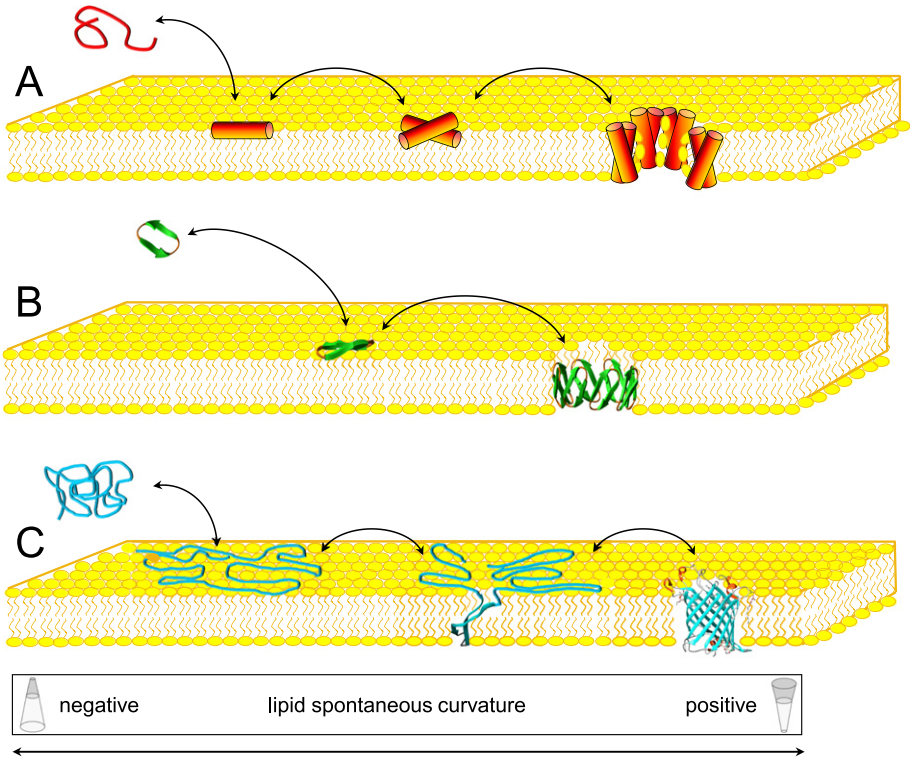

Fig. 3. Insertion of AMPs and OMPs is facilitated in membranes composed of lipids with positive spontaneous curvature, which is related to the shape of the lipid molecules. (A) Amphiphilic α-helical peptides always remain on the membrane surface when bound to lipids with a negative spontaneous curvature, probably as monomers. With increasing positive spontaneous curvature, more deeply inserted orientations are found, in which peptides presumably form dimers and higher oligomers. (B) The cyclic β-stranded peptide gramicidin S has also been shown to insert only into membranes with positive spontaneous curvature. (C) Also for β-barrel proteins the folded inserted state is promoted in lipids with positive spontaneous curvature. The example illustrated here is OmpLa, and the figure of the folded protein was made in Chimera [110] using Protein Data Bank file 1DQ5 [111].

membranes containing PE lipids [35]. This equilibrium of binding and re-alignment into a pore is schematically illustrated in Fig. 3B. Also the β-hairpin peptide protegrin-1 was studied using OCD, and indeed a higher proportion of inserted peptides was found in DPhPC compared to DPhPC/DPhPE (at the same P/L ratio and hydration level), showing again that positive spontaneous curvature promotes an inserted state [73]. Therefore, the role of spontaneous curvature seems to be valid also for other types of membrane-active peptides that are not α-helical.

4. Studies of β-barrel outer membrane proteins

Outer membrane proteins (OMPs) represent a distinctive class of proteins that form transmembrane β-barrels in the outer membranes of Gram-negative bacteria. At first sight, they are completely different from the amphiphilic α-helices discussed above, as they are much larger, have an entirely different secondary structure, and have a very different evolutionary origin. However, in both cases the peptides/proteins consist of amphiphilic segments, which assemble into transmembrane pores or channels, so both types of molecules must insert some charged and polar residues into the hydrophobic core of the membrane to become active. Here, we will consider only the un-assisted folding and membrane-insertion of OMPs into membranes, to find out whether there are any similarities that are dictated by the physicochemical properties of the lipid bilayer.

In several studies, unfolded OMPs have been shown to spontaneously fold and insert into lipid vesicles, as recently reviewed by Mitchell [74]. When unfolded protein is added to the vesicles in solution, it can often be observed to become reconstituted into the membranes with a proper β-barrel structure. In these experiments, the proportion of folded and unfolded protein is quantitatively determined by SDS-PAGE, as the two species show up as different bands. The initial binding of an unfolded OMP to the membrane surface must be mediated by the local amphiphilic character of its β-strands, which typically have a consensus sequence of alternating polar and nonpolar residues. Fig. 3C illustrates schematically how one or more hydrogen-bonded hairpins of the polypeptide chain must then (cooperatively) reach across the membrane to form the correct β-barrel fold.

In an early study, OmpA from *Escherichia coli* was solubilized in detergents and reconstituted into DMPC small unilamellar vesicles (SUVs), where it was shown to have the correct fold and to be active [75]. In a later study, OmpA was unfolded in water/urea, and refolding was induced by dilution of urea and the simultaneous addition of DMPC SUVs. Also in this case the native β-barrel structure of the protein was observed [76]. In another study of OmpA, large unilamellar vesicles (LUVs) were used, which have a curvature more similar to that of cell membranes. The protein was found to insert and take on its native fold in LUVs in a membrane thickness dependent fashion. The highest degree of folding was observed in di-10:0-PC, and also in di-11:0-PC and di-12:0-PC (DLPC) some folding occurred, whereas no folding was seen in di-14:0-PC (DMPC) and di-18:1-PC (DOPC) LUVs [77].

In another study, OmpA was reconstituted in POPC/POPG (12.3:1) where some POPC was replaced with other lipids [78]. It was found that the free energy of unfolding, ΔG_u, decreased when short-chain unsaturated PC lipids were added, while it increased for longer-chain PC. Folding was clearly enhanced in thin membranes, but can this effect be attributed to the intrinsic lipid curvature? Remarkably, for saturated and unsaturated chains of similar hydrophobic thickness, L_h (like di-14:1-PC with $L_h = 19.2$ Å, and di-12:0-PC with $L_h = 21.0$ Å), the unsaturated lipids were found to destabilize the folded state, even though they had slightly shorter chains. Also when POPE was added, ΔG_u increased, showing that lipids with a negative spontaneous curvature were indeed unfavorable for folding, as postulated above. A more recent study of Opa60 and Opa50, two 8-stranded β-barrel OMPs from *Neisseria gonorrhea* MS11, showed a similar behavior [79]: in a series of saturated PC lipids, more folding was observed for those with shorter chains. However, when DPoPC (with $L_h = 23.0$ Å) was added to DMPC (with $L_h = 25.4$ Å), folding was reduced, indicating that again the negative spontaneous curvature of the unsaturated DMoPC had reduced the folding despite its decreased membrane thickness.

A very systematic folding study used nine different β-barrel OMPs from *E. coli* with sizes from 8 to 16 β-strands, and a wide variety of lipid systems was investigated [80]. Using large unilamellar vesicles, it was initially found that in POPE/POPG (3:1), mimicking the *E. coli* membrane, almost no folding was observed, while in DLPC most OMPs folded strongly. In this case, the PE/PG system has both longer chains and a negative spontaneous curvature. In an extended experiment with a series of saturated PC lipids, folding was then found to be favored in shorter lipids, with the least folding seen in DMPC and most in di-10:0-PC. The same trend was found for unsaturated chains, with almost no folding in DOPC, some folding in DPoPC, and even more folding in DMoPC. All these findings were interpreted by the fact that the energy barrier for insertion is higher in thicker membranes, as would be expected, since charged residues have to be taken through the membrane to the other side. However, the odd deviations can be elegantly explained now by the effect of lipid curvature. When the hydrophobic thickness is compared between saturated and unsaturated chain PC lipids, it is clear that for a similar thickness there is more folding in saturated lipids. Namely, di-11:0-PC (with $L_h = 18.8$ Å) gives much more folding than di-14:1-PC (which has almost the same hydrophobic thickness of $L_h = 19.2$ Å), and di-14:0-PC ($L_h = 25.4$ Å) gives much more folding than di-16:1-PC ($L_h = 23.0$ Å), even though the latter has a smaller hydrophobic thickness [80]. Since the spontaneous curvature of saturated PC lipids is positive, and that of unsaturated PC lipids is negative, we can clearly see here the effect of spontaneous curvature.

Folding experiments were also done in DLPC LUVs with differing amounts of DLPE, which should not change the hydrophobic thickness but only the spontaneous curvature. In most cases, less folding was seen with a higher amount of PE, indicating that folding was inhibited by the increase in negative spontaneous curvature induced by the PE lipids. No such effect was seen when DLPG was added to the DLPC LUVs; for some OMPs more, for others less folding was observed in this case upon addition of charged PG lipids [80].

In nature, OMPs require special assembly machineries to fold into their native bacterial membranes, despite being capable of folding quickly and efficiently in suitably chosen lipid systems *in vitro*. Obviously, these proteins can fold more easily in model systems with short acyl chains and positive curvature, compared to their natural environment, in which spontaneous insertion would be rather unfavorable. Therefore, it is not surprising that many OMPs utilize a special "β-barrel assembly machinery subunit A" (BamA), the central subunit of the BAM complex (or analogous systems), which accelerates OMP folding by lowering the kinetic barrier imposed by the PE head groups in the outer bacterial membrane [81]. The same study also showed that PE lipids, with a negative spontaneous curvature, inhibit OMP folding.

From these results it is clear that the spontaneous curvature has an important effect on the membrane interactions not only of amphiphilic α-helical peptide but also of β-barrel proteins. In both cases, the energy barrier is lower for the peptides to insert into membranes with a positive spontaneous curvature. The thickness of the membrane also plays a role, which is well known for the β-barrel proteins and has also been noted for cationic α-helical peptides [82]. However, as shorter acyl chains exhibit an increased effect of positive curvature (i.e. they have smaller values of R_0, such as +50 Å for DMPC [48] compared to +147 Å for DPPC [46]), the spontaneous curvature can be considered as the dominant parameter.

In summary, Fig. 3 illustrates the different states of membrane-active peptides and outer membrane proteins, showing which side of the equilibrium is promoted by lipids with positive or negative spontaneous curvature. Amphiphilic α-helices (Fig. 3A) bind to the membrane surface and will remain there in lipids with negative spontaneous curvature, even at high peptide concentration, whereas they can get inserted in a tilted or transmembrane orientation in lipids with positive

spontaneous curvature. The cyclic β-stranded gramicidin S has a well-defined structure already in solution and binds to the membrane surface according to its amphiphilic character. At high concentration it can insert into membranes, but only when they are composed of lipids with a positive spontaneous curvature (Fig. 3B). OMP sequences (Fig. 3C), which also have a pronounced amphiphilic character along their individual β-strands, initially bind to the membrane surface in a two-dimensionally "disordered" state. In lipids with a negative spontaneous curvature they will remain there or become aggregated, whereas they can favorably insert and obtain their correct fold in lipids with a positive spontaneous curvature.

5. Conclusions

We have demonstrated that peptides and proteins can insert more readily into membranes composed of lipids with a positive spontaneous curvature, compared to lipids with a negative spontaneous curvature. This is the case, both, for amphiphilic antimicrobial peptides (AMPs) with either α-helical or β-stranded structures, as well as for β-barrel outer membrane proteins (OMPs), at least for the examples discussed here, and most likely also for other similar peptides and proteins. In the light of these findings, we propose that it will be more interesting and illustrative to perform studies of membrane-bound peptides and proteins in lipid systems with positive spontaneous curvature, like DMPC/DMPG, because membrane insertion processes and the resulting structures can be comprehensively studied only under these conditions. Even though bacterial membrane compositions tend to be more similar to POPE/POPG model bilayers, the latter systems with an intrinsic negative curvature do not favorably permit membrane insertion *in vitro*. In nature, this physico-chemical disadvantage is of course outweighed by the strong concentration gradient of the incoming peptide, and transient pores may be all that is needed to fulfil their biological function.

Some examples of relevant biophysical experiments may include the following. Given the assumption that an inserted cationic α-helical peptide (e.g. in the I-state) can be taken as a genuine sign of pore formation, it would be interesting to make modifications on this peptide to see whether this leads to an abolished I-state, as this would mean the peptide is no longer able to form pores. At the same time, a more stable I-state could indicate a higher pore-forming propensity and thus a more potent AMP candidate. Even if only a small population of peptides actually form the pores *in vivo*, their actual structures should then be responsible for the desired antimicrobial effect. However, in bacterial-type POPE/POPG model membranes the peptides are always found in an S-state, as documented above, and any promising changes in their propensity to reach an I-state cannot be noticed. Therefore, it is more appropriate to examine changes in the I-state propensities in DMPC/DMPG model membranes, where the peptides can insert more easily. The actual proportion of peptides in the I-state is probably much higher in this model system than in a native bacterial membrane, but all that matters is the fact that the I-state can be observed at all. On this basis, it is then possible to compare different peptides in terms of their pore-forming potential, and to develop more active peptides. It may even be useful to add lyso-lipids to this system, in order to further increase the positive spontaneous curvature and make the I-state even more accessible. Also in future experiment on β-barrel proteins it would make sense to reconstitute them in lipid vesicles with a strong positive spontaneous curvature and short chains. So far, to the best of our knowledge, no study of β-barrel proteins has ever actively included lyso-lipids or detergents, but we predict that this would further promote and stabilize the folded state. It is even conceivable that the effect of incomplete detergent removal has—unwittingly—lead to more stable samples of OMPs compared to fully purified proteins.

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