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Extending the Hydrophobic Mismatch Concept to Amphiphilic Membranolytic Peptides

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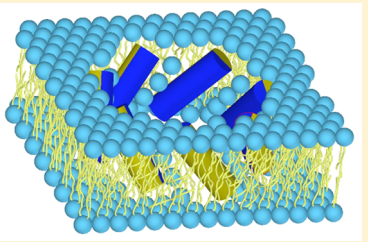
Supporting Information

ABSTRACT: A series of nine amphiphilic, pore-forming α-helical KIA peptides (KIAGKIA repeats) with lengths between 14 and 28 residues were studied by solid-state ^{15}N NMR to determine their alignment in oriented lipid bilayers. In a 2:1 mixture of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylglycerol (DMPC) with its corresponding 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lyso-MPC), which has a highly positive spontaneous curvature, the helix tilt angle was found to vary steadily with peptide length. The shortest peptide was aligned transmembrane and upright, while the longer ones successively became tilted away from the membrane normal. This behavior is in agreement with the hydrophobic matching concept, conceived so far only for hydrophobic helices. In 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine, with a negative spontaneous curvature, all KIA peptides remained flat on the bilayer surface, while the cylindrical DMPC lipids permitted a slight tilt. Peptide insertion thus depends critically on the intrinsic lipid curvature, and helix orientation is then fine-tuned by membrane thickness. A refined toroidal pore model is proposed.



Hydrophobic matching is a well-known concept that has been widely applied to hydrophobic transmembrane segments of proteins.[1] However, its potential relevance for amphiphilic helices has been largely ignored, because many other parameters limit the ability of an amphiphilic peptide to insert into a lipid bilayer in the first place. These include the peptide-to-lipid ratio,[2−5] temperature,[6] sample hydration,[7] pH,[8] lipid composition,[9−11] as well as peptide amphiphilicity and interfacial activity.[12] Among the various physical properties of the bilayer, the spontaneous curvature of the lipids has been recently suggested to play a pivotal role in allowing peptides to flip from a surface-bound to a tilted or inserted transmembrane state.[9−11] Here, we have utilized bilayers with a highly positive spontaneous curvature to promote the ability of amphiphilic peptides to insert and assemble as stable toroidal pores. These data prove that (i) the intrinsic lipid curvature is a critical parameter that allows membrane insertion to occur (rather than membrane thickness) and (ii) once inserted, the helix tilt angle is governed by hydrophobic mismatch, as had been described only for hydrophobic segments so far.[13,14] Bilayer thickness thus determines only the "fine-tuning" of the helix tilt angle, and based on these findings, a new and refined model of a toroidal pore can be presented.

A series of nine ideally amphiphilic α-helical peptides, called KIA peptides (whose sequences consist of KIAGKIA repeats, see Table 1), were synthesized with lengths between 14 and 28 amino acids and were shown to be highly helical when bound to membranes (Table 1).[15] We had previously found that these peptides show antimicrobial and hemolytic activity and can induce leakage in small unilamellar vesicles depending on peptide length. Leakage is induced only when the length of the peptide is at least as long as the hydrophobic thickness of the membrane, indicating that the mechanism of action invokes genuine pore formation with peptides spanning the membrane, with the peptide axis parallel to the membrane normal.[15]

The orientations of the KIA peptides in membranes were determined by solid-state ^{15}N NMR in aligned samples, as previously described.[15] The helix tilt angle was estimated from the chemical shift of a ^{15}N-Ala label at position 10 in the backbone,[9,16] and ^{31}P NMR was used to monitor any perturbations of the phospholipid bilayers. The NMR experiments were performed in different lipid systems with varying spontaneous curvature, using typically 1 mg of labeled peptide per sample. Considering the different lengths of the KIA peptides, their concentrations were chosen such that the number of amino acid residues per lipid was kept constant, with a peptide-to-lipid molar ratio (P/L) of 1:50 for KIA21 as a reference point. When bound to the membrane surface, all peptides should thus cover a comparable area and cause a similar degree of membrane perturbation.

The NMR spectra in Figure 1A show that all peptides gave a peak close to 90 ppm in 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC), indicating a flat, surface-bound state. In a mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) with the anionic 1-palmitoyl-2-oleoyl-*sn*-glycero-

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Table 1. Peptides Used in This Study[a]

peptide	sequence	helicity (%)[b]	P/L[c]
KIA14	KIAGKIA KIAGKIA-NH_2	74.1	1:34
KIA15	KIAGKIA KIAGKIA K-NH_2	68.7	1:37
KIA17	KIAGKIA KIAGKIA KIA-NH_2	82.7	1:41
KIA19	KIAGKIA KIAGKIA KIAGK-NH_2	81.7	1:46
KIA21	KIAGKIA KIAGKIA KIAGKIA-NH_2	83.0	1:50
KIA22	KIAGKIA KIAGKIA KIAGKIA K-NH_2	67.7	1:54
KIA24	KIAGKIA KIAGKIA KIAGKIA KIA-NH_2	72.6	1:58
KIA26	KIAGKIA KIAGKIA KIAGKIA KIAGK-NH_2	71.1	1:62
KIA28	KIAGKIA KIAGKIA KIAGKIA KIAGKIA-NH_2	76.2	1:66

[a]The underlined Ala-10 was labeled with ^{15}N in the backbone amide. [b]Percentage α-helix according to a deconvolution of circular dichroism spectra.[15] [c]Peptide-to-lipid molar ratio in samples with a constant peptide-to-lipid mass ratio.

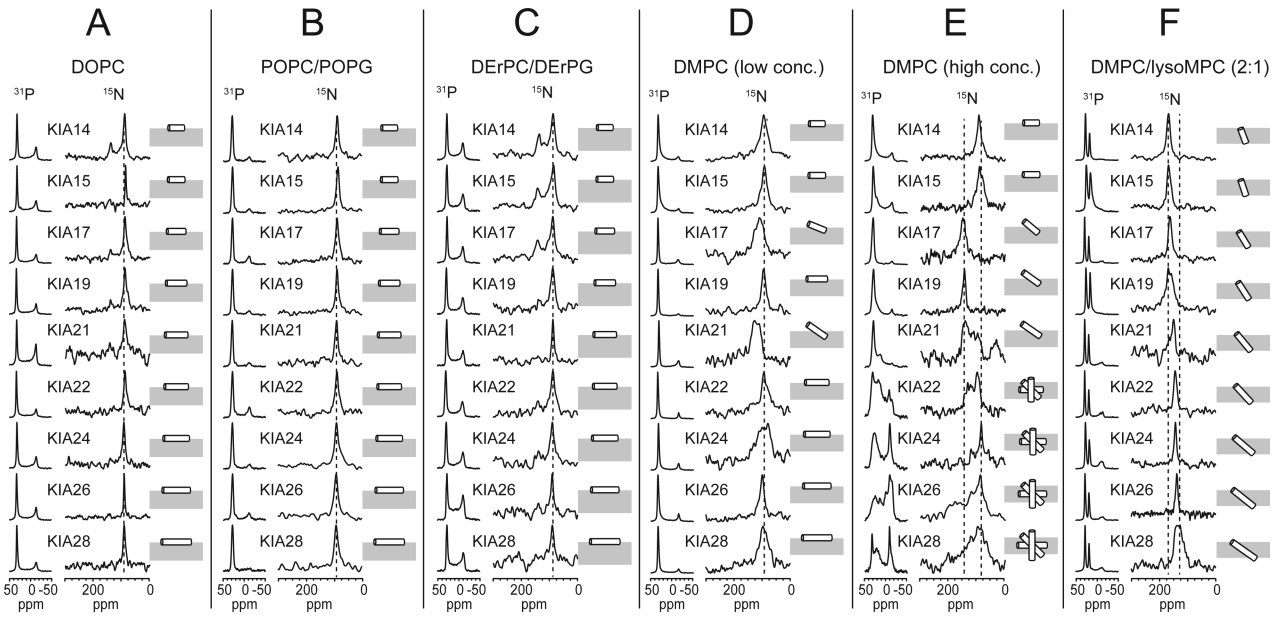

Figure 1. Solid-state ^{31}P and ^{15}N NMR of KIA peptides with varying lengths, measured in oriented samples composed of lipids with different spontaneous curvatures. (A) DOPC (20 mg, 1.2 mg peptide); (B) POPC/POPG (6.5 mg each, 0.8 mg peptide); (C) DErPC/DErPG (10 mg each, 1.1 mg peptide); (D) DMPC (15 mg, 1.1 mg peptide); (E) DMPC at a high P/L ratio of 1:20; (F) DMPC/lyso-MPC (2:1) (12 mg DMPC, 5 mg lyso-MPC, 1.2 mg peptide). Except for column E, the peptide-to-lipid mass ratio was kept constant (and corresponds to a commonly used molar P/L ratio of 1:50 for the medium-length KIA21). Vertical dotted lines are included in the spectra to guide the eye, and the estimated peptide orientation is illustrated for each case.

3-phosphatidylglycerol (POPG) (1:1), the same behavior was found (Figure 1B). It has been suggested that negative spontaneous curvature (i.e., voluminous acyl chains with relatively small head groups) is unfavorable for peptide insertion.[10] Hence, it is not surprising that none of the KIA peptides would insert into these lipids.[17] Also in 1,2-dierucoyl-*sn*-glycero-3-phosphatidylcholine (DErPC)/1,2-dierucoyl-*sn*-glycero-3-phosphatidylglycerol (DErPG) (1:1), with long unsaturated chains (22 carbon atoms), no insertion was observed. All these lipid systems support our previous findings that amphiphilic peptides (in which multiple cationic residues evenly distributed along the length of the helix) generally remain surface-bound in unsaturated PC bilayers.[9,10,18]

In 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylglycerol (DMPC), a lipid with a small positive spontaneous curvature, the KIA peptides were also found to lie more or less flat on the membrane surface (Figure 1D). Only a slight insertion was expected, and indeed the peaks of KIA17 and KIA21 are seen to be shifted to ~120 ppm. Samples were therefore also prepared with a higher P/L of 1:20 to promote overall insertion. This set of data shows a clear dependence on peptide length, as seen in Figure 1E. For KIA14 and KIA15, the signal at 90 ppm indicates a surface orientation, while KIA17 and KIA19 with a chemical shift around 150 ppm exhibit a more inserted state. KIA21 and KIA22 gave broad line shapes, where the signal at 150 ppm represents inserted peptides, while the 90 ppm peak arises from large unoriented aggregates that give rise to a powder spectrum with a 90 ppm maximum. KIA24 also has a maximum around 90 ppm, yet both KIA24 and KIA26 show very poor orientation in the membrane, as seen from the ^{31}P spectra (Figure 1E). KIA26 and KIA28, finally, gave mostly powder spectra in both ^{31}P and ^{15}N NMR , indicating that these peptides at such high P/L ratio formed immobilized aggregates, which perturbed the membranes and destroyed the bilayer orientation.

After comparing these conventional lipids, which do not tend to readily support peptide insertion, we prepared another series of samples with DMPC and its corresponding lyso-lipid (1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, lyso-MPC), which has a very high positive spontaneous curvature, at a

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2:1 molar ratio. This optimized lipid mixture facilitated insertion of the KIA peptides remarkably well, even at low concentration (using the same mass of peptide in each sample, as above). Under these conditions, we observed for the first time an exemplary length-dependent realignment of amphiphilic peptides. Namely, a distinct, gradual change in the ^{15}N chemical shift is seen in Figure 1F, without any indication of problematic peptide aggregation or membrane perturbation, as confirmed by ^{31}P NMR. The shortest peptide, KIA14, gave a signal at 167 ppm, indicating a fully inserted transmembrane state in which the helix axis is essentially parallel to the membrane normal (much more upright than what was seen in DMPC, Figure 1E). The longer peptides then showed gradually smaller chemical shifts down to 134 ppm for KIA28. This series indicates that the shortest peptides are almost fully upright in the membrane, while the longer ones exhibit a steady decrease in tilt angle, until the longest ones end up in an obliquely tilted state. The latter scenario resembles that of KIA21 (also called MSI-103) in DMPC at P/L = 1:50, where a tilt angle of 125° was previously found by ^{2}H NMR.[9,10,19]

The same type of tilting response has been reported and extensively discussed for completely hydrophobic, designated transmembrane segments.[13,14] The new data suggest that also for amphiphilic peptides a larger tilt angle seems to be necessary for the longer helices to avoid hydrophobic mismatch. A more detailed evaluation of the quantitative relationship between chemical shift and peptide orientation, supporting the conclusions and illustrations drawn here, can be found in the Supporting Information. We briefly note that our samples were also measured at a vertical alignment, showing that the peptides rotated rapidly around the membrane normal; this was the case for both long and short peptides in all lipid systems (see Figure S1 in the Supporting Information). Such behavior had been previously observed for surface-bound and tilted amphiphilic peptides[9,10,20] but was unknown so far for their oligomeric pore-forming transmembrane states. A fast rotation around the membrane normal has also been observed for other types of membrane-spanning peptides in liquid crystalline lipid bilayers.[21−23]

Interestingly, in DMPC the abrupt change in chemical shift upon going from KIA15 (90 ppm) to KIA17 (150 ppm) indicates that the peptides can get inserted only provided that they are long enough to span the membrane. This observation suggests that membrane lysis by these amphiphilic peptides invokes the formation of genuine oligomeric transmembrane pores.[15] In a recent study, the orientations of KIA peptides found from ^{15}N NMR did not correlate directly with membrane leakage in unsaturated lipids,[15] but they could nevertheless be reconciled with activity. Leakage occurs only when pores are formed, but when a small population of peptides is assembled as transient, short-lived pores, this is already sufficient to cause considerable leakage. On the other hand, ^{15}N NMR provides a steady-state, time-averaged picture of all peptides, and if only a small fraction exists in the transmembrane state at any given time, these pores are hardly visible in the NMR spectra. Here, the unconventional DMPC/lyso-MPC lipid mixture allowed the entire peptide population to insert into the membrane and to evidently assemble as stable toroidal pores, which could be conveniently studied using solid-state NMR. Under these conditions, the α-helical KIA peptides were found to readily adjust their tilt angle according to their respective lengths, in full agreement with the concept of hydrophobic matching. This study demonstrates that the straightforward biophysical description of hydrophobic mismatch not only applies to transmembrane protein segments but also can now be extended toward amphiphilic peptides.

The pores formed by KIA peptides are most likely of the toroidal type which has been proposed for similar amphipathic peptides like magainin 2,[24,25] with the hydrophilic face of the peptides lining the pore, and lipid head groups being interspersed between the peptides (Figure 2). As discussed in the Supporting Information, the ^{31}P NMR spectra are compatible with such pores (see Figure 1, and Figure S2). The mean curvature in the interior of a toroidal pore can be rather small, corresponding to a saddle with a negative Gaussian modulus. It has nevertheless been suggested that lipids with a positive spontaneous curvature, such as the lyso-lipids used here, are preferentially localized in these regions, facilitating and stabilizing toroidal pores.[26−28] It was originally suggested that negatively charged lipid head groups are necessary inside the pore to neutralize the positive charges of the peptides and to counteract their electrostatic repulsion,[25] but recent results indicate that this is not the case. For example, in pure PC lipid systems, we have observed the cationic amphiphilic antimicrobial peptide PGLa in a distinct transmembrane orientation, assembled with magainin 2 into a pore-forming complex,[9] and here we also find KIA peptide pores in PC/lyso-PC lipids, where no negatively charged lipids are present. A recent molecular dynamics simulation also indicated that pores of

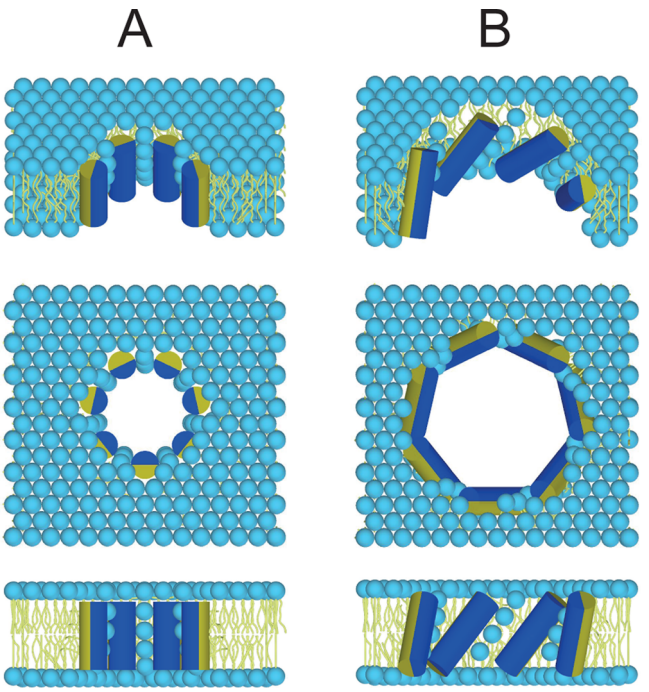

Figure 2. Model of a transmembrane toroidal pore formed by KIA peptides. The lipids are represented with light blue head groups and yellow acyl chains. The peptides are shown as cylinders with one hydrophobic face (dark blue) and one polar, hydrophilic face (gold). The inside of the pore is lined by the polar faces of the peptides and presumably also by lipid head groups that are intercalated between the helices. (A) Short peptides matching the hydrophobic thickness of the membrane have a straight upright orientation. (B) Longer peptides adapt to the membrane thickness by tilting, forming an iris-like ring of helices lining the pore, presumably together with some lipid head groups.

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melittin were more stable in DMPC than in DMPC/DMPG bilayer, in line with experimental results.[29]

The idea of hydrophobic mismatch was conceived for "classical" transmembrane segments of integral membrane proteins, i.e., when the helices are completely hydrophobic (and obviously have no pore-forming function). This concept was found to apply ideally when a single hydrophobic peptide crosses the membrane, while helix bundles in polytopic membrane proteins tend to interact with one another to stabilize their individual tilt angles.[13,30] In the case of the amphiphilic pore-forming peptides, however, a soft oligomeric assembly has to be considered, where the individual components can readily slide along each other like the segments of an iris, "greased" by the intercalated lipid head groups.

Depending on the global or local point of view, the (global) "transmembrane" alignment of the KIA peptides within the pore is fully consistent with the statement that the peptides are at the same time (locally) floating on the amphiphilic "surface" of the lipid monolayer lining the toroidal wormhole. If the peptides were free to diffuse within the continuous monolayer leaflet, one might expect that the helices could assume any tilt angle simply by shifting their position along the surface within the toroidal pore. However, this is not observed by NMR, because there is not a broad distribution of NMR signals. Instead, a single sharp peak (Figure 1F) with a characteristic chemical shift is observed, indicating that all peptides have very similar orientations and that they do not undergo averaging by rapid passages across the toroidal pore. It thus seems there are two stable orientations for KIA peptides in lipid bilayers: either flat on the (global) membrane surface or more or less tilted within the center of the pore, but nothing in between. This would fit with a "classical" toroidal pore as illustrated for example by Brogden.[31] Under the equilibrium conditions of the NMR experiment, we conclude that the ideally amphiphilic α-helical KIA peptides are able to assemble into a stable pore complex, containing an unknown number of helices, as shown in Figure 2. When the peptides are just long enough to span the bilayer, they are aligned fully upright with the helix axis parallel to the membrane normal (Figure 2A). When the peptides are longer (Figure 2B), they will tilt within the complex like an iris, such that the hydrophobic residues (e.g., Ile-2) are still retained within the hydrophobic region of the bilayer. This refined toroidal pore model can account for the length-dependent differences in helix tilt angles, observed here for the first time in a suitable lipid matrix. In these pores, peptides have a global transmembrane orientation along the membrane normal and their tilt angle depends on the length of the helix and the hydrophobic thickness of the bilayer, in a similar way as the tilt of hydrophobic helices is known to depend on hydrophobic matching with the membrane. This mismatch dependence of amphiphilic helical peptides had not been observed previously.

By using lipids with a high positive spontaneous curvature, we have shown that it is possible to stabilize transmembrane pores made up of amphiphilic peptides. In most other lipid systems and under biological conditions, such pore structures should be just as plausible, but are obviously only short-lived. These transient events are hard to study with biophysical methods, unless lipids with a high positive spontaneous curvature are included, such as the lyso-MPC in the present case. The use of lipids with a high positive spontaneous curvature had so far been explored only sporadically.[18] The present study implies that the simple idea of including lyso-lipids should be rather useful in future studies, where other putative pore-forming amphiphilic peptides need to be characterized and compared in a stable transmembrane state.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.6b00136.

Experimental methods, a discussion of peptide orientations determined from ^{15}N chemical shifts, data on rotational diffusion of peptides, and an analysis of ^{31}P NMR data (PDF)

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Notes

The authors declare no competing financial interest.

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