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Lipid saturation and head group composition have a pronounced influence on the membrane insertion equilibrium of amphipathic helical polypeptides

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Key words: helix topology, lipid order parameter, lipophobic effect, hydrophobic mismatch, molecular shape, magainin, PGLa, solid-state NMR, supported lipid bilayer

Running title: lipid unsaturation drives helix insertion

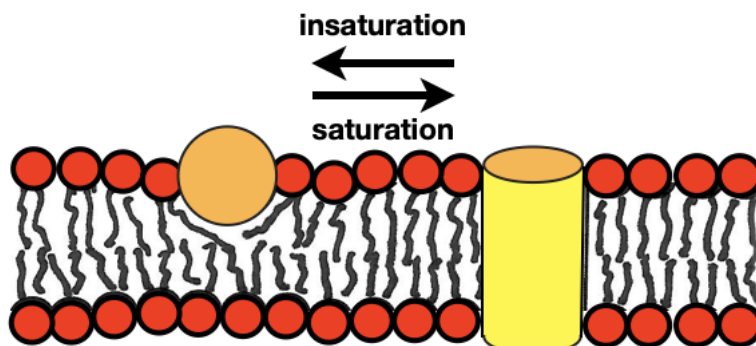
Abstract

The histidine-rich peptides of the LAH4 family were designed using cationic antimicrobial peptides such as magainin and PGLa as templates. The LAH4 amphipathic helical sequences exhibit a multitude of interesting biological properties such as antimicrobial activity, cell penetration of a large variety of cargo and lentiviral transduction enhancement. The parent peptide associates with lipid bilayers where it changes from an orientation along the membrane interface into a transmembrane configuration in a pH-dependent manner. Here we show that LAH4 adopts a transmembrane configuration in fully saturated DMPC membranes already at pH 3.5, i.e. much below the pK_a of the histidines whereas the transition pH in POPC correlates closely with histidine neutralization. In contrast in POPG membranes the in-planar configuration is stabilized by about one pH unit. The differences in pH can be converted into energetic contributions for the in-plane to transmembrane transition equilibrium, where the shift in the transition pH due to lipid saturation corresponds to energies which are otherwise obtained by the exchange of several cationic with hydrophobic residues. A similar dependence on lipid saturation has also been observed when the PGLa and magainin antimicrobial peptides interact within lipid bilayers suggesting that the quantitative evaluation presented in this paper also applies to other membrane polypeptides.

Highlights

- *Lipid saturation strongly drives the interface-to-transmembrane realignment
- *The energetic contribution of lipid saturation corresponds to removing numerous charges from the amino acid sequence
- *Anionic lipids stabilize the interfacial localization of cationic amphipathic peptides.

TOC graphics



Abbreviations used

ATR FTIR	attenuated total reflection Fourier transform infra red
CP	cross polarization
DMPC	1,2-dimyristoyl- <i>sn</i> -glycero-3-phosphocholine
DMPE	1,2-dimyristoyl- <i>sn</i> -glycero-3-phosphoethanolamine
IP	in-plane
MALDI-TOF	matrix-assisted laser desorption ionization – time of flight
NMR	nuclear magnetic resonance
PC	1,2-diacyl- <i>sn</i> -glycero-3-phosphocholine
PE	1,2-diacyl- <i>sn</i> -glycero-3-phosphoethanolamine
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
POPE	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphoethanolamine
POPG	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phospho-(1'-rac-glycerol) sodium salt
RP-HPLC	reversed-phase high performance liquid chromatography
TFA	trifluoroacetic acid
TFE	trifluoroethanol
TM	transmembrane

Introduction

Helical polypeptide sequences constitute important building blocks of membrane proteins [1-3] and membrane-associated peptides are also active as independent units [4, 5]. When polypeptide sequences interact with lipid bilayers they can adopt transmembrane or surface-associated orientations, with important consequences for their function [6]. These topologies exchange with each other through reversible chemical equilibria and topological variations that depend on the environmental conditions have been observed [7-10]. The topologies of membrane polypeptide domains have been tested qualitatively using accessibility measurements [11] or in a quantitative manner by biophysical methods such as ATR FTIR and solid-state NMR spectroscopies [12-14]. Biophysical experiments allow one to also determine intermolecular interactions, supramolecular arrangements and oligomerization of polypeptides within liquid crystalline bilayers. For example, biophysical studies have resulted in important insight into the functioning of peptides [15-18] and on the aggregation behavior of transmembrane or membrane-proximal sequences [19-21].

As expected, very hydrophobic sequences have been found to preferentially adopt transmembrane alignments whereas amphipathic sequences tend to interact with the interface of lipid bilayers [6, 22-25]. However, depending on the detailed conditions, alamethicin, a peptide devoid of any charges has also been found at surface orientations [26], and cationic amphipathic sequences have been observed in transmembrane configurations [8, 27].

This data are indicative that the membrane topologies of these sequences are not determined by the amino acid composition alone [28]. In contrast, there are many more interaction contributions that govern the equilibria of polypeptides where the location and topology changes from buffer \Leftrightarrow membrane interface (in-plane) \Leftrightarrow membrane inserted (transmembrane). These transitions are accompanied by structural transitions including the formation of folded structures when random coil sequences in aqueous solution adopt helical structures in the presence of lipid bilayers [29, 30]. Related topological transitions are also observed for beta-sheet structures [31, 32]. Furthermore, oligomerization and supramolecular organisation can occur which extends the simplified scheme mentioned above [10, 33]. The interactions that govern topological equilibria of helical sequences have been tested including Leu-Ala model sequences with lysines introduced at strategic positions, polyalanines, hydrophobic sequences of different lengths or membranes of different hydrophobic thickness [34-36].

When the membrane alignment of the antimicrobial peptide PGLa (Table 1) is investigated the peptide always aligns parallel to the surface of unsaturated membranes [8, 27,

37]. However, in fully saturated lipid bilayers the detailed tilt angle not only depends on the peptide-to-lipid ratio, the hydrophobic thickness and the bilayer hydration but also shows a pronounced dependence on the lipid composition [8, 38]. A wide range of angles is observed for PGLa which even adopts transmembrane orientations in the presence of magainin 2 (Table 1), another antimicrobial peptide produced by the same frog [8, 27, 37]. When studied in saturated membranes the magainin 2 sequence also shows a somewhat different tilt due to the presence of PGLa although the effects are much less pronounced than for PGLa in the same lipid/peptide mixture [39]. Notably, in an environment of saturated membranes the realignment of a protein domain in the presence of another could have regulatory functions [8] thus a better understanding of such processes is of potential biomedical importance.

TABLE 1: Sequences of peptides discussed in this paper

LAH4	KKALL ALALH HLAHL ALHLA LALKK A-NH ₂
magainin 2	GIGKF LHS AK KFGKA FVGEI MNS-NH ₂
PGLa	GMASK AGAIA GKIAK VALKA L-NH ₂

In the past much focus had been given to the composition of the lipid head group in order to explain how these peptides selectively interact with bacterial cell membranes [40]. Later on, the saturation of the lipid fatty acyl chains has been found to make a pronounced difference when the PGLa interactions with membranes are studied [8, 27, 37]. The LAH4 sequence was initially designed (Table 1) to better understand and test the orientation-dependence and activity of antimicrobial peptides [41]. Thereafter, the LAH4 peptides were also shown to exhibit potent antimicrobial and cell penetrating activities for nucleic acids, proteins, peptides, vaccines, nanodots, adeno associated viruses and more [42]. Furthermore, a derivative of the sequence is explored in clinical settings for its lentiviral transduction enhancement activities [43]. The LAH4 peptides are made up of a leucine-alanine hydrophobic core which is interrupted by four histidines. In an α -helical conformation these line up along one face of the helical surface thus an amphipathic structure is formed (Figure S1). At both termini two lysines each increase solubility and serve as interfacial anchoring residues. In solution the histidines are characterized by a pK_a around 6, therefore the hydrophobic moment and the membrane alignment of the LAH4 peptides is strongly pH dependent [12, 41].

Oriented ¹⁵N solid-state NMR and ATR FTIR spectroscopies of uniaxially oriented POPC lipid bilayers have demonstrated a reversible topological transition of LAH4 from an

in-planar ($\text{pH} < 5.5$) to a transmembrane state ($\text{pH} > 7$) with a midpoint of 6.1 ± 0.2 [12, 41]. Single amino acid substitutions have been shown to change transition pH and thereby have allowed the evaluation of the energetic contribution of individual amino acids when they move from the membrane interface to its hydrophobic interior [44, 45]. When more than one labelled site is explored more accurate tilt angle information has been obtained also for LAH4 at pH 5.3 [46].

In this paper, we investigated in quantitative detail how the transition of LAH4 is affected by the lipid composition, in particular its fatty acyl chain saturation. Therefore, the pH-dependent topological transition of this helical peptide was investigated in different membrane environments. In particular, the role of the lipid saturation and head group composition was analyzed by investigating in quantitative detail the transmembrane or in-planar alignment of LAH4 using ^{15}N solid-state NMR spectroscopy [47]. In parallel information on the membrane macroscopic phase properties and the packing of the lipid fatty acyl chains is obtained from ^{31}P and ^2H solid-state NMR spectra, respectively [48].

Materials and Methods

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) in its natural abundance or with the palmitoyl chain deuterated were purchased from Coger (Paris, France), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) and ^2H -depleted water ($<1\text{ppm}$) were from Sigma-Aldrich / Merck KGaA (St Quentin Fallavier, France). F-moc protected amino acids were from NovaBiochem / Merck KGaA (Darmstadt, Germany), isotope labeled amino acids from Cortecnet (Voisins les Bretonneux, France), Euriso-top (Paris, France) or Isotec® Sigma-Aldrich/Merck KGaA (St Quentin Fallavier, France), the TentaGel-S-RAM resin used during peptide synthesis was from Rapp Polymer GmbH (Tübingen, Germany).

The peptide sequence of LAH4 is KKALL ALALH HLAHL ALHLA LALKK A-CONH₂ where the underlined positions Ala6 and Ala16 were labelled with ^{15}N one at a time. The sequence was prepared by solid-phase peptide synthesis using a Millipore 9050 automatic peptide synthesizer and a four-fold excess of Fmoc protected amino acid as described previously [49].

The peptides were purified by reversed phase HPLC (Gilson, Villiers-le-Bel, France) using an acetonitrile/water gradient and a preparative C-18 column (Luna, C18-300Å-5μm, Phenomenex, Le Pecq, France). The identity and purity of the product ($> 90\%$) were analyzed

by HPLC and MALDI mass spectrometry (MALDI-TOF Autoflex, Bruker Daltonics, Bremen, Germany). Before usage the peptides were lyophilized from a solution in 4% acetic acid to exchange the trifluoroacetic acid counter ions [50].

Sample Preparation for ^2H -solid-state NMR measurements

Non-oriented samples were prepared by mixing POPC/POPC- d_{31} (4.3 mg/1.5 mg, 3/1) or DMPC/DMPC- d_{54} (4.3 mg/1.5 mg, 3/1) in trifluoroethanol (TFE). TFE was evaporated with a stream of nitrogen and exposure to high vacuum overnight such that a film forms along the walls of the glass tube (6 mm outer diameter). LAH4 (0.38 mg) in 140 μl milliQ water, or for comparison the same amount of water without peptide, was added to each vial, and the pH was adjusted to the desired value by addition of 50 mM solutions of either NaOH or HCl. Thereafter, the samples were lyophilized and the lipids suspended in 25 μl of the appropriate buffer by vortexing and bath sonication, followed by three freeze/thaw cycles at -20°C and 40°C , respectively, to produce multilamellar vesicles. The buffers were 100 mM Tris (pH 7.5), 100 mM acetate (pH 5.1), or 100 mM citric acid at either pH 5.0 or pH 3.0. For solid-state NMR measurements the samples were inserted into the solenoidal coil of the solid-state NMR probe. The spectra were recorded at 298K for POPC and 310K for DMPC samples.

Preparation of supported lipid bilayers for solid-state NMR spectroscopy

Typically, 3.7 mg of LAH4 labelled with ^{15}N at either the alanine 6 or alanine 16 position was dissolved in 300 μL milliQ water. The pH was adjusted by addition of a few microliters of 1 M solutions of NaOH or HCl. Thereafter the peptide solution was added to 55 mg lipid, vortexed, bath-sonicated for 5 minutes, exposed to three cycles between 40°C and -20°C . Thereafter, the pH was controlled and readjusted if necessary. The suspension was loaded onto 23 ultrathin glass plates (8 x 18 mm, thickness 00; Marienfeld, Lauda-Königshofen, Germany), left overnight under the hood to dry and equilibrated at 93% relative humidity at 37°C for two days. The pH value of the lipid/peptide samples was again tested from a small part of the sample that was mixed with 300 μl milliQ water. After equilibration, the glass plates were stacked on top of each other, wrapped with Teflon tape and sealed in plastic wrapping [50].

Solid-state NMR spectroscopy

Proton-decoupled ^{31}P solid-state NMR spectra at 121.576 MHz were recorded using a Bruker Avance wide-bore 300 solid-state NMR spectrometer equipped with a commercial double-

resonance flat-coil probe (Bruker, Rheinstetten, Germany). A Hahn-echo pulse sequence [51] was used where the $\pi/2$ pulse was 7 μ s, the spectral width 100 kHz, the echo delays 100 μ s, the acquisition time 10.2 ms, and the recycle delay 3 s. 85% H_3PO_4 at 0 ppm was used as an external reference. The temperature was set to 25 °C for POPC, and to 37 °C for DMPC and POPG samples. A Lorentz apodization of 50 Hz was applied before the Fourier transform.

^2H solid-state NMR spectra of deuterated POPC- d_{31} or DMPC- d_{54} were recorded by applying a quadrupolar pulse-echo sequence [52]. The recycle delay was 0.3 s, the echo time 100 μ s, the dwell time 0.5 μ s and the $\pi/2$ pulse 5 μ s. An exponential apodization with line broadening of 300 Hz was applied before Fourier transformation of the free induction decay. The temperature was set to 25 °C for POPC and 37 °C for DMPC samples.

Proton-decoupled ^{15}N cross-polarization (CP) spectra were recorded either at 76.016 MHz or at 30.432 MHz on Bruker Avance wide bore NMR spectrometers using a cross polarization pulse sequence and an e-free (at the 17.6 Tesla magnet [53]) or a standard (at 7 Tesla) flat-coil double-resonance probe [54]. The spectral width, acquisition time, CP contact time, and recycle delay time were 100 kHz, 3.9 ms, 0.4 ms, and 2 s, respectively. The B_1 field strength was 35 kHz during CP, for the ^1H $\pi/2$ pulse and the SPINAL-64 heteronuclear decoupling [55]. A Lorentzian apodization of 200 Hz was applied before the Fourier transform. An external reference of $^{15}\text{NH}_4\text{Cl}$ was used for calibration of the ^{15}N chemical shift scale (39.3 ppm; [56]). The temperature was set to 25 °C for POPC and 37 °C for DMPC samples.

Quantitative evaluation of the LAH4 topological transition

The transmembrane fraction was obtained from oriented ^{15}N solid-state NMR spectra by comparing the integrals corresponding to the transmembrane region (140 to 240 ppm) with the total signal intensity (40 to 240 ppm). The corresponding pH titration curves were analyzed according to [45]:

$$P_{TM} = \frac{[TM]}{[TM] + [IP^o] + [IP^{ch}]} = \frac{1}{1 + e^{\frac{\Delta G}{RT}} (1 + e^{2.3(pK_a - pH)})^4} \quad (\text{equation 1}).$$

The pK_a value was fixed to 6.1 except for POPG where an apparent pK_a of 7.1 was taken into consideration.

Deuterium order parameters

The deuterium order parameters (S_{CD}) of the CD_2 and CD_3 groups was calculated by dePake-ing [57] according to: $S_{CD}^i = \frac{4}{3} \frac{h}{e^2 q Q} \Delta^i \nu$, where $\Delta^i \nu$ is the quadrupolar splitting of segment i

and (e^2qQ/h) is the static quadrupole coupling constant (167 kHz) for C–D deuterons [58]. When establishing order parameter profiles, based on early studies of pure lipid bilayers labelled with $^2\text{H}_2\text{-C}$ at individual segments one at a time, it is assumed that the order parameters diminishes in a continuous fashion from the membrane interface to the membrane interior.

Statistical considerations: The quality of the spectra allows one to determine the quadrupolar splitting with about 1 kHz accuracy. This corresponds to an error of 0.01 for the order parameters and 0.014 for the relative order parameters. Here we compare order parameter profiles encompassing up to $n=13$ data points rather than individual order parameters. In this case Student's t can be calculated from the sample average (\bar{D}) and the standard deviation (S_d) of the pair-wise differences between corresponding points of two data sets according to $t = \frac{\bar{D}}{S_d} \sqrt{n}$, where n is the number of data points/differences [59]. From reference t -values it can be estimated that relative order parameters profiles that are at least 0.02 units apart are different with high probability.

Results

Here we investigated the effect of lipid fatty acyl chain and head group composition on the topological transition of the LAH4 peptide from in-planar to transmembrane alignments. In order to monitor peptide orientation relative to the membrane normal the LAH4 sequence was prepared with a single ^{15}N label at either the Ala-6 or the Ala-16 position. The peptide was reconstituted into supported lipid bilayers and studied by proton-decoupled ^{15}N solid-state NMR spectroscopy. When the sample is inserted with its normal parallel to the magnetic field direction of the NMR spectrometer ^{15}N chemical shifts < 100 ppm occur at helix alignments approximately parallel to the membrane surface (in-plane, IP state) whereas ^{15}N chemical shifts around 200 ppm are associated with transmembrane orientations (TM state). Because the chemical shift scale follows a continuous trend intermediate chemical shifts are observed at other tilt angles [47].

The ^{15}N solid-state NMR spectra of 2 mole% LAH4 in DMPC exhibit a predominant peak in the range of transmembrane alignments also at acidic pH with chemical shifts of 218 ± 11 ppm at pH 5.3 and 210 ± 10 ppm and pH 4.9 (Figure 1A,B), where the width of the spectral intensity at half-height is also indicated. When the pH is further lowered the spectrum at pH 3.8 reveals a close to equal distribution of two intensities at 202 ± 20 ppm and 77 ± 19

ppm (Figure 1C). At pH 3.0 a dominant intensity in the in-planar range (79 ± 16 ppm) (Figure 1D). The ^{31}P solid-state NMR spectra of the same samples exhibit predominant peaks around 30 ppm with intensities extending to -15 ppm indicative of phospholipid bilayers that are well aligned along the glass plates [48] despite the highly acidic pH and the orientational dispersion of the peptide (Figure 2A,B).

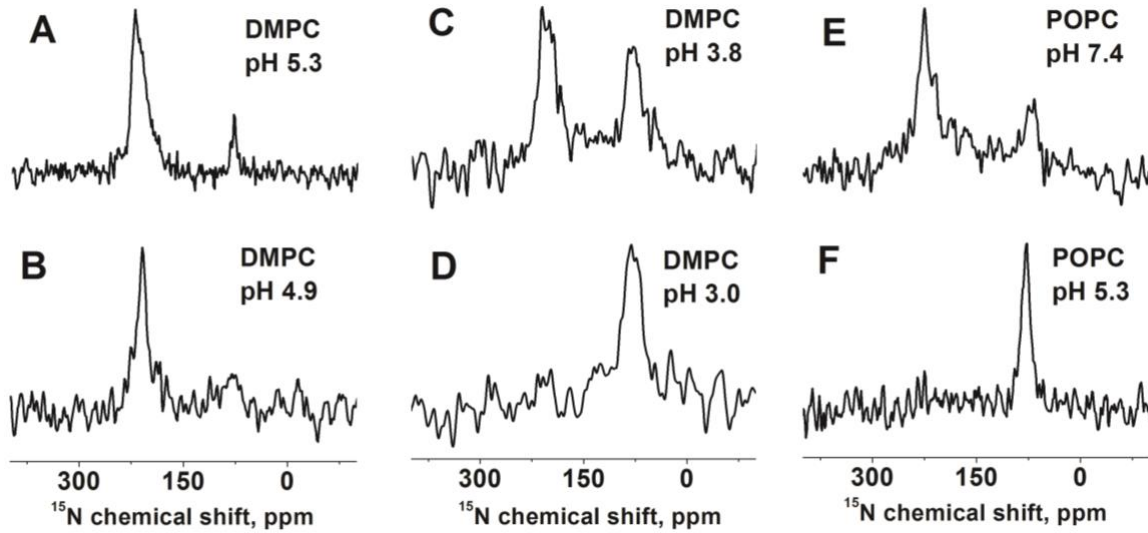


Figure 1: ^{15}N proton-decoupled spectra of 2 mole% ^{15}N -Ala16-LAH4 in mechanically supported DMPC (A-D) or POPC (E, F) bilayers. The pH was adjusted to 5.3 (A), 4.9 (B), 3.8 (C), 3.0 (D), 7.4 (E), and 5.3 (F). The sample normal is parallel to the magnetic field direction of the NMR spectrometer. The spectrum in panel E is from [60].

Because a large amount of data have already been obtained for LAH4 in POPC at different pH [12, 41, 60], here we only investigated a limited number of samples as control (Figure 1E,F). Indeed, the peptide adopts transmembrane alignments at pH 7.4 (225 ± 15 ppm; Figure 1E) and in-planar orientations at pH 5.3 (79 ± 8 ppm for Ala 16, Figure 1F and 73.5 ± 2.5 ppm for Ala 6 [46]) in agreement with the previously measured transition pH of 6.1 ± 0.2 [12, 41].

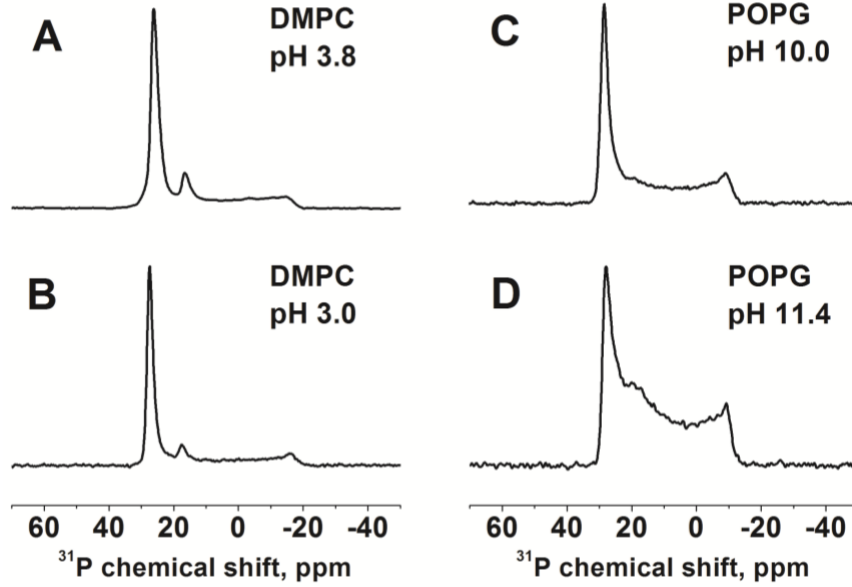


Figure 2. ^{31}P proton-decoupled spectra of 2 mole% LAH4 in oriented DMPC (A and B) or POPG (C and D) bilayers. The pH was adjusted to 3.8 (A), 3.0 (B), 10.0 (C), and 11.4 (D). The spectra were recorded with the sample normal parallel to the magnetic field direction from the same samples also shown in Figures 1C,D and 4C,D.

This data is summarized in Figure 3 where the transmembrane fraction is shown as a function of pH. The midpoint of the topological transition is $\text{pH}_{50} = 3.5$ for LAH4 in DMPC and 6.1 in POPC oriented lipid bilayers.

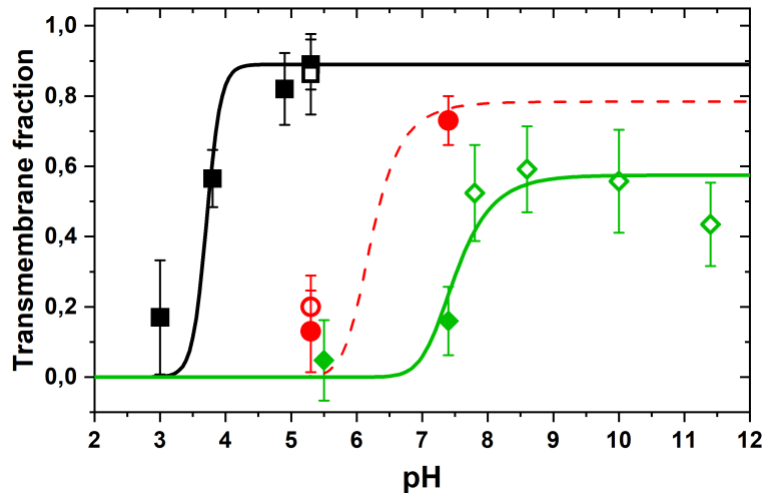


Figure 3: The experimentally measured contribution of the ^{15}N signal that corresponds to a transmembrane orientation of LAH4 as a function of sample pH in DMPC (black squares), POPC (red circles) and POPG (green diamonds). Open symbols indicate the results obtained with the ^{15}N label at position of Ala6, closed ones with ^{15}N -Ala16. The continuous lines show simulations with equation 1 (cf. Methods section) scaled by 0.87 to take into account the

maximum for the observed transmembrane fraction in DMPC. The pH was measured from a suspension of 240 mM lipid and the corresponding amount of peptide.

In a next step the LAH4 transition from in-plane to transmembrane was investigated in uniaxially oriented liquid crystalline POPG lipid bilayers. Also, in this case the ^{31}P solid-state NMR spectra indicate that the membranes are reasonably well aligned (Figure 2C,D). Previously, ^{15}N chemical shifts of 2 mole% ^{15}N -Ala16-LAH4 in POPG of 72.5 ± 3 ppm were observed when investigated at pH 5.5 or pH 7.4 indicative that the in-planar alignment is more stable when compared to POPC [60]. Indeed, significant proportions of transmembrane signal intensities were only observed at very basic pH (Figures 3 and 4). The broad intensities < 100 ppm are indicative of either peptides which do not strongly interact with the oriented membranes and/or contributions of peptides adopting a large range of tilt angles (Figures 4B-D). This fraction has a different appearance when compared to the peptide spectrum at pH 7.8, where a narrow ^{15}N peak at 72.5 ppm is indicative of a population aligned along the bilayer surface in a rather homogenous manner (Fig. 4A).

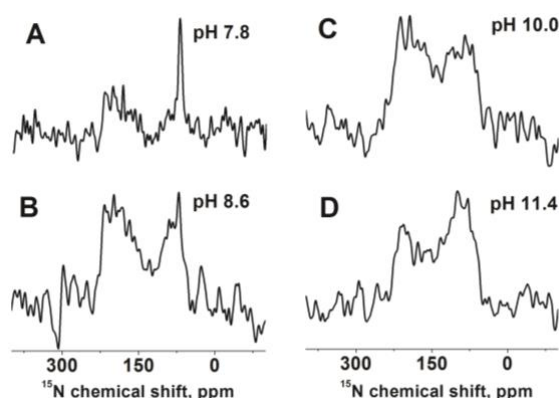


Figure 4: ^{15}N proton-decoupled spectra of 2 mole% ^{15}N -Ala16]-LAH4 in oriented POPG bilayers. The pH was adjusted to 7.8 (A), 8.6 (B), 10.0 (C), and 11.4 (D) at 5.5 μl water per mg of lipid (i.e. at a concentration of 240 mM).

Finally, the effect of the peptides on the lipid fatty acyl chain order parameters was investigated. These provide valuable information about the packing of the lipids and the effect LAH4 exerts at its different pH-dependent topologies. The order parameters are obtained from ^2H solid-state NMR spectra of phospholipids labelled uniformly with ^2H at one or both fatty acyl chains (Fig. 5A,D). In this study either a single palmitoyl chain of POPC or both myristoyl chains of DMPC were deuterated. To analyze the effect of LAH4 on the lipid chain mobility and conformation the quadrupolar spectra that compose the ^2H -NMR spectra were

converted into an order parameter profile (Fig. 5B,E). In order to visualize the segment-dependent changes upon peptide addition the ratio of the order parameter in the presence and in the absence of LAH4 was calculated. These relative order parameters are shown in Figure 5C and F.

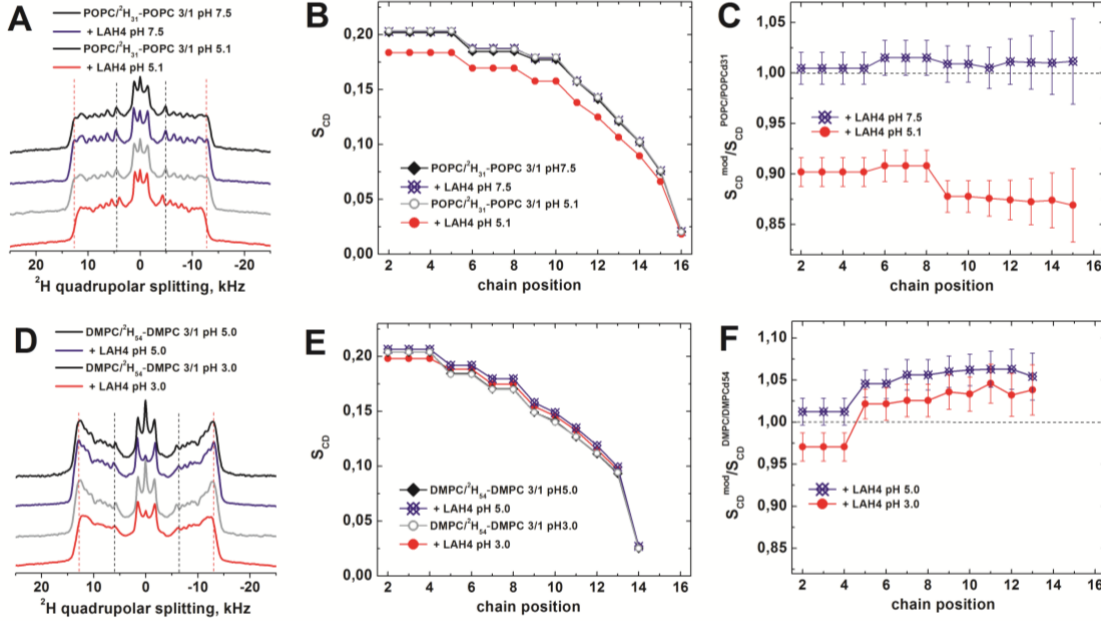


Figure 5. ^2H solid-state NMR spectra (A, D) and order parameter profiles (B, C, E, and F) for POPC/ $^2\text{H}_{31}$ -POPC 3:1 (A-C) and DMPC/ $^2\text{H}_{54}$ -DMPC 3:1 (D-F) in the absence and presence of 2 mole% LAH4 at pH 7.4 or pH 5.1 (A-C), and at pH 5.0 or pH 3.0 (D-F). The temperature was set to 25°C (298 K) for POPC (panels A-C), and 37°C (310K) for DMPC (D-F). Dashed lines in panels A and D are shown to guide the eye.

The plateau region of the POPC- d_{31} order parameters assigned to segments 2 to 5 occurs at 0.20. The order parameter profile hardly changes upon addition of LAH4 at pH 7.5 (Fig. 5B,C) where the peptide adopts transmembrane alignments (Figs. 1E and 3). In contrast at pH 5.1 when the peptide is in-plane (Figs. 1F and 3) a 10-15% decrease in the order parameters is observed (Fig. 5B,C). These observations are in agreement with previous investigations of a related peptide LAH4-L1 in POPE/POPG 3/1 membranes [61].

The order parameter plateau of DMPC occurs at 0.20 (Fig. 5E). While at pH 5, where LAH4 adopts a transmembrane orientation (Figs. 1A,B and 3), the plateau region remains unaffected by the peptide a 5% increase in the order parameter is observed upon addition of 2 mole% LAH4 (Fig. 5F). At pH 3.1 a decrease by 3 % in the order of the plateau region and an increase by about the same amount at positions 5-13 is observed (Fig. 5F). Because it has been shown that full hydration favors the transmembrane alignment of the antimicrobial

peptide PGLa in dimyristoyl phospholipids [8] it cannot be excluded that the transition also shifts for LAH4 thus under the conditions of the ^2H solid-state NMR experiments the peptide potentially adopts transmembrane alignments already at pH 3.1. Because it is difficult to maintain chemically stable samples at even more extreme pH values especially at full hydration this has not been investigated further.

Discussion

Lipids are important determinants for peptide topology: The prediction of the membrane topology of proteins focuses on the amino acid composition of the sequences [28, 62, 63] including previous investigations of helical model sequences using solid-state NMR [34, 36, 41]. This approach was refined by measuring the energies of transferring amino acid residues from the membrane interface to the hydrophobic interior [28, 45].

When the topology of PGLa and its synergistic interactions with magainin 2 were investigated a pronounced influence of the fatty acyl chain composition was observed where the transition of PGLa from in-planar to transmembrane only occurs in fully saturated membranes [8, 27]. Here the influence of fatty acyl chain and phospholipid head group composition was investigated in quantitative detail for LAH4, a designer peptide resembling PGLa in its hydrophobic angle when analyzed by helical wheel representations (Figure S1). Indeed, also in the case of LAH4 the lipid head group and fatty acyl chain composition have a major effect on the pH-dependence of its in-planar or transmembrane alignments (Figure 3).

Interaction contributions that influence IP-TM equilibria: A thermodynamic framework has previously been established to describe such transitions of helices from in-planar to transmembrane orientations. Importantly, even if exchange between the two configurations is slow on the 10^{-4} second time scale thus both topologies are observed simultaneously in ^{15}N solid-state NMR spectra (e.g. spectra shown in Figures 1 and 4) previous experiments using ATR FTIR have shown that the transition is reversible [12].

The in-plane – to – transmembrane transition of a helical polypeptide domain comes along with changes in the Gibbs free energy ΔG that arise from hydrophobic interactions (ΔG_h), from placing polar side-chains into the hydrophobic interior (ΔG_p), a pH-dependent energy of discharge of the four histidine side chains (ΔG_d), conformational changes (ΔG_c) and hydrophobic mismatch (ΔG_m) ([41] and references cited therein). For example, the helical outlines of LAH4 in DPC micelles have been shown to change in a pH dependent manner

[30] while the random coil to helical transition of magainin has been associated with a -0.5 kJ/mole per residue contribution to membrane partitioning [64].

Furthermore, it has been demonstrated that LAH4 peptides interact with each other and arrange in a mesophase arrangement when oriented along the surface of phosphatidylcholine membranes [33]. Therefore, interactions between the peptides also change during the transition and have to be taken into consideration (ΔG_i). In addition, interactions between the peptides and the lipids have an impact (ΔG_j). Another layer of complexity arises from lipophobic (entropic) [41, 65] and van der Waals interactions ($\Delta G_l + \Delta G_w$) which are related to the lipid curvature strain, the lipid structure and peptide-lipid interactions ([41] and references cited therein). Therefore, the total Gibbs free energy is influenced by a set of contributions well beyond the changes in environment of individual amino acid residues within the helical structure:

$$\Delta G = \Delta G_h + \Delta G_p + \Delta G_d + \Delta G_m + \Delta G_l + \Delta G_w + \Delta G_c + \Delta G_i + \Delta G_j \text{ (equation 2) [37, 41].}$$

LAH4 is carrying four histidines that change their protonation state during the pH titration and the experimentally accessible proportion of transmembrane peptide P_{TM} (Figure 3) has been calculated as [45]:

$$P_{TM} = \frac{[TM]}{[TM] + [IP^0] + [IP^{ch}]} = \frac{1}{1 + e^{\frac{\Delta G}{RT}} (1 + e^{2.3(pK_a - pH)})^4} \text{ (equation 1),}$$

where the total peptide concentration encompasses transmembrane [TM] and in-planar states with the histidines all neutral [IP^0] or some of them charged [IP^{ch}]. The pK_a describes the acid-base equilibrium of the histidines. Notably, the slope of the transition is a function of ΔG where close to zero or positive values result in an only partially populated transmembrane state even when the pH is much increased [45]. Furthermore, this theoretical framework shows that the transition midpoint is shifted to higher values due to the degeneracy of the in-planar state. The observations made with LAH4 in POPG are thus in line with this previously simulated behavior (Figure 3).

For situations where ΔG is strongly negative, such as very hydrophobic peptide sequences, the transition occurs at pH values below the pK_a namely at $pK_a + \frac{\Delta G}{4 \cdot 2.3RT}$ (equation 3) and the sigmoidal curve can be approximated by [41, 45]

$$P_{TM} \approx \frac{1}{1 + e^{4 \cdot 2.3(pK_a + \frac{\Delta G}{2.3 \cdot 4RT} - pH)}} \text{ (equation 4).}$$

Thus, according to equation (1) the midpoint of transition, pH_{50} , where 50% of the molecules are in the transmembrane state ($P_{TM}=1/2$) is: $pH_{50} = pK_a - \frac{1}{2.3} \ln \left(e^{-\frac{\Delta G}{4RT}} - 1 \right)$ (equation 5).

From these considerations, for very negative ΔG a linear correlation between the transition pH and the Gibbs free energy governing the in-plane – transmembrane equilibrium is obtained [45]: $\Delta pH = -\frac{\Delta \Delta G}{4 \cdot 2.3 \cdot RT}$ (equation 6).

Thereby the transition-pHs observed in bilayers of different lipid composition can be used to obtain information on the relative lipid contributions to ΔG . For example, in the fully saturated DMPC lipid the transition occurs at pH 3.5 whereas it happens at pH 6.1 in POPC where one of the fatty acyl chains is unsaturated. This ΔpH translates into a difference in the driving forces for peptide realignment of these two bilayers $\Delta \Delta G$ of about 60 kJ/mole. This is an energy comparable with exchanging several hydrophobic amino acids with residues that prefer an interfacial or aqueous localization [28, 34, 45]. For example, the transfer of 4-5 lysines from water to octanol has been associated with a penalty of about this size [28].

The IP to TM transition in POPG: In contrast, the in-planar topology is stabilized in POPG when compared to the PC membranes with a pK_{50} around 7.1 (Figure 3). Notably, in POPG the transition to transmembrane is incomplete even at pH 11 indicating that the ΔG of the transition is close to zero once the histidines have lost their charges. This has been analyzed in quantitative detail [45] and the corresponding pH-dependent transition curves have been simulated in Figure 3. The in-plane topology is probably stabilized by the negative surface charge density and the additional hydrogen bonding possibilities of the PG bilayers that favorably interact with the histidines (and lysines). Indeed, molecular dynamics calculations show an accumulation of close contacts between cationic amino acids of membrane-inserted antimicrobial peptides and the PG phosphate groups [66, 67]. Furthermore, due to the negative surface charge density of the PG bilayer there may indeed be a pH gradient even in the reduced volume of the stacked membranes (Figure S2).

LAH4 and PGLa respond to lipid saturation: Notably, not only LAH4 but also the antimicrobial peptide PGLa, which exhibits related hydrophobic angles in helical wheel representations (Figure S1), exhibits a range of different tilt angles when studied in the presence of saturated lipids of very different hydrophobic thickness but not when one chain of the phospholipid is unsaturated [8, 38]. The topological transition of PGLa is most pronounced in the presence of equimolar amounts of magainin where PGLa adopts tilt angles that agree with transmembrane configurations [8, 27]. This observation could be reproduced with bilayers of different head group composition such as PE and PC membranes arguing against curvature being the major determinant for the PGLa membrane insertion [37]. In contrast magainin 2, which adopts a smaller hydrophobic angle (Figure S1) and a less

pronounced membrane insertion [68, 69], adopts more stable in-plane configurations [8, 27, 70].

Order parameters correlate with helix alignment: For LAH4, magainin 2 and PGLa a profound decrease in the ^2H order parameters is observed when the peptides are aligned parallel to the membrane surface, an effect which disappears for transmembrane alignments (Figure 5A-C) [37]. This observation suggests that the lipid contributions $\Delta G_i + \Delta G_w$ play an important role for the helix orientation. An amphipathic helical peptide residing at the membrane interface takes more space at the lipid head group region when compared to the hydrophobic interior [71, 72]. Thereby, the membrane monolayer exhibits positive curvature strain, an increased disorder of the lipid fatty acyl chains (Fig. 5A-C) concomitant with membrane thinning [73]. This strain is released when the peptide adopts a transmembrane alignment concomitant with ^2H order parameters close to the ones in the pure lipids (Fig. 5C). Indeed, the disordering of the lipid fatty acyl chains in the presence of amphipathic helices oriented along the membrane surface has been recognized as a driving force for TM realignments [71]. Thus the LAH4 helix orientation determined by ^{15}N solid-state NMR spectroscopy correlates well with the disordering of the palmitoyl chain of POPC and the myristoyl chains of DMPC at pH 5 (Figs. 3 and 5C,F). A good correlation between fatty acyl chain order parameters and helix alignment has also been observed for the antimicrobial peptide PGLa when investigated in a variety of membranes [37]. Because the LAH4 alignment in fully hydrated DMPC bilayers at pH 3 remains uncertain (cf. above) this data set cannot be taken into consideration in the present discussion and the ultimate test of consistency with above hypothesis not be obtained.

Notably, for PGLa the transmembrane insertion in the presence of magainin 2 occurs in saturated PC membranes of very different thickness, in DMPC or in DMPE bilayers. This indicates that the hydrophobic thickness and the shape of the lipids are only of secondary importance in driving the helix realignment [8, 27, 37]. Therefore, while DMPC and POPC also differ slightly in thickness and overall shape the degree of saturation seems a more important parameter to explain the observed differences in the LAH4 topological transition (Fig. 3).

Quantitative considerations POPC versus DMPC: Here (Fig. 5) and in previous investigations [37] the palmitoyl chain of palmitoyl-oleoyl phospholipids and of the two fatty acyl chains in di-myristoyl phospholipids exhibit a similar degree of disorder in the presence of in-plane oriented amphipathic helices. This is somewhat surprising because PGLa and LAH4 exhibit a quite different behavior in DMPC and POPC lipids (Figure 3) [8, 27, 37].

Therefore, more data are needed to fully understand why when compared to POPC the fully saturated membranes favor transmembrane alignments to such an extent. Notably, there are two saturated chains in DMPC but only one in POPC. The saturated chains may act in a highly cooperative manner when aligned side-by-side in a fully saturated lipid bilayer. In contrast, in POPC the *cis* double bond of the unsaturated oleoyl chain probably ‘decouples’ the palmitoyl chains from each other by introducing considerably disorder already in the case of pure lipid bilayers. Furthermore, the *per se* more disordered unsaturated oleoyl chain may help to compensate for the disruption of the lipid packing upon peptide insertion.

Conclusions

The pH-dependent realignment of LAH4 occurs at a much lower pH in DMPC than in POPC membranes and at pH values well below the histidine pK_a. Thus, the data indicate that favorable driving forces for the in-plane – transmembrane transition arise from the saturated lipids where the in-plane alignment of amphipathic peptides causes significant membrane disorder. Theoretical analysis has shown that such disordering is a strong driving force for topological changes, oligomerization and pore formation [71, 72]. Indeed, a strong disordering of the deuterated myristoyl and palmitoyl chains by magainin 2 and PGLa has been demonstrated in a previous publication where the changes due to the membrane-associated magainin is driving PGLa realignment [37]. Here we used the LAH4 model antimicrobial peptide to monitor the pH₅₀ of the transition which allowed us to quantitatively evaluate the Gibbs free energy difference for the peptide realignment when DMPC and POPC membranes are compared to each other. We suggest that similar contributions from lipid saturation arise also for the magainin-driven topological change of PGLa where related observations have been made but could not be evaluated in a quantitative manner [37]. Notably, the topological rearrangements that are modulated by lipid composition, another peptide or a protein domain can have an important role in the regulation of membrane polypeptides.

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