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## Review

Special Issue

Meeting of the Membrane Study Group GEM of the French Biophysical Society

# Effect of lipid saturation on the topology and oligomeric state of helical membrane polypeptides.

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Keywords: helix topology, antimicrobial peptide, supported lipid bilayer, MHC II receptor, hydrophobic mismatch, magainins, alamethicin

### Highlights:

- Lipid saturation favors monomeric form of transmembrane peptide.
- Lipid saturation strongly drives the interface-to-transmembrane realignment.
- High energetic contributions of lipid fatty acyl chain to polypeptide realignment.

## **Abstract**

Natural liquid crystalline membranes are made up of many different lipids carrying a mixture of saturated and unsaturated fatty acyl chains. Whereas in the past considerable attention has been paid to cholesterol content, the phospholipid head groups and the membrane surface charge the detailed fatty acyl composition was often considered less important. However, recent investigations indicate that the detailed fatty acyl chain composition has pronounced effects on the oligomerization of the transmembrane helical anchoring domains of the MHC II receptor or the membrane alignment of the cationic antimicrobial peptide PGLa. In contrast the antimicrobial peptides magainin 2 and alamethicin are less susceptible to lipid saturation. Using histidine-rich LAH4 designer peptides the high energetic contributions of lipid saturation in stabilizing transmembrane helical alignments are quantitatively evaluated. These observations can have important implications for the biological regulation of membrane proteins and should be taken into considerations during biophysical or structural experiments.

## ABBREVIATIONS:

|       |   |
|-------|---|
| Aib   | $\alpha$ -aminobutyric acid   |
| C16:0 | fatty acyl chain of 16 C atoms (saturated; palmitic acid)                       |
| C16:1 | fatty acyl chain of 16 C atoms carrying one unsaturation                        |
| C18:0 | fatty acyl chain of 18 C atoms (saturated; stearic acid)                        |
| C18:1 | fatty acyl chain of 18 C atoms carrying one unsaturation                        |
| C20:4 | fatty acyl chain of 20 C atoms carrying 4 unsaturations (arachidonic acid)      |
| DHPC  | 1, 2-dihexanoyl- <i>sn</i> -glycero-3-phosphocholine                            |
| DMPC  | 1, 2-dimyristoyl- <i>sn</i> -glycero-3-phosphocholine                           |
| DMPG  | 1, 2-dimyristoyl- <i>sn</i> -glycero-3- phospho-(1'- <i>rac</i> -glycerol)      |
| NMR   | nuclear magnetic resonance  |
| PC    | phosphatidylcholine   |
| PE    | phosphatidylethanolamine  |
| PG    | phosphatidylglycerol  |
| 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'- <i>rac</i> -glycerol) |
| PS    | phosphatidylserine  |
| TMD   | transmembrane domain  |

## 1. Introduction

Helical domains are building blocks of membrane proteins [1-3] and they also occur as independent units with important biological activities such as cell signaling, antibiotic action and cell penetrating activities [4, 5]. One of the first steps when new genes are sequenced consists in the prediction if these are membrane or soluble proteins. To this end the protein sequence is analyzed for hydrophobic stretches long enough to span the membrane while additional parameters such as the nature and composition of the respective lipid environment are much less taken into consideration [6]. However, studies with helical peptides indicate that the membrane environment, the polypeptide concentration and the presence of other sequences all can have profound influence on the topology and lipid interactions of such helical domains [7-9].

Natural membranes contain a large portion of unsaturated chain lipids [10], which exhibit a much lower phase transition temperature when compared to the corresponding saturated chains. For example, while a DMPC bilayer (two saturated C14 chains) exhibits a phase transition at  $23.6 \pm 1.5$  °C the one of POPC (C16 saturated and C18 unsaturated) changes from the gel to the liquid crystalline phase at  $-2.5 \pm 2.4$  °C [11]. Indeed, a palmitoyl chain at the glycerophospholipid 1-position and an oleoyl-chain at the 2-position are considered to represent membranes of average hydrophobic thickness and saturation [12-15]. For example, *E.coli* membranes have been found to contain 32% C16:0, 35% C16:1 and 28% C18:1 lipids [13]. The phospholipid composition of mitochondria found in rat heart, red and white muscles consists to about 50% of unsaturated fatty acid chains and the average chain length is 16.7 carbon atoms [12]. The phospholipid fatty acid composition of cultured mammalian cells encompasses C16:0 (near 20%), C18:0 (15%), C18:1 (around 20-30 %), C16:1 and C20:4 (taken together ca. 10-15%) [14]. Such and related lipid compositions assure a liquid crystalline nature of the resulting lipid bilayers at physiological temperature. In fact, the lipid composition has been found to adapt to temperature variations thus the membrane is kept in the fluid phase [16].

In laboratory experiments, for practical reasons saturated phospholipids have been widely used as membrane mimetics because they are easier to prepare and store. In particular DMPC occurs in the liquid crystalline state at physiological temperatures and is generally considered to be good membrane mimetic [8, 9, 17]. Furthermore, magnetically oriented bicelles provide valuable additional information in NMR structural investigations and these have been established in the presence of saturated lipids [18, 19]. However, over the years our

work using oriented solid-state NMR spectroscopy has shown that considerable differences exist when the membrane interactions of polypeptide domains with saturated and unsaturated lipid bilayers are compared to each other.

The present review presents a number of polypeptide sequences (Table 1) for some of which lipid chain saturation is crucial. The phenomenon may be more common than generally expected, therefore, the lipid systems used for membrane biophysical investigations should be chosen with care.

Table 1: Amino acid sequences of polypeptides discussed in this paper

The one-letter code is used for peptides made from conventional amino acids. For the alamethicin sequence the three-letter code with the following non-standard residues is used: Aib:  $\alpha$ -aminoisobutyric acid, Phl: L-phenylalaninol, Ac- for acetyl- and -NH<sub>2</sub> for the carboxamide termini, respectively. DQA1-TMD are amino acids 215-244 of UniProt entry P01909 (including two additional lysines at the both termini); DQB1-TMD represents residues 165-193 of UniProt entry Q15363 (including two additional lysines at the amino-terminus).

|                     |  |
|---------------------|--|
| magainin 2          | GIGKF LHS AK KFGKA FVGEI MNS   |
| PGLa                | GMASK AGAIA GKIAK VALKA L-NH <sub>2</sub>  |
| LAH4                | KKALL ALALH HLAHL ALHLA LALKK A-NH <sub>2</sub>                                    |
| DQA1-TMD            | KK TETVV CALGL SVGLV GIVVG TVFII RGLRS KK-NH <sub>2</sub>                          |
| DQB1-TMD            | KK QSKML SGIGG FVLGL IFLGL GLIIH HRSQK K-NH <sub>2</sub>                           |
| alamethicin (F50/7) | Ac-Aib-Pro-Aib-Ala-Aib-Aib-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Gln-Gln-Phl |

## 2. Solid-state NMR spectroscopy provides detailed topological information

Solid-state NMR spectra obtained from polypeptides labelled with <sup>15</sup>N at the peptide bonds provide direct information about the approximate tilt angle of helical domains relative to the membrane surface. When the labelled peptides are reconstituted into liquid crystalline bilayers oriented with the membrane normal parallel to the magnetic field direction <sup>15</sup>N chemical shifts around 200 ppm are indicative of transmembrane helices whereas values < 100 ppm are obtained from helix topologies parallel to the surface [20]. A more detailed tilt/rotational pitch angle analysis is obtained when multiple <sup>15</sup>N labels are analyzed in two-dimensional <sup>15</sup>N chemical shift / <sup>15</sup>N-<sup>1</sup>H dipolar coupling correlation spectra [19, 21]. Furthermore, deuterium NMR spectra from alanines carrying <sup>2</sup>H<sub>3</sub>-labelled methyl groups

provide highly complementary information [22] which has been used for detailed topological analyses including information about rocking and wobbling motions [23-26].

Uniaxially oriented lipid bilayers are obtained when the lipid/peptide mixtures are applied onto solid supports. For the measurements described in this review these are typically flat ultra-thin microscope cover glasses of a size that fits into specially designed NMR coils (e.g. 8x 20 mm). However, polymers have also been used and these can be wrapped into other shapes such as cylinders [27, 28]. Thousands of lipid bilayers are deposited onto these surfaces, hydrated, about 20-30 glass plates are stacked on top of each other, the samples sealed and inserted into the NMR coil [29]. These uniaxially oriented membrane samples can be investigated at different alignments relative to the NMR magnetic field, preferentially with the normal parallel to the membrane normal as in this configuration rotational and lateral diffusion within the membrane plane have no effect on the spectral line shape [30].

Alternatively, large bicelles (> 30 nm) are prepared where patches of bilayer are stabilized by an amphiphilic rim [31-34]. The rim can be made up of detergents, short chain lipids, polymers, amphipathic peptides or proteins [18, 19, 35, 36]. Because of the anisotropy of the magnetic susceptibility of the fatty acyl chains experimental conditions can be found where the bicelles align in the magnetic field of the NMR spectrometer. At well-chosen experimental conditions where all of temperature, hydration and lipid/detergent ratio have to be adjusted, the bilayer normal is aligned perpendicular to the magnetic field direction (cf. scheme next to Figure 2F) but the bicelle alignment can be flipped by the addition of paramagnetic ions [37]. The resulting solid-state NMR spectra tend to exhibit relatively sharp and well-resolved lines of good sensitivity because the magnetic field drives the alignment and acts as the reference. Furthermore, intrinsic motions of and within bicelles such as wobbling, rotation of the bicelle around its normal and diffusion within the bicelle result in averaging of inhomogeneities and of some of the anisotropies. Such motions should be taken in account during detailed structure calculations [38]. The bicelle technique has been very powerful during the solid-state NMR investigations of transmembrane proteins (e.g. [19]), but it is less suitable for amphipathic peptides which can partition not only into the bilayer part but also into the rim of the bicelle [36]. Finally, a considerable amount of saturated fatty acyl chains is required for magnetic alignment and therefore oriented bicelles are often based on DMPC lipids. Thereby, the choice of lipids is more limited when magnetically well-oriented bicelles are needed for high-resolution solid-state NMR studies.

In contrast, mechanically oriented samples have been prepared for many different lipids including complex mixtures (e.g. [8, 17]), but when compared to bicelles there is less

motional averaging in these samples. Therefore, from a spectroscopic standpoint the investigation of these preparations is more challenging. Their investigation by solid-state NMR requires higher power pulses, and the relaxation rate of the peptide (and protein) resonances is significantly faster, resulting in significantly broader lines. The latter has prevented the implementation of many of the novel pulse sequence that in recent years have much advanced the field for bicellar samples [39, 40].

The lipid dependence of magnetically oriented bicelle samples is illustrated in Figure 1 showing the  $^{31}\text{P}$  solid-state NMR spectra of mixtures of long-chain and short chain phospholipids. The conditions were chosen to be favorable to the formation of bicelles that align in the magnetic field of the NMR spectrometers. The  $^{31}\text{P}$  solid-state NMR resonance position of phospholipids is an indicator of their molecular alignment relative to the magnetic field direction. A phosphatidylcholine in a liquid crystalline bilayer exhibits a chemical shift of about 30 ppm when being oriented with its long axis parallel to the magnetic field and of -15 ppm at perpendicular alignments. Isotropic motions result in values close to 0 ppm, a value also observed for phospholipids in solution.

Figure 1A exhibits a narrow main signal intensity at about -10 ppm showing that DMPC in the mixture with DHPC is well-oriented with the bilayer normal perpendicular to the magnetic field as illustrated next to Figure 2F. The smaller peak at about -5 ppm is from the rim structure formed by the short chain DHPC where diffusion along the rim results in additional averaging of the orientational anisotropy. In a next step half of the DMPC is replaced by POPC (Fig. 1B). The  $^{31}\text{P}$  solid-state NMR spectra is indicative that also this mixture shows magnetic alignment albeit the peaks are somewhat broadened. In the presence of POPC, the temperature range where alignment takes place is shifted to low temperatures in line with the shift of the main transition temperature ( $T_m$ ) of the long-chain lipid mixture [41]. Attempts to increase the amount of unsaturated-chain lipids leads to  $^{31}\text{P}$  solid-state NMR spectra that are indicative of mixtures of vesicles and micelles/small bicelles (see Fig. 1C), and without the characteristic features of bicelles that are oriented in the magnetic field [42].

Previous investigations of the phase diagram of DMPC/DHPC mixtures and the corresponding lipid distribution show that oriented (large) bicelles form in the  $T_m < T < T_v$  temperature range (Figure 1A), where  $T_m$  is the main transition temperature of the long-chain lipid mixture [42]. At temperatures below  $T_m$  the long-chain lipid are in the gel phase and nearly all short-chain lipids accumulate in the rim (see the left column in the Figure 1 for a schematic representation). This results in the formation of small isotropic bicelles with diameters in the 10 nm range. Above  $T_m$  of the long-chain lipids better mixing of the lipids



reduces the rim contributions and larger bicelles form. When the bicelles interact with each other in a cooperative manner magnetic alignment of these structures is obtained (cf. Figure 1A). At even higher temperatures  $^{31}\text{P}$  NMR signals at -15 ppm are indicative of the presence of lamellae of large size and/or vesicles ( $T > T_v$ ). These signal have been suggested to correspond to extended lamella with considerable perforations which are stabilized by the short chain lipids [42], however, to our knowledge no electron microscopic images of such structures have so far been published and as one reviewer pointed out other topologies could be invoked to account for the corresponding X-ray and NMR data. For POPC/DHPC mixtures the vesicle-associated  $^{31}\text{P}$  NMR signal is observed even below  $T_m$  (see Fig. 1C) suggesting a preference for extended bilayer structures rather than large bicelles that align in the magnetic field.

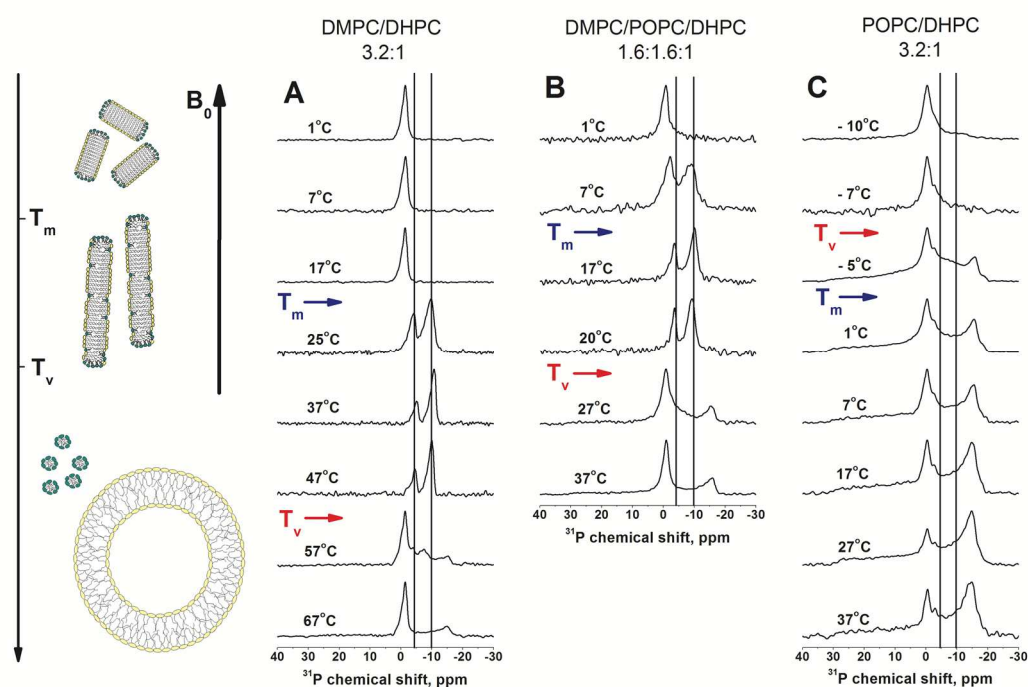


Figure 1. Proton-decoupled  $^{31}\text{P}$  spectra of the DMPC/DHPC 3.2:1 molar ratio (A), DMPC/POPC/DHPC 1.6:1.6:1 (B), and POPC/DHPC 3.2:1 (C) at the indicated temperatures. Samples are prepared in 10 mM Tris buffer at pH 7 with the lipid concentration of 28% (w/v).  $T_m$  is the main transition temperature for the long chain lipids (in the absence of DHPC). Above  $T_v$  intensities also appear at -15ppm suggesting the presence of lamellae of large size and/or the formation of vesicles. A schematic presentation of the possible structures is shown in the left column with DHPC molecules labeled in green and long chain lipids in yellow for better visibility.

### 3. The transmembrane helical domains of the MHC II receptor

The MHC class II membrane receptors are key players of the adaptive immune response and their malfunctioning can result in diabetes type I, chronic inflammatory and other autoimmune diseases [43, 44]. The DQ alpha 1 (DQA1) and the DQ beta 1 chains (DQB1) assemble as a heterodimer through GXXXG-mediated protein-protein interactions [45] to constitute the transmembrane region of the MHC class II protein. DQA1 carries a highly specific sphingomyelin-C18 interaction motif [46] and a cholesterol-recognition motif has been identified within the C-terminal regions of the transmembrane domains of both DQA1 and DQB1 [47, 48]. However, structural data about the oligomerization processes and the interactions of these sequences with lipids remain sparse. A better understanding of the structure and lipid interactions of MHC class II receptors promises novel insight into regulatory processes that are potentially also involved in disease development and therapeutic intervention.

In this context we have investigated the structure and topology of the MHC II transmembrane domains individually, together and their interactions with lipids [49-51]. When DQB1 carrying a  $^{15}\text{N}$  at either the valine-14 or the leucine-15 positions is investigated (Table 1) the  $^{15}\text{N}$  solid-state NMR spectra shown in Figure 2 are obtained. When reconstituted into POPC two contributions are observed (Figure 2E), one with  $^{15}\text{N}$  chemical shifts between 180 and 220 ppm representing a distribution of alignments in slow exchange and a sharp peak at 196 ppm for which fast averaging occurs. When the valine-14 position is studied and analyzed for the same topology and motional regime a much sharper resonance is obtained which makes the separation of two different contributions difficult (Fig. 2B). In a related manner, the resulting  $^{15}\text{N}$  solid-state NMR spectra of the DQA1 transmembrane domain exhibit two concentration-dependent membrane topologies, suggesting an equilibrium between a monomeric sequence and homodimers that form through its GXXXG motif [49, 52].

When DQB1 was reconstituted into DMPC/DHPC bicelles the  $^{15}\text{N}$  chemical shifts were  $85 \pm 7$  and  $90 \pm 5$  ppm for the valine-14 and the leucine-15 positions, respectively (Fig. 2C,F). The bicelles spontaneously orient with the normal perpendicular to the magnetic field direction and are thus aligned different by 90 degrees when compared to the spectra shown in Figure 2A,B,D,E. While the chemical shift measurements all agree with a transmembrane alignment of the DQB1 helix, the bicellar spectra only show a single peak. It cannot be excluded that this is due to the intrinsic motions of the bicelle and the 90-degree tilted

orientation relative to the NMR magnetic field. Therefore, the peptides were also reconstituted into stacks of DMPC lipid bilayers which were mechanically supported on glass surfaces (Fig. 2A,B) and thereby directly comparable to the spectra obtained in POPC (Fig. 2D,E). The chemical shift measured for the leucine-15 site is 191.5 ppm (Fig. 2B), i.e. only 4.5 ppm reduced when compared to POPC (Fig. 2E) but the second contribution exhibiting a range of different alignments is missing. Therefore, the bicellar membranes and the mechanically supported DMPC bilayers lack the evidence for a monomer- oligomer equilibrium that is visible in the POPC sample (Fig. 2E).

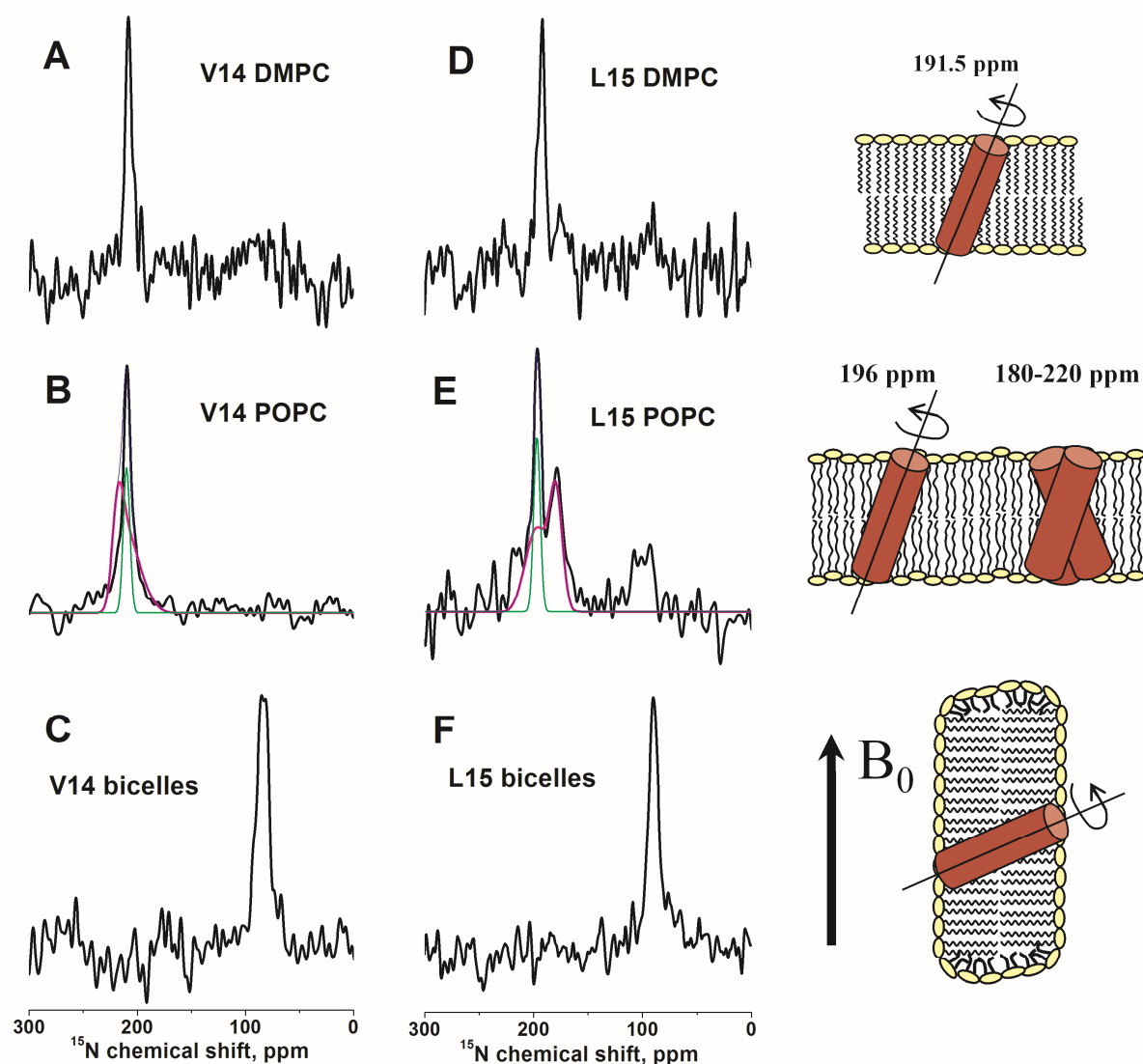


Figure 2. Proton-decoupled  $^{15}\text{N}$  solid-state NMR spectra of DQB1-TMD peptide reconstituted into oriented DMPC (A, D), and POPC (B, E) bilayers, and DMPC/DHPC ( $q=3.2$ ) bicelles at 37°C. Labeled residues are  $^{15}\text{N}$ -Val14 (A-C) and  $^{15}\text{N}$ -Leu15 (D-F). Adapted from [50] for panels A, C-E, the panel F spectrum is from [51] and the data in the panel B was not published previously.

#### 4. Alamethicin

Alamethicins constitute a family of very hydrophobic peptides of fungal origin with potent antimicrobial activities. Alamethicins are made up of 20 residues many of which are alpha-aminobutyric acid (Aib). The presence of Aib amino acids characterizes the family of peptaibols and many sequence variants of alamethicin have been identified [53]. Being hydrophobic and long enough to cross the lipid bilayer alamethicin has been extensively investigated being a paradigm for pores formed by bundles of helical polypeptides [54-56]. Therefore, also the alignment and structure of alamethicin in membrane environments has been studied by a multitude of biophysical techniques, including its supramolecular organization and oligomerization by solid-state NMR spectroscopy and molecular modelling [57-59].  $^{19}\text{F}$  MAS solid-state NMR measurements show that the oligomerization is dependent on the peptide-to-lipid ratio and distances were obtained to establish a pentamer structure at  $\text{P/L} = 1/13$  in POPC [59]. Such a model agrees with the step-wise increase in conductance levels [56] which is thought to arise from the variable number of helices forming its channel structure [54]. These previous investigations also indicated that hydrophobic (mis)match between peptide hydrophobic length and bilayer hydrophobic thickness is an important parameter determining the alignment of hydrophobic helical sequences relative to the membrane [58, 60-62].

Proton decoupled  $^{15}\text{N}$  solid-state NMR spectra of uniformly  $^{15}\text{N}$ -labeled alamethicin in macroscopically oriented bilayers made of POPC or of DMPC are shown in Figure 3. A predominant signal intensity around 200 ppm is observed for alamethicin reconstituted in either lipid bilayer clearly indicating transmembrane helix orientations. However, a detailed analysis reveals interesting differences. When reconstituted in POPC (Fig. 3B) the  $^{15}\text{N}$  chemical shifts arising from 19 labelled peptide bonds exhibit a maximal intensity at 213 ppm and a half width of 18 ppm. The higher chemical shift values when compared to DMPC (197 ppm; Fig. 3A) indicate a less tilted peptide topology in POPC membranes. To obtain more detailed information two-dimensional spectra were recorded where the intensities are better resolved and  $^{15}\text{N}$  chemical shifts and  $^{15}\text{N}$ - $^1\text{H}$  dipolar interactions correlate with each other (Fig. 3C) [58, 63]. Both of these NMR parameters are orientation dependent and the patterns obtained from multiple labelled sites provides information on secondary structure details and the corresponding tilt angles (e.g. recent work by [19]). The experimental spectra were compared to various models that had been proposed for membrane-associated alamethicin including  $\alpha$ - and 3.10-helical secondary structures (e.g. Fig. 3C) and the results of such

simulations are summarized in the Table 2. Whatever secondary structure is taken into consideration, alamethicin exhibits a more tilted arrangement in DMPC membranes when compared to POPC, the difference being around 7°. Because the hydrophobic thickness of DMPC was found to be 25.7 Å at 30°C, which is 3 Å less than that of POPC at the same temperature (28.8 Å, [64]) this difference can be explained with an adaptation of the helix tilt angle to match the hydrophobic thickness of the membrane [61, 62].

Figure 3. Experimental proton-decoupled  $^{15}\text{N}$  (A, B) and 2D  $^{15}\text{N}$  chemical shift /  $^1\text{H}$ - $^{15}\text{N}$  dipolar coupling correlation (C, grey lines) solid-state NMR spectra of alamethicin labelled uniformly with  $^{15}\text{N}$ . (A) in DMPC (peptide/lipid molar ratio 1:8) recorded at 37°C, and (B, C) in POPC (1/100) recorded at 21°C. The peptides were reconstituted into uniaxially oriented lipid bilayers and inserted into the NMR spectrometer with the membrane normal parallel to the magnetic field. The simulation of an ideal  $3_{10}$ -helix at reduced line broadening is shown in black in panel C. Adapted from [58].

Table 2. Alamethicin tilt angles in DMPC [63] and in POPC [58] bilayers from simulations of two-dimensional  $^{15}\text{N}$  chemical shift /  $^1\text{H}$ - $^{15}\text{N}$  dipolar coupling correlation spectra.

| Secondary structure          | DMPC | POPC |
|------------------------------|------|------|
| $\alpha$ -helix              | 17°  | 6.3° |
| $3_{10}$ -helix              | 21°  | 14°  |
| mixed $\alpha/3_{10}$ -helix | 12°  | 8.7° |

## 5. PGLa and magainin 2 antimicrobial peptides

Magainin antimicrobial peptides have been found in the skin of *Xenopus laevis* frogs and were among the first for which the potential usefulness of their antimicrobial activity was described [65]. These amphipathic peptides interfere with the barrier function of bacterial membranes and their interactions with lipid bilayers have therefore been extensively investigated by biophysical approaches including structural studies by solution and solid-state NMR spectroscopy [5, 66, 67]. From such investigations a view emerges [68] where at high concentrations the peptides form a dense carpet on the membrane surface which results in membrane disintegration, whereas at low concentrations single channel conductivities are observed which have been associated with disordered supramolecular arrangements of peptides and lipids [69-72].

While magainins have been found to be random coil in aqueous solution they adopt amphipathic helical structures upon membrane-association [73]. Solid-state NMR and optical spectroscopies are indicative that the peptide helices reside within the membrane interface and align with the helix long axes parallel to the membrane surface [74-76]. This topology was later confirmed for magainin analogues and for several other linear cationic antimicrobial peptides (e.g. [5, 23, 77-79]).

$^{15}\text{N}$  solid-state NMR spectra of labelled PGLa or labelled magainin 2 (Table 1) reconstituted into oriented lipid bilayers are shown in Figure 4 where the approximate helix topologies of the labelled peptides becomes obvious from the  $^{15}\text{N}$  chemical shift values and line shapes [20]. The magainin 2 helix has been found to partition into the membrane parallel to its surface regardless of membrane lipid composition [75, 80]. This becomes obvious also from the two  $^{15}\text{N}$  solid-state NMR spectra shown in Figure 4E and F which exhibit well-defined  $^{15}\text{N}$  chemical shifts < 100 ppm in DMPC/DMPG as well as in POPC/POPG membranes, respectively.

In contrast, the alignment of its relative PGLa is strongly dependent on the lipid saturation and composition [62, 80, 81]. In fully saturated lipid bilayers PGLa has been observed to align parallel to the membrane surface but at higher peptide concentrations it tends to adopt a somewhat more tilted orientation, which is thought to be due to homodimer formation [9]. The broad resonance observed in Figure 4A from labelled PGLa in DMPC thus represents a range of different topologies roughly in the plane of the membrane surface. Still in fully saturated membranes and in the presence of magainin 2  $^{15}\text{N}$ -labelled PGLa even adopts transmembrane alignments as evidenced by the  $^{15}\text{N}$  chemical shift around 178 ppm (Figure 4C) [8, 62, 81, 82].

However, this behavior much changes when lipid unsaturations are present. In membranes made of *E. coli* lipid extracts [83] or of POPC, POPE and/or POPG both PGLa and magainin 2 have always been observed close to perfectly parallel to the bilayer surface [8, 62, 81]. This is also evident from the inspection of the  $^{15}\text{N}$  chemical shift spectra shown in Figure 4B,D,F which are all characterized by sharp resonances < 100 ppm.

An additional level of complexity has been detected during fluorescence self-quenching investigations which show that magainin 2, PGLa and mixtures thereof form higher order supramolecular structures along the membrane surface. The data agree with nematic phase arrangements at the membrane surface as a function of lipid composition and salt concentration of the surrounding buffer [84, 85]. Notably, these investigations also show that the nematic phases of the PGLa/magainin 2 mixture pack more densely and importantly

that the membrane-association of one peptide increases by an order of magnitude in the presence of the other peptide [85]. It is interesting to note that the peptide mixture is about an order of magnitude more active in antimicrobial and calcein release assays when compared to the same total concentration of the individual peptides [86-88]. These observations are suggestive that the packing of the nematic phases is responsible for a much increased membrane-association, correspondingly a much higher peptide density in the membrane which ultimately results in a much more efficient bacterial killing by the peptide mixture. Notably, such synergistic effects are observed in the presence of PE but not PC lipids [85, 88], which increases the selectivity for bacterial when compared to eukaryotic membranes.

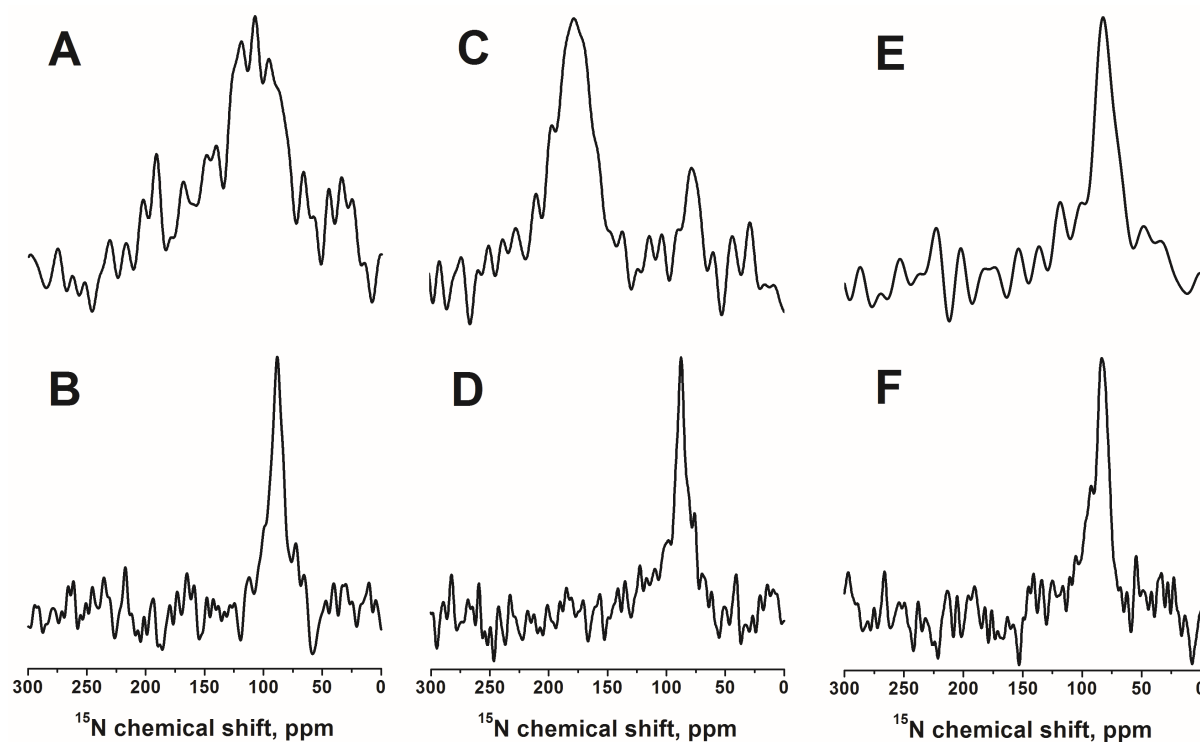


Figure 4. Proton-decoupled  $^{15}\text{N}$  solid-state NMR spectra of single peptide (A, B) and of peptide mixtures (C-F) reconstituted into oriented DMPC (A), DMPC/DMPG 3/1 (C and E), POPC (B) or POPC/POPG 3/1 (D, F) membranes at 310 K. (A and B) [ $^{15}\text{N}$ -Ala14]-PGLa. (C and D) [ $^{15}\text{N}$ -Ala14]-PGLa and unlabeled magainin 2. (E and F) Unlabeled PGLa and [ $^{15}\text{N}$ -Ala15]-magainin 2. The molar ratios of PGLa in DMPC and POPC are 1:50, and of PGLa/magainin 2/DMPC/DMPG and PGLa/magainin 2/POPC/POPG 1:1:75:25. Adapted from [62].

## 6. The LAH4 designer peptide provides quantitative insight into lipid-polypeptide interactions.

Taking magainin antimicrobial peptides as a template the LAH4 sequence was designed (Table 1) to test and understand how the activity of antimicrobial peptides is influenced by their membrane alignment [89]. The LAH4 peptides consist of leucines and alanines which form a hydrophobic core. The sequence is interrupted by four histidines in such a manner that in an  $\alpha$ -helical secondary structure these polar residues all line up along one face of the helical surface thereby an amphipathic structure is obtained. Two lysines at each terminus serve as interfacial anchoring residues and help in the better handling of the peptides in aqueous environments.

As expected from the initial design LAH4 and related sequences show potent antimicrobial activities [90]. Furthermore, cell penetrating activities for DNA, siRNA, polypeptides, vaccines, nanodots and adeno associated viruses have been demonstrated [91]. Members of the LAH4 family of peptides much enhance the lentiviral transduction of human cells a feature with potential clinical applications for *ex vivo* gene therapy [92]. Because the histidines exhibit a pK value around 6 the polarity and thereby the hydrophobic moment of the LAH4 peptides can be easily modulated by merely changing the pH of the environment [89, 93].

Indeed, when reconstituted into uniaxially oriented POPC lipid bilayers the membrane alignment of LAH4 is pH-dependent and the peptides can flip from in-plane ( $\text{pH} < 5.5$ ) to transmembrane topologies ( $\text{pH} > 7$ ) in a reversible manner with a midpoint of  $6.1 \pm 0.2$  [89, 93]. Because a multitude of interactions contribute to the total Gibbs free energy which characterizes the topological equilibrium these can be quantitatively evaluated from the transition curves. For example, it has been possible to establish the energies involved during their transfer of amino acid residues from the membrane interface to the membrane interior [94, 95].

This same concept was recently applied to investigate the effect of lipid saturation on the topological transition [96, 97]. Notably, the peptide is oriented along the surface of POPC but transmembrane in DMPC when supported lipid bilayers are investigated at pH 5.3 (Figure 5A-D). This effect is visible in oriented  $^{15}\text{N}$  solid-state NMR spectra regardless if the sequence was labelled with  $^{15}\text{N}$  at the alanine-6 (Fig. 5C,D) or the alanine-16 position (Fig. 5A,B). With additional information from deuterium labelled  $^2\text{H}_3$ -alanine-16 more accurate tilt angle information has been obtained for this peptide [25].



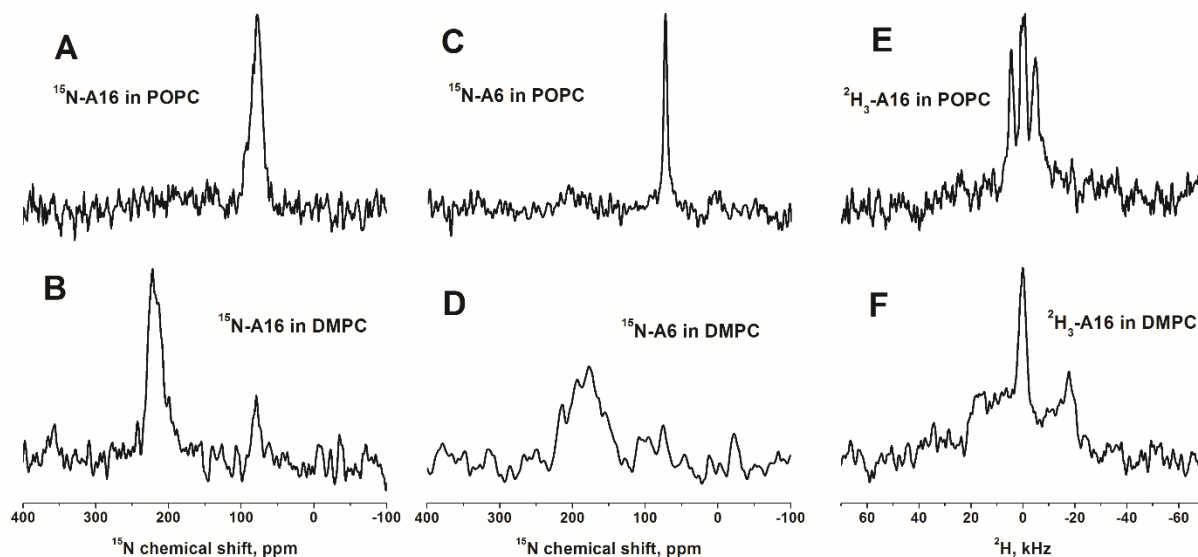


Figure 5. Proton-decoupled  $^{15}\text{N}$  (A-D) and  $^2\text{H}$  (E,F) solid-state NMR spectra of 2 mole% LAH4 in supported POPC bilayers at 23°C (A,C,E) and DMPC membranes at 37°C (B,D,F). The pH was adjusted to 5.3. The normal of the glass plates was aligned parallel to the external magnetic field of the NMR spectrometer. The spectra are from LAH4 peptides labeled with  $^{15}\text{N}$  at alanine 16 (A,B), alanine 6 (C,D) or with  $^2\text{H}_3$ -alanine at position 16 (E,F). An exponential apodization function of 100 Hz was applied before Fourier transformation of the free induction decay for the spectrum shown in A, 50 Hz for B, and 500 Hz for C. Panels A,C,E are from [25]. B is from [96], panels D and F show unpublished data.

Whereas the transition in POPC has previously been investigated in quantitative detail [89, 93] a more extensive data set was obtained only recently for DMPC [96] showing a major shift in the transition pH upon lipid saturation (Figure 6). In DMPC membranes the transmembrane state is more easily obtained and when compared to POPC and the topological transition is shifted by 3.6. units far into the acidic range in DMPC (Figure 6). This difference can be converted into an energy difference thereby allowing to compare the effect of saturation with other energy contributions relevant during the topological transition.

When a helical peptide changes its membrane interactions from an in planar to a transmembrane orientation many structural features change including the environment of amino acids, the lipid packing or the conformation of the peptide. All of these are associated with changes in the standard Gibbs free energy  $\Delta G$  of the transition which can be decomposed in contributions including from hydrophobic interactions ( $\Delta G_h$ ), peptide conformational changes ( $\Delta G_c$ ) [98] or changes involving the lipid structure and interactions ( $\Delta G_l$ ) [8, 89]. Furthermore, it is assumed that the four histidines of the LAH4 peptide insert

into the membrane interior only after being deprotonated. This is the case under neutral conditions but requires additional energy at acidic pH [95]. When the transmembrane insertion is favored e.g. by strong hydrophobic interactions and therefore, the overall  $\Delta G$  of the in-plane to transmembrane transition strongly negative, the shift in pH is proportional to the changes in the Gibbs free energy [95]:  $\Delta pH = -\frac{\Delta\Delta G}{4 \cdot 2.3 \cdot RT}$ . Therefore, the differences in the transition observed between POPC and DMPC can be associated with an energy that arises from saturation from one of the two fatty acyl chains. In DMPC the transition occurs at pH 3.7 whereas a transition pH 6.2 is measured for POPC, which corresponds to a  $\Delta\Delta G$  of about 50 kJ/mole. To put this energy into perspective, similar contributions to  $\Delta G$  are obtained when 4-5 hydrophobic amino acids are exchanged with lysines [6, 95, 99]. To explain this large effect of lipid saturation it has been suggested that the considerable disordering of the fatty acyl chain packing when amphipathic sequences partition into the membrane interface is a major driving force for the transmembrane insertion of the peptides [8, 25, 100].

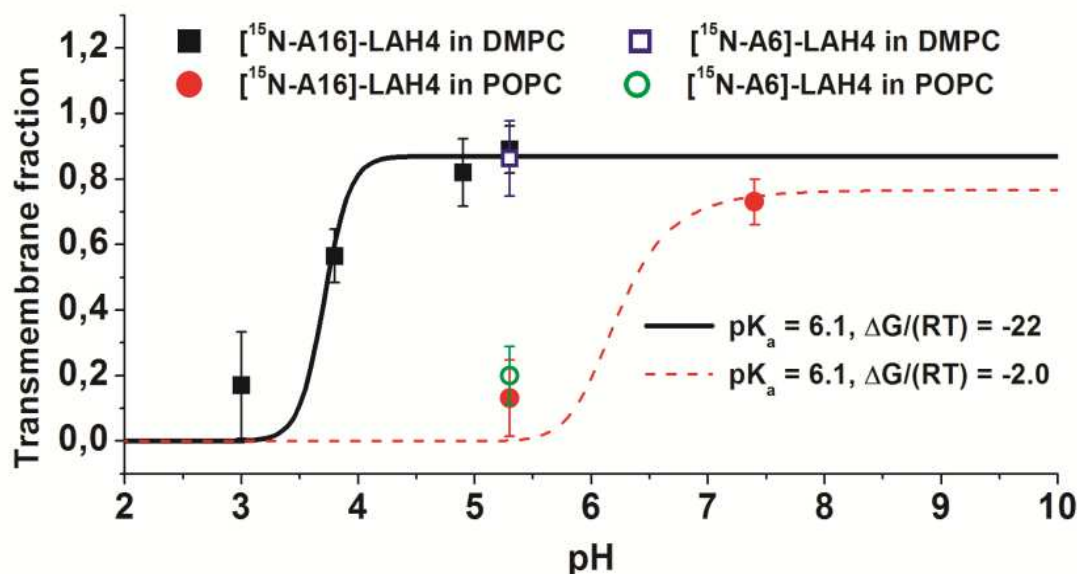


Figure 6. The experimentally measured contribution of the  $^{15}\text{N}$  signal that corresponds to a transmembrane orientation of LAH4 as a function of sample pH in DMPC (black squares) and POPC (red circles). Open symbols indicate the results obtained with the  $^{15}\text{N}$  label at position of Ala6, closed ones with  $^{15}\text{N}$ -Ala16. The continuous lines show the theoretical pH-dependence of the transmembrane fraction according to Eq. (1). The corresponding  $\Delta G$  values are  $-2 \cdot RT$  for LAH4 in POPC, and  $-22 \cdot RT$  in DMPC. From [96].

The observations presented in this paper on the peptide oligomerization and alignment in POPC or DMPC lipid bilayers are summarized in Table 3.

Table 3. Membrane orientations in DMPC and POPC membranes of the peptides presented in this work from solid-state NMR investigation. For DQB1 the oligomeric state is also indicated.

|                          | DMPC                  | POPC                           |
|--------------------------|-----------------------|--------------------------------|
| alamethicin              | transmembrane         | transmembrane                  |
| DQB1TMD                  | transmembrane monomer | transmembrane monomer/oligomer |
| PGLa                     | tilted topology       | surface                        |
| PGLa in presence of Mag2 | transmembrane         | surface                        |
| Mag2                     | surface               | surface                        |
| Mag2 in presence of PGLa | surface               | surface                        |
| LAH4 at pH 5             | transmembrane         | surface                        |

## 7. Head group and specific interactions

Whereas focus of this review is on the often neglected influence of fatty acyl chain composition during polypeptide-membrane interactions the importance of the phospholipid head group has been well established in previous work and should only be shortly mentioned here. For example, electrostatic interactions have been shown to promote the membrane-association of cationic peptides and to contribute to the selective killing by antimicrobial peptides of bacteria, which expose anionic surface changes to the environment, but not of eukaryotic cells [73, 101]. This effect has been investigated in a quantitative manner using biophysical approaches [102] and the release of peripheral membrane proteins when cationic peptides bind to bacterial membranes in a competitive manner has been suggested to be a cause of antimicrobial action [103]. While for many multicationic peptides the electrostatic attraction by PC/PG anionic surfaces has provided a suitable model to analyze the membrane-association isotherms [102, 104] a more specific association with PS lipids has provided explanation for data sets obtained for a limited number of peptides [105]. Furthermore the sialic acid-rich GM1 gangliosides have been found important in directing the way of amyloid aggregation, membrane disruption and toxicity [106]

When magainin2 and PGLa were investigated the membrane curvature has been found an important membrane property to explain their synergistic action. A considerably enhanced

calcein release activity by the peptide mixture was observed for membranes with negative intrinsic curvature such as PE lipid bilayers, but not for PC membranes [88]. Furthermore, fluorescence spectroscopy investigations show that the presence of equimolar PGLa enhances the association of magainin 2 to POPE/POPG membranes by an order of magnitude and vice versa, an effect which is not observed for POPC/POPG [85]. Because PE is more abundant in bacterial membranes when compared to eukaryotes the antibacterial synergism potentially also increases the therapeutic window of such peptide mixtures.

Finally, it should be mentioned that an amino acid motif has been described that is involved in highly specific lipid interactions. It recognizes not only the sphingomyelin head group but requires also a C18 fatty acyl chain [46, 107].

While various cholesterol interaction motifs have also been described these occur stochastically at quite high frequency and have been found uncorrelated to the cholesterol - protein association sites identified during structural studies [108-110]. However, this lipid has been shown to considerably modify the lipid bilayer physico-chemical properties and to be involved in the lateral phase separation of liquid ordered and liquid disordered domains with important implications to biological function [111].

## **8. Conclusions**

Whereas the fatty acid chain composition of phospholipids determines the hydrophobic thickness of membranes and therefore also the detailed tilt angle of a number of transmembrane domains [58, 112] more recent investigations show that lipid saturation has a profound influence on the membrane interactions of amphipathic helical sequences. While lipid saturation has been shown to result in a pronounced driving force for membrane insertion (Figs. 4-6) it also affects the mono- oligomer equilibria of a number of transmembrane anchoring sequences (Fig. 2). It has been speculated that the lipid cooperative order of two saturated fatty acyl chains are at the basis of the underlying driving forces for the realignment of membrane domains [96]. In analogy to the hydrophobic interactions in water such lipophobic effects can have an important role in the regulation of membrane-associated processes, but to our knowledge these have so far not been investigated in molecular detail by experimental approaches.

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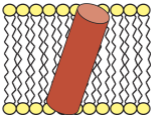
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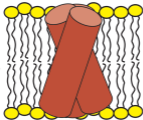
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**Saturated lipids**



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