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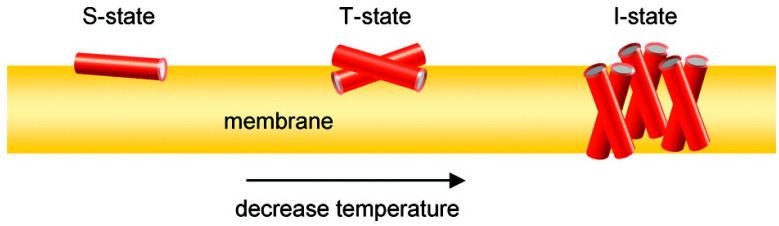

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Temperature-Dependent Transmembrane Insertion of the Amphiphilic Peptide PGLa in Lipid Bilayers Observed by Solid State ^{19}F NMR Spectroscopy

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Crucial for the activity of many antimicrobial peptides is their interaction with the lipid membrane of the target cell. To characterize this interaction, we studied the membrane-alignment of the α-helical amphiphilic antimicrobial peptide PGLa (GMASKAGAIAGKIAKVALKAL-amide) in DMPC/DMPG bilayers using solid state ^{19}F NMR. Here, it is demonstrated that the helix alignment depends not only on peptide concentration,[1,2] but that it changes even more dramatically with the temperature and lipid phase state. Below the main lipid phase transition, PGLa was found to insert upright with the helix axis almost parallel to the bilayer normal (I-state), suggesting for the first time that this peptide is able to form a transmembrane pore *per se*. In the liquid-crystalline phase, PGLa adopts a tilted T-state orientation as known from earlier studies.[1,2] This state, however, changes to an alignment parallel to the membrane surface (S-state) at elevated temperatures.

Several mechanistic models have been suggested as to how membrane-active antimicrobial peptides are able to permeabilize bacterial membranes.[3,4] The disruption or penetration of the lipid bilayer is attributed to a high density of peptides localized on the surface ("carpet model"), or to peptides forming transbilayer pores ("barrel stave", or "toroidal wormhole").[5−8] Since the peptide alignment differs for these various models, measurement of the peptide orientation in the bilayer provides important clues about the mechanism. Previous studies have demonstrated that amphiphilic peptides can be aligned parallel to the surface ("S-state"), they can be obliquely tilted in the membrane ("T-state"), or they can be inserted in a transmembrane alignment ("I-state"), depending on the conditions.[1−4,9−14] Specifically PGLa has been shown by solid state ^{2}H and ^{19}F NMR to be able to adopt all three different orientational states. Upon increasing the peptide concentration beyond a peptide:lipid ratio (P:L) of about 1:50 in liquid crystalline DMPC/DMPG mixtures, the α-helix realigns from the S-state to the T-state, which has been suggested to reflect dimerization.[1,15−17] More recently, PGLa was also found to be able to insert into the bilayer in a nearly upright I-state, but only when paired up 1:1 with the partner peptide magainin 2 (MAG2).[18] The I-state was proposed to represent an oligomeric transmembrane pore, i.e. the functionally relevant step of membrane permeabilization. The involvement of PGLa/MAG2 heterodimers can explain the known synergistic action of these two structurally related peptides, which are secreted from the same gland of *Xenopus laevis*. Notably, it has not been possible so far to find any experimental conditions under which PGLa would insert all the way into a transmembrane I-state on its own, even though a wide concentration regime has been systematically searched (P:L of 1:3000 to 1:20), the charge of the membrane has been varied, and samples with different hydration levels have been examined.[2,16,19]

In numerous studies it has been shown that the peptide alignment can change with the P:L,[1,10−12,14,20−24] lipid composition,[25−27] sample hydration,[6,25,28,29] or pH.[30,31] However, little is known about the behavior of amphiphilic peptides at different temperatures and in different lipid phase states.[6] We have thus used ^{19}F NMR to determine the orientation of PGLa in DMPC/DMPG bilayers as a function of temperature and bilayer phase, as this lipid system undergoes a chain-melting phase transition around 23 °C. Orientational NMR constraints were measured for four individually ^{19}F-labeled PGLa analogues, in which the position of Ile9, Ala10, Ile13 or Ala14 was substituted by 4-trifluoromethyl-*L*-phenylglycine (CF_3−Phg). The peptides labeled this way have been shown to possess the same functional and structural properties as the unmodified PGLa.[15] Each peptide was synthesized, purified and reconstituted into mechanically oriented bilayers of DMPC/DMPG (3:1 mol:mol) at a P:L of 1:50 as described previously.[1,2,15,16,32] The peptide alignment could then be calculated from the orientational constraints derived from the ^{19}F NMR signals of each trifluoromethyl-label, which give rise to triplet signals with orientation-dependent dipolar splittings and chemical shifts.[33−36]

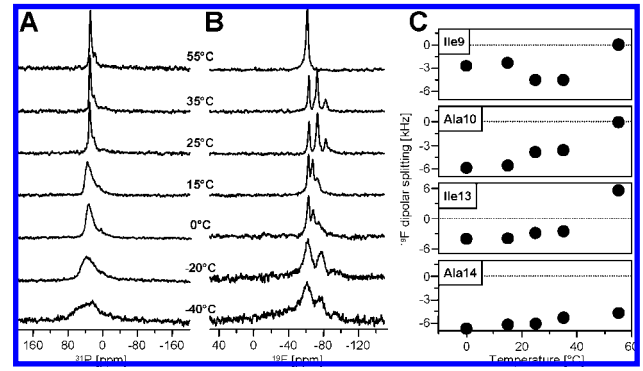

Figure 1. (A) In the oriented peptide/lipid samples ^{31}P NMR was used to assess the quality of phospholipid alignment and the lipid phase state. The bilayers were well aligned (downfield signals of DMPC and DMPG) in all samples and for all temperatures. (B) Representative ^{19}F NMR spectra are shown for PGLa labeled with CF_3−Phg at the position of Ile9, reconstituted at P:L of 1:50 into oriented DMPC/DMPG (3:1) bilayers. Spectra were acquired with the sample normal parallel to the magnetic field for a series of successive temperatures (30 min pre-equilibration) as indicated. Identical spectra were obtained in heating and cooling scans. (C) The temperature dependence of the ^{19}F dipolar splittings for each of the CF_3-labels in positions 9, 10, 13, and 14 shows discrete steps. All ^{19}F NMR experiments were performed on a 500 MHz NMR spectrometer (Bruker-Biospin, Karlsruhe), using ^{1}H-decoupling of ∼20 kHz, acquisition times of ∼10 ms, recycle delays of 2 s. Temperature settings were calibrated using methanol ^{1}H chemical shifts, with an accuracy of <1 °C.

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Figure 1B shows representative ^{19}F NMR spectra of PGLa labeled with CF_3−Phg in the position of Ile9, acquired at different temperatures between −40 and 55 °C. (The data for the peptides labeled at Ala10, Ile13, and Ala14 are provided in Suppl. Figure 2). Figure 1A shows the corresponding ^{31}P NMR spectra. First, we note that the lipids are well oriented, with the two downfield ^{31}P NMR signals reflecting DMPC and DMPG molecules aligned parallel to the membrane normal at all temperatures, even in frozen membranes (Figure 1A). Furthermore, the (i) gel-to-liquid crystalline and the (ii) subgel-to-gel lipid phase transitions, as well as (iii) the arrest of rotational diffusion, are clearly seen in the ^{31}P NMR spectra to occur between (i) 25 and 15 °C, (ii) 0 and −20 °C, and (iii) −20 and −40 °C, respectively. All ^{19}F NMR spectra (Figure 1B) exhibit the expected triplet signals, but they have distinctly different splittings over the different ranges of temperature. (The splitting at 55 °C happens to be zero for geometric reasons,[1,15,16] and it does not reflect an isotropically tumbling peptide, given the sizable splittings of the other labels at this temperature as seen in Figure 1C.) The splittings of all four CF_3-labeled PGLa analogues are displayed in Figure 1C as a function of temperature (and the raw data are listed in Suppl. Table 1). Each panel shows nearly horizontal plateau regions, meaning that PGLa changes its helix alignment or its conformational state over four discrete stages. The data at 25 and 35 °C confirm that the PGLa helix is aligned in the T-state as previously found at ambient temperature for this concentration (P:L of 1:50) in DMPC and DMPC/DMPG in the liquid crystalline phase. However, at temperatures above and below this range, the ^{19}F NMR splittings and hence the peptide alignment and/or conformation are clearly seen to change.

To obtain dynamic information about the lateral mobility of PGLa in the lipid bilayer, the oriented ^{19}F NMR samples were measured with their membrane normal parallel (Figure 1B) and perpendicular (see Suppl. Figure 3) to the magnetic field.[34] In the liquid crystalline state (>23 °C) each of these pairs of spectra is scaled by a factor of $-1/2$, indicating that PGLa is motionally averaged by rotational diffusion around the membrane normal faster than the NMR time scale (∼1 ms). Below the fluid-to-gel lipid phase transition this motion is significantly slowed down or stopped, according to the powder-like lineshapes observed for the perpendicular sample alignment. Finally, at −20 and −40 °C powder spectra are obtained, with the characteristic maximum splitting of ∼8 kHz at both sample alignments. This observation suggests an unoriented and immobilized peptide (Figure 1B, Suppl. Figure 3), meaning that the otherwise well-defined helix orientation does not persist in the subgel phase. The peptide thus seems to get expelled from the bilayer when the lipid acyl chains adopt a densely packed *all-trans* conformation (even though the lipids themselves remain oriented, as seen by ^{31}P NMR in Figure 1A).

To characterize the peptide alignment in detail, we have determined the helix tilt angle τ, its azimuthal rotation angle ρ, and the order parameter S quantifying the motional averaging, according to the definitions described in more detail elsewhere (and in Suppl. Figure 1).[16] Figure 2A illustrates the combined analysis of all four ^{19}F data points as a "dipolar wave"[37] for each temperature (see Suppl. Equation 1). In brief, assuming an α-helical peptide conformation, the theoretical splittings for the four labels were calculated upon systematically varying the values of τ, ρ, and S. The deviation from the experimental data was quantified by a root-mean-square deviation (rmsd) to obtain the best fit for each temperature, as illustrated in Figure 2B. Only in the frozen state this analysis was not possible, since the peptides did not show any oriented line shapes with discrete dipolar splittings.

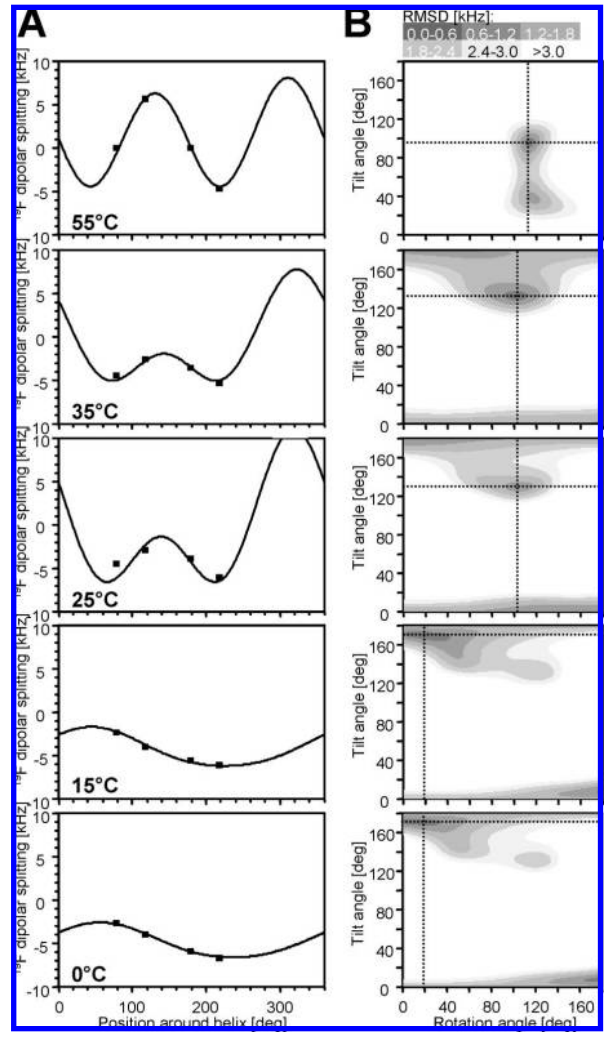

Figure 2. (A) CF_3 dipolar couplings, taken from the splittings in the ^{19}F NMR spectra of the four labeled positions, vary in a wave-like pattern as a function of the position around the helix (residue number). The amplitude and frequency of such "dipolar wave" is determined by the helix tilt angle τ, the azimuthal rotation ρ, and the order parameter S. (B) The $\rho-\tau$ maps display the rmsd minimum between the experimentally obtained dipolar couplings and the values calculated for a helix with systematically varied tilt and rotation angles. Shown here are the respective best-fit values of S (see Suppl. Table 2).

To discuss the temperature-dependent changes in the alignment of PGLa, we will set off in Figure 3 with the biologically relevant liquid crystalline phase before examining the less obvious effects of the gel state and the subgel phase. In liquid crystalline DMPC/DMPG at temperatures above 45 °C, a helix tilt angle of $\tau \sim 96°$ was found, which changed to ∼130° at ambient temperature (25 to 35 °C), and finally to ∼170° between 0 and 15 °C. PGLa is thus aligned parallel to the membrane surface (S-state) at elevated temperature, it is tilted (T-state) when moderately above the lipid phase transition, and it is found here for the first time to insert nearly upright into the bilayer (I-state) in the gel phase. (Angular values and S are listed in Suppl. Table 2.) An azimuthal rotation angle of $\rho \sim 100°$ was observed for both the S- and T-state, which places the charged face of the amphiphilic helix toward the aqueous layer, as expected. The numerically best-fit value of $\rho \sim 20°$ for the I-state is less precise and has less meaning, as ρ becomes degenerate in a perfectly upright alignment ($\tau = 0°$ or 180°).

The order parameter S describes the peptide wobble as a factor between 0 (for a fully averaged system) and 1.0 (complete absence

of wobble). In liquid crystalline bilayers, the realignment of PGLa from the S- to the T-state is accompanied by a slight increase in S from ~0.6 to 0.7. S approaches its maximum of 1.0 at 0 °C, which reflects the absence of wobble in the I-state, like it has been reported also for PGLa/MAG2 heterodimers in the I-state at ambient temperature.[18] Rotational diffusion of the peptide around the bilayer normal is manifest only in the S- and T-states, but not in the I-state (Figure 1B, Suppl. Figure 3). All of these observations indicate a much lower mobility of the peptide in the lipid gel phase than in the fluid bilayer, as expected. In the subgel phase, finally, the peptide is completely immobile and has moreover lost any preferred alignment.

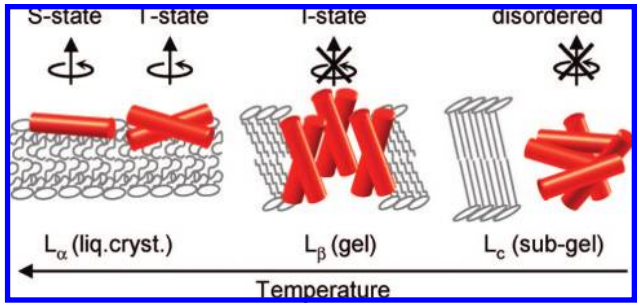

Figure 3. Four different states are found for PGLa, depending on the temperature and lipid phase state. At elevated temperatures (>45 °C) PGLa adopts a monomeric state, with the helices aligned parallel to the membrane surface (S-state). Closer to the lipid chain melting transition (temperature range 45 to 25 °C), a tilted alignment (T-state) prevails, where PGLa appears to assemble as homodimers. In the lipid gel phase (15 to 0 °C), PGLa inserts in a nearly upright transmembrane orientation (I-state) lacking rotational diffusion, where it presumably also forms dimers which line an aqueous pore. In frozen membranes in the subgel phase (<0 °C), the peptide is immobile and assumes no defined alignment, even though the lipid molecules are well oriented.

In summary, three distinct states of alignment have been found for PGLa in lipid bilayers, which can be reversibly interconverted by changing the temperature. Notably, these states are structurally discrete, and they do not reflect a gradual change of the helix tilt angle with temperature (Figure 1C). For high concentrations of PGLa (P:L ≥ 1:50) the tilted T-state has been well characterized in previous studies at ambient temperature. Here, we have observed with increasing temperature a realignment into the surface-bound S-state with a slightly higher mobility. This transition of the peptide does not correlate with any change in the lipid phase. The existence of the T-state has been previously rationalized in terms of dimerization above a critical peptide:lipid ratio to explain the tilted helix orientation.[2] It is thus reasonable to speculate that such PGLa dimers are no longer stable above a critical temperature. The realignment of PGLa from the T- to the I-state, on the other hand, seems to be related to the lipid phase, as the presence of local defects in a gel-state bilayer may facilitate the full insertion of peptides into the hydrophobic core.[38] When the amphiphilic peptide helices adopt a transmembrane orientation, the homodimers presumably assemble into higher oligomeric structures, such as a toroidal wormhole with an aqueous pore.[22] The present study has thus shown that these oligomeric I-state assemblies can be conveniently trapped in the lipid gel phase. In a similar way, the presence of MAG2 had been previously shown to stabilize a heterodimeric I-state of PGLa at ambient temperature. The present observation, namely that the lipid gel phase favors the I-state of PGLa *per se*, is highly relevant even for natural fluid membranes. It demonstrates the fundamental propensity of PGLa to insert into a lipid bilayer, emphasizing this functional step in both peptide-induced permeabilization of the target membrane and/or its translocation into the cell, which is essential for antimicrobial activity.

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Supporting Information Available: Supplementary Figures 1−3, Tables 1−2, and eq 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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