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Investigations of Polypeptide Rotational Diffusion in Aligned Membranes by ^2H and ^{15}N Solid-State NMR Spectroscopy

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Abstract: Transmembrane and in-plane oriented peptides have been prepared by solid-phase peptide synthesis, labeled with 3,3,3-$^2H_3$-alanine and ^{15}N-leucine at two selected sites, and reconstituted into oriented phophatidylcholine membranes. Thereafter, proton-decoupled ^{15}N and ^2H solid-state NMR spectroscopy at sample orientations of the membrane normal parallel to the magnetic field direction have been used to characterize the tilt and rotational pitch angle of these peptides in some detail. In a second step the samples have been tilted by 90°. In this setup the spectral line shapes are sensitive indicators of the rate of rotational diffusion. Whereas monomeric transmembrane peptides exhibit spectral averaging and well-defined resonances, larger complexes are characterized by broad spectral line shapes. In particular the deuterium line shape is sensitive to association of a few transmembrane helices. In contrast, the formation of much larger complexes affects the ^{15}N chemical shift spectrum. The spectra indicate that in liquid crystalline membranes an amphipathic peptide of 14 amino acids exhibits fast rotational diffusion on both the ^2H and ^{15}N time scales ($>10^{-5}$ s). Extending the sequences to 26 amino acids results in pronounced changes of the ^2H solid-state NMR spectrum, whereas the signal intensities of ^{15}N solid-state NMR spectra degrade. Below the phase transition temperature of the phospholipid bilayers, motional averaging on the time scale of the ^2H solid-state NMR spectrum ceases for transmembrane and in-plane oriented peptides. Furthermore at temperatures close to the phase transition the total signal intensities of the deuterium solid-state NMR spectra strongly decrease.

Introduction

Although there is widespread interest to better understand the structure and dynamics of membrane proteins, our knowledge about this important family of biomolecules remains very limited. This is due to the difficulties encountered during their biochemical preparation and structural investigations. For this class of proteins the classical diffraction[1−5] and solution NMR techniques[6,7] have so far succeeded only in a few exceptional cases. In addition, the picture obtained by high-resolution X-ray diffraction techniques remains static, and neither solubilized membrane proteins nor crystals thereof represents well their natural bilayer environment.

Solid-state NMR spectroscopy has emerged as an additional method to study the structure of proteins by using either static oriented samples[8] or magic angle sample spinning techniques.[9] The technique has been designed to study immobilized samples, such as peptides and proteins which are strongly associated with extended lipid bilayers (reviewed, e.g., in refs 8, 10−13). In previous work, solid-state NMR spectroscopy has been used to analyze secondary structures in membrane environments as well as their orientation with respect to the membrane normal.

In particular, proton-decoupled ^{15}N solid-state NMR spectroscopy has been shown to provide information on the alignment of α-helices within oriented membrane samples in a straightforward manner (reviewed in refs 8, 12, and 14). Whereas the ^{15}N chemical shift alone has proven to be a good indicator of the helical tilt angle,[14] the combination of ^{15}N and ^2H solid-state NMR spectroscopy makes accessible also the rotational pitch angle of polypeptides labeled at a few selected sites.[15,16] Furthermore, deuterium solid-state NMR spectroscopy has a proven record during the investigation of membrane-associated lipids (e.g., refs 17−20), polypeptides,[8,16,21−27] or water.[28−30]

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A major advantage of solid-state NMR techniques is their applicability to the study of the structure and dynamics of polypeptides when associated with hydrated liquid crystalline bilayers, where a high degree of lateral and rotational diffusion occurs. However, the polypeptides not only move within and along the membrane surface in a highly dynamic fashion, in addition some of them are believed to temporarily and reversibly associate into oligomeric structures.[31] Whereas optical methods allow one to study lateral diffusion (FRAP, refs 32 and 33) as well as distances between peptides (FRET, ref 34), these techniques require that the polypeptides carry fluorophores either naturally or by modification.

Although these dynamic properties of membrane-associated polypeptides are essential for their function,[35] the underlying conformational and configurational equilibria complicate the detailed interpretation of the structural data obtained by solid-state NMR techniques. Indeed an unambiguous analysis of these data would require a detailed knowledge of the aggregation state of the polypeptides within the sample. In previous investigations structural models have, therefore, been constructed from solid-state NMR data on the basis of evidence and assumptions about the aggregation state.[36−38] Evidently, the analysis of solid-state NMR data would very much profit from having at hand at least a good indication on the distribution of aggregation states and on the equilibria that govern peptide oligomerization.[39] To avoid that modifications of the peptides shift these association equilibria, additional investigations are best performed on the same samples used during the structural studies.

To perform solid-state NMR structural analyses using static oriented samples, the polypeptides are reconstituted into oriented lipid bilayers and the sample introduced into the magnet of the NMR spectrometer with the normal parallel to the magnetic field direction (Figure 1A). With this arrangement lateral or rotational diffusion around the membrane normal does not change the alignment of the polypeptide relative to the magnetic field direction. Therefore the resulting solid-state NMR signal is a direct function of the molecular alignment relative to the bilayer normal/B_o-vector and can be used to obtain orientational constraints for the molecule. However, wobbling and vibrational motions,[40−43] conformational changes, or rotation around the molecular axes results in averaging of the interaction anisotropies, and this needs to be analyzed in detail for high-precision structural analyses.[44]

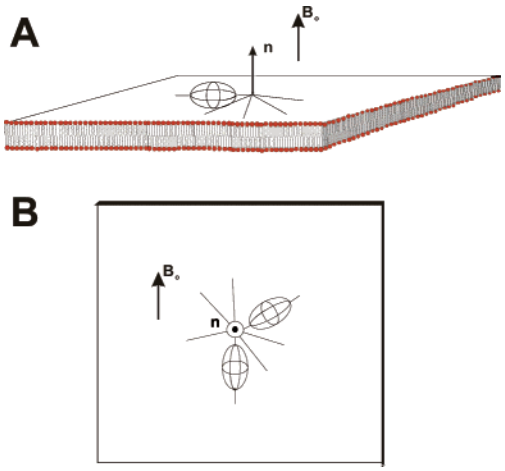

Figure 1. Model of an elongated molecule oriented along the surface of the membrane at sample orientations of the normal parallel (A) or perpendicular (B) to the magnetic field direction B_o. The ellipsoid can also be taken to represent a tensor, which describes the anisotropy of NMR interactions relative to the magnetic field.[14]

Here we extend such a structural study using ^{2}H and ^{15}N solid-state NMR spectroscopy by also investigating the same samples tilted by 90°. In this second sample setup, rotational diffusion around the membrane normal results in many different alignments of the molecule with respect to the magnetic field direction (Figure 1B) concomitant with changes in the NMR signals. The possible spatial alignments are illustrated in Figure 1B, where an elongated ellipsoidal molecule, such as, for example, an α-helix, is shown. Alternatively, the ellipsoidal shape can be taken to represent the tensor describing a given NMR interaction of a labeled site with the magnetic field.[14]

This tensor has been well studied for peptide bonds labeled with ^{15}N.[14] Whereas the ^{15}N chemical shift of a peptide bond within a helical molecule exhibits values around 220 ppm when the helix long axis is oriented approximately parallel to the magnetic field direction, ^{15}N chemical shifts in the 60−80 ppm range are measured at perpendicular alignments. A priori at sample orientations with the normal perpendicular to B_o, chemical shift values covering a wide range of values within the 60−230 ppm region are obtained (Figure 1B). However, quantitative estimates suggest that the rotational diffusion of short peptides is fast enough to cause motional averaging, and a single line is observed (cf. below). Fast rotation is also observed for lipids or glycolipids when being inserted into

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oriented phospholipid bilayers.[45,46] On the other hand larger molecules or peptide aggregates exhibit reduced rotational correlation times. Therefore, the resulting NMR line shapes are direct indicators of the rates of rotational diffusion and concomitantly the peptide aggregation state in the membrane. In this paper, helical peptides, labeled with [$^2H_3$]-alanine and with ^{15}N at one of the backbone amides, are reconstituted into liquid crystalline phospholipid bilayers and investigated by solid-state NMR spectroscopy. The experiments aim to test in a systematic manner the effect of motional averaging on the ^2H and ^{15}N spectral line shapes.

Theory

At room temperature the methyl group of alanine exhibits fast rotational motions around the $C_\alpha-C_\beta$ bond. As a result the three methyl deuterons are equivalent and exhibit overlapping resonances. The resulting ^2H tensor is axially symmetric with respect to the $C_\alpha-C_\beta$ bond vector, and the measured splitting $\Delta\nu_Q$ is directly related to the angle Θ describing the orientation of the $C_\alpha-C_\beta$ bond relative to the magnetic field direction:

$$\Delta\nu_Q = \frac{3}{2}\frac{e^2qQ}{h}\frac{(3\cos^2\Theta - 1)}{2} \qquad (1)$$

The factor e^2qQ/h is the static quadrupolar coupling constant.[47] As the C_α carbons are an integral part of the polypeptide backbone, the orientation of the $C_\alpha-C_\beta$ bond also reflects the overall alignment of the peptide.

In hydrated liquid crystalline membranes, the lipids and peptides freely diffuse about the membrane normal. Therefore, the $C_\alpha-C_\beta$ bond moves on a cone of semiangle φ relative to this direction. Furthermore, the orientation of this cone relative to the magnetic field direction and, thus, the deuterium quadrupolar splitting also depend on the angle β between the membrane normal and the magnetic field direction. The averaged quadrupolar splitting is given by[47]

$$\Delta\nu_Q = \frac{3}{2}\frac{e^2qQ}{h}\frac{(3\cos^2\varphi - 1)}{2}\frac{(3\cos^2\beta - 1)}{2} \qquad (2)$$

Equation 2 illustrates that, under conditions of rotational diffusion around the membrane normal, the deuterium quadrupole splitting at bilayer orientations with the normal perpendicular to the magnetic field direction ($\beta = 90°$) is half of the value at parallel alignments ($\beta = 0°$). Additional motions of the peptide relative to the membrane normal are taken into consideration by calculating the time average of $(3\cos^2\varphi - 1)$.

A priori the orientation-dependent chemical shifts, quadrupolar and dipolar splittings of the peptide vary depending on the actual alignment of the peptide with respect to the magnetic field direction (Figure 1B). When rotational diffusion is slow, separate signal intensities are apparent for different peptide alignments. However, fast rotational diffusion around the membrane normal leads to motional averaging of the signal intensities. Whether superposition of signals arising from different orientations or averaging is observed depends on the relationship between the anisotropy of the tensor and the rotational diffusion constant. At room temperature the peak positions of a deuterium-labeled alanine methyl group shift as a function of the molecular alignments within a range of ≤ 80 kHz. In contrast, at a magnetic field strength of 9.4 T (40.5 MHz Larmor frequency) the anisotropy of the ^{15}N-chemical shift of the peptide bond amounts to about ≤ 8 kHz. Signal averaging of deuterium lines separated by 80 kHz requires correspondingly higher rotational diffusion rates as averaging of the 1 order of magnitude reduced ^{15}N chemical shift dispersion.

The rotational diffusion rate is a function of the size and the shape of the peptide as well as the number of monomers associated with an aggregate. For an object with surface S in an environment characterized by the viscosity coefficient η, the rotational diffusion coefficient D_{pep} at absolute temperature T is given by

$$D_{pep} = \frac{1}{4}\frac{k_B T\cdot F}{S\cdot\eta\cdot h} \qquad (3)$$

where k_B is the Boltzmann constant, F, the shape factor, and h, the thickness within the membrane.[48−50] For an ellipse with major and minor axes $2a$ and $2b$, respectively, F amounts to $2/(1+a^2/b^2)$. The rotational diffusion constant is a function of the rotational correlation time:[22] $D_{pep} = \tau_c^{-1}$.

The radius of the molecule a relative to the membrane normal, therefore, provides a first approximation to model the rotational diffusion of a peptide in the lipid bilayer. Thus assuming $\eta = 5$ poise,[51] the limiting radius which corresponds to rotational diffusion at a 80 kHz rate amounts to approximately the peptide diameter. As a consequence, the corresponding ^2H solid-state NMR spectrum of peptides labeled with $^2H_3$-alanine should be sensitive to size variations that are also observed when peptides aggregate in the membrane, a prediction which is tested experimentally in this paper.

Materials and Methods

Phosphatidylcholines were purchased from Avanti Polar Lipids (Birmingham, AL). The peptides KL14 (KKLLKKAKKLLKKL), KL26 (KKLLKLLKKLLKALLKLLKKLLKLLK), and hΦ19W (KKK-ALLALLALAWALALLALLAKKK) were prepared by solid-phase peptide synthesis on a Millipore 9050 automatic peptide synthesizer using Fmoc (9-flourenylmethyloxycarbonyl) chemistry. At the double underlined positions the ^{15}N-labeled analogue of leucine and the (3,3,3-$^2H_3$)-labeled analogue of alanine were incorporated. The synthetic products were purified using reversed-phase high performance liquid chromatography. The identity of the products was confirmed by MALDI mass spectrometry.

Sample Preparation: 10 mg of peptide and 200 mg of lipid were codissolved in trifluoroethanol (TFE). The mixtures were applied onto 30 ultrathin cover glasses (9×22 mm^2, Marienfeld, Lauda-Königshofen, Germany), first dried in air and thereafter in high vacuum overnight. After the samples have been equilibrated at 93% relative humidity, the glass plates were stacked on top of each other, and the samples were sealed with Teflon tape and plastic wrappings.

Nonoriented lipid bilayer samples were prepared from 20 mg of peptide and 300 mg of lipid. These were codissolved in TFE and spread on a glass surface. The samples were dried and rehydrated under the

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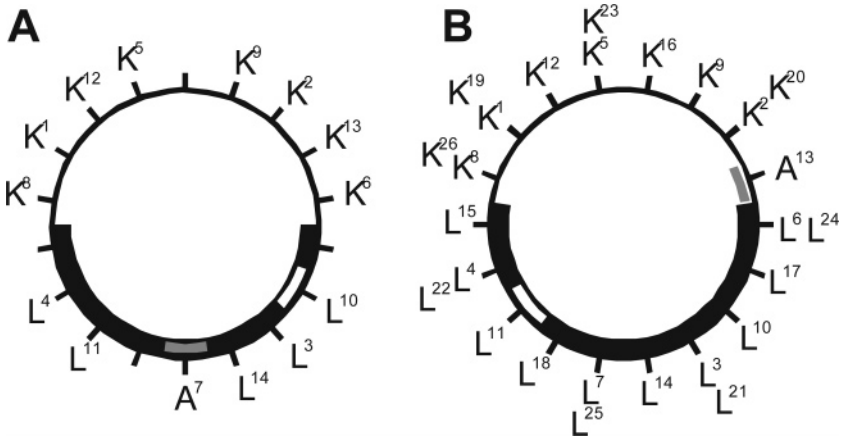

Figure 2. Edmundson helical wheel diagram of KL14 (A) and KL26 (B) illustrating the perfect amphipathic separation of leucine and lysine residues. The positions labeled with (3,3,3-$^{2}H_3$)-alanine are shown in gray; the ^{15}N-labeled positions are marked in white.

same conditions as the oriented samples. The equilibrated membranes were scratched off the glass plates and transferred into round test tubes.

Solid-State NMR Measurements: Solid-state NMR spectra were recorded on a Bruker AMX400 wide-bore NMR spectrometer using a commercial double-resonance solid-state NMR probe modified with flattened coils of inner dimensions $15 \times 4 \times 9$ mm^3. The spectra of samples rotated by 90° relative to the magnetic field directions were acquired using a second coil of similar geometry. Unless indicated otherwise the samples were cooled by a stream of air during the solid-state NMR measurements. The temperature was set to 310 K or 295 K for samples made of DMPC (1,2-dimyristoyl-*sn*-glycero-3 phosphatidylcholine) and POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3 phosphatidylcholine), respectively.

Proton-decoupled ^{15}N solid-state NMR spectra were acquired using cross polarization.[52] Typical acquisition parameters were the following: spin lock time 1.6 ms, recycle delay 3 s, ^{1}H B_1 field 31 kHz, 256 data points, 20 000 acquisitions, and spectral width 40 kHz. Before Fourier transformation an exponential apodization function corresponding to a line broadening of 300 Hz was applied. NH_4Cl was used as a reference (41.5 ppm).

Deuterium solid-state NMR spectra were recorded using a quadrupolar echo pulse sequence[53] with the following parameters: ^{2}H B_1 field 42 kHz, echo delay 48 μs, spectral width 100 kHz, 4096 data points, 20 000 acquisitions, and a repetition time of 1.5 s. The spectra were referenced relative to $^{2}H_2O$ (0 ppm). An exponential apodization function corresponding to a line broadening of 300 Hz was applied before Fourier transformation.

Results

To test for the effects of peptide geometry and orientation on the rotational diffusion and consecutively the solid-state NMR line shapes of membrane-associated polypeptides, two amphipathic and one hydrophobic sequences were prepared by solid-phase peptide synthesis. The ^{15}N-labeled analogue of leucine and the (3,3,3-$^{2}H_3$)-labeled analogue of alanine were incorporated at specific sites in the central regions of the sequences.

It has been shown previously that membrane insertion and α-helix formation are tightly linked processes.[54] As a consequence a wide variety of similar or closely related polypeptides exhibit α-helical conformations when inserted into the membrane (refs 38, 55, and 56). The Edmundson helical wheel representations of KL26 and of KL14 indicate that the α-helical secondary structures result in perfect amphipathic distributions of amino acids, with all the leucines being aligned on one face of the helix and all the lysines aligned at the opposite side (Figure 2).

When reconstituted into oriented DMPC bilayers, the proton-decoupled ^{15}N solid-state NMR spectra of KL14 labeled with ^{15}N-leucine-10 and (3,3,3-$^{2}H_3$)-alanine-7 exhibits ^{15}N chemical shifts of 75 ppm (Figure 3A). These NMR measurements have been performed at 310 K, i.e., well above the gel-to-liquid crystalline phase transition of DMPC (296 K). The functional dependence between the ^{15}N amide chemical shift and the alignment of peptides has been investigated previously in considerable detail.[14] The measured chemical shift value < 100 ppm is indicative of peptide orientations parallel to the membrane surface, in excellent agreement with the amphipathic design of the peptide.

The ^{2}H NMR spectrum of the same sample is shown in Figure 4B. A single well-resolved quadrupolar spitting of 16.1 kHz is observed at 310 K. The alanine methyl group exhibits fast rotational averaging around the $C_\alpha-C_\beta$ bond.[57] This motion ensures fast exchange of the methyl deuterons and results in a single quadrupolar splitting for all three labeled sites. The sharp spectral resonances indicate that the peptide is well aligned with respect to the magnetic field direction. Furthermore water deuterons (natural abundance 0.015%) make considerable contributions to the spectral intensity in the central region of the deuterium NMR spectrum. The observed deuterium quadrupole splitting provides an additional orientational constraint to align the peptide relative to the membrane normal. To evaluate the peptide orientations that agree with the experimental spectra, a Cartesian coordinate system was defined in which the helix long axis defines the *z*-axis and the plane dividing hydrophobic and hydrophilic sections of amphipathic helices defines the *x*-direction.[16] By successively rotating the peptide molecule around the initial *z*- and the *y*-axis (50×50 steps), the three-dimensional orientational space was systematically screened. For each peptide alignment the ^{15}N chemical shift and $\Delta\nu_Q$ were calculated assuming a magnetic field direction parallel to the original *z*-axis. Contour plots mark the tilt and rotational pitch angles that agree with the experimental results (Figure 5A). Although the experimental data are in agreement with several peptide alignments relative to B_o, only one result

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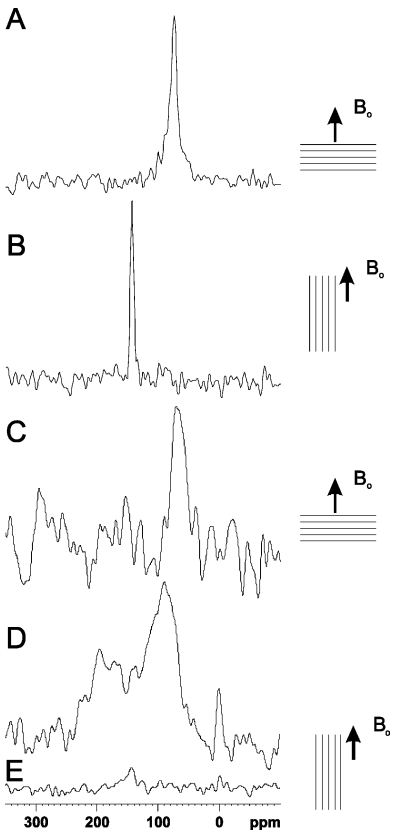

Figure 3. Proton-decoupled ^{15}N spectra of (A) KL14 ^{15}N-labeled at the leucine-10 position and reconstituted into oriented DMPC phospholipid membranes at orientations of the normal parallel to the magnetic field direction. (B) The sample shown in panel A tilted by 90°. (C) KL26 labeled with ^{15}N at the leucine-11 position and reconstituted into aligned POPC phopsholipid membranes at sample orientations of the membrane normal parallel to B_o. (D) The 26-residue peptide LAH4[55] labeled with ^{15}N at alanines 6, 16, and 22 at sample orientations with the POPC bilayer normal perpendicular to B_o. The spectrum was recorded at −10 °C. (E) Same sample as D recorded at ambient temperature.

positions the hydrophobic and hydrophilic residues in an energetically favorable manner within the membrane environment. In this case the peptide tilt angle deviates by approximately 10° (±7°) from a perfect in-plane alignment, and the rotational pitch angle is 160° (±8°). When the alignment of KL14 has been investigated in other phopspholipid bilayers, small differences in chemical shift and quadrupolar splitting have been observed corresponding in differences of 5° and 15° in tilt and rotational pitch angle, respectively.[16]

After reconstitution of [^{15}N-Leu11, 3,3,3-$^2H_3$-alanine-13]-KL26 into POPC bilayers oriented with the normal parallel to the magnetic field direction the observed ^{15}N chemical shift is 71 ppm (Figure 3C). At the same time the deuterium quadrupole splitting of the $^2H_3$-labeled site is 25 kHz (Figure 4E). These data are in agreement with helix orientations described by tilt/rotational pitch angular pairs of 102 ± 3° / 145 ± 2° and 67 ± 2° / 152 ± 5°, respectively (Figure 5B). The first result seems to be in better agreement with the perfect separation of charged and apolar residues (Figure 2B). The small deviation from perfect in-plane alignments reflects the non-negligible asymmetry of the three-dimensional peptide structure including the opposite charges of the N- and C-terminus.

Figure 4C and F show simulations of the experimental deuterium NMR line shapes of [$^2H_3$-Ala7]-KL14 and [$^2H_3$-Ala13]-KL26, respectively. The spectra indicate that at the labeled position the alanine $C_\alpha-C_\beta$ bonds exhibit, respectively, alignments of 46° and 41° relative to the magnetic field direction and orientational mosaic spreads <1°.

After having established the alignment of the two polypeptides, the samples were rotated by 90°. Both the ^{15}N and the ^2H solid-state NMR spectra of KL14 continue to exhibit well-resolved peaks intensities. When tilting the sample, the ^{15}N chemical shift has moved to 143 ppm and the quadrupolar splitting has reduced to 7.8 kHz (Figures 3B and 4A). Such features of the ^{15}N and ^2H solid-state NMR spectra are indicative of fast motional averaging around the membrane normal. Within experimental error the deuterium quadrupole splitting exhibits half the value when compared to parallel sample orientations in excellent agreement with eq 3. On the other hand the ^{15}N chemical shift values observed at 75 and 143 ppm correspond to $\sigma_{||}$ and $\sigma_\perp$ of the averaged nitrogen tensor.[14] In the case of helical peptides, the latter is close to the average between σ_{33} and a second value around σ_{11}/σ_{22}. The solid-state NMR results are indicative of fast rotational diffusion around the membrane normal concomitant with efficient averaging on both the ^{15}N chemical shift and the ^2H NMR time scales (10^{-5} s).

When the KL26 sample is tilted by 90°, the deuterium solid-state NMR spectrum shows a considerable spread of the ^2H spectral intensities (Figure 4D). This indicates that rotational diffusion of this polypeptide around the membrane normal is insufficient for averaging the anisotropy of the deuterium quadrupolar interactions, which requires rotational correlation times faster than 10^{-5} s. Furthermore, despite prolonged acquisition times the signal observed for [^{15}N-Leu11]-KL26 remains too weak to be analyzed. The situation did not improve even when the sample was heated to 310 K to augment rotational diffusion (not shown). Therefore, the 26-residue peptide LAH4,[55] which has been labeled with ^{15}N at three distinct amide positions, was also investigated by proton-decoupled ^{15}N NMR spectroscopy. At orientations of the membrane normal parallel to B_o, intensive and well-resolved resonances are observed which are indicative of in-plane orientations (not shown). However, the same sample exhibits only minor signal intensities at about 143 ppm when investigated at room temperature and at sample orientations perpendicular to the magnetic field direction (Figure 3E), thereby confirming the result obtained with KL26. When the sample is frozen, the full spectral intensity is recovered (Figure 3D).

Previously it has been demonstrated that interferences between electromagnetic irradiation of the NMR experiment and motions within the sample can occur.[58] Therefore, it remains possible that wobbling, libration, and rotational diffusion motions interfere with efficient cross polarization and decoupling using a ^1H field of 31 kHz. Furthermore, exchange processes on the 10^{-4} or 10^{-5} s scale result in fast T_2- and $T_{1\rho}$-relaxation. Indeed the ^2H NMR spectra as well as the solid-state NMR spectra obtained at orientations of the membrane normal parallel to the B_o direction indicate rotational diffusion rates > 10^{-5} s.

When the samples encompassing the KL14 peptides are

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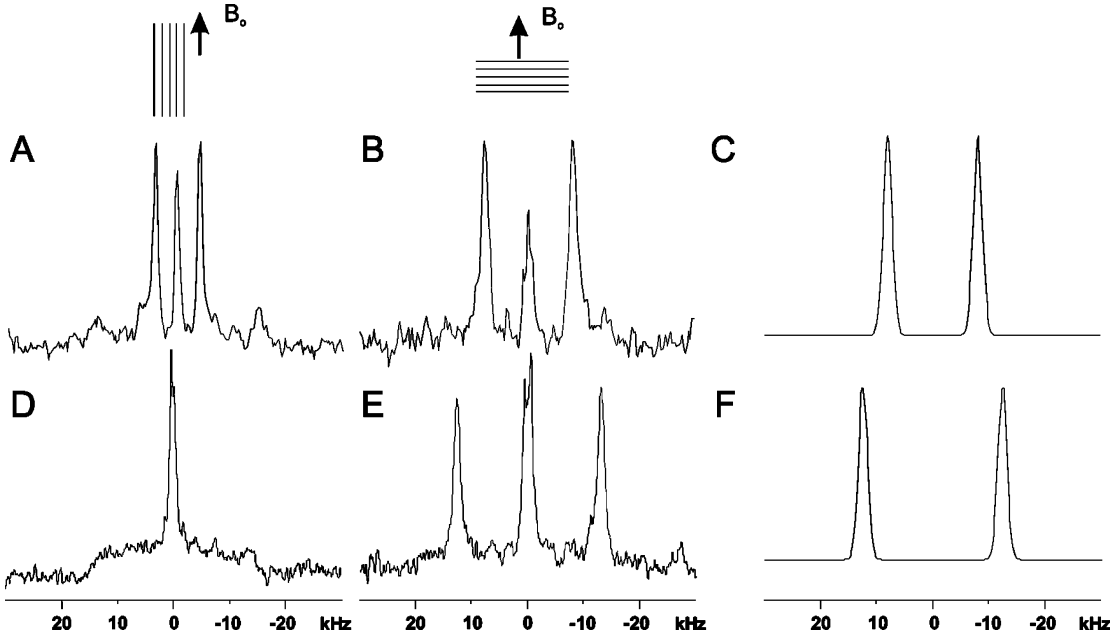

Figure 4. Deuterium solid-state NMR spectra of KL14, labeled with (3,3,3-$^{2}H_3$)-alanine at residue 7 and reconstituted into DMPC phospholipid bilayers (A,B) and of KL26 labeled with (3,3,3-$^{2}H_3$)-alanine at residue 13 and reconstituted into POPC phospholipid bilayers (D,E). The sample orientations of the membrane normal were either parallel (B,E) or perpendicular (A,D) to the magnetic field direction. To reproduce the spectral line shapes of the experimental spectra shown in panels B and E, Lorentzian lines exhibiting an orientation of the $C_\alpha-C_\beta$ direction relative to the magnetic field of 46° and 41.3° and a mosaic spread of 0.7° are simulated and shown in panels C and F, respectively.

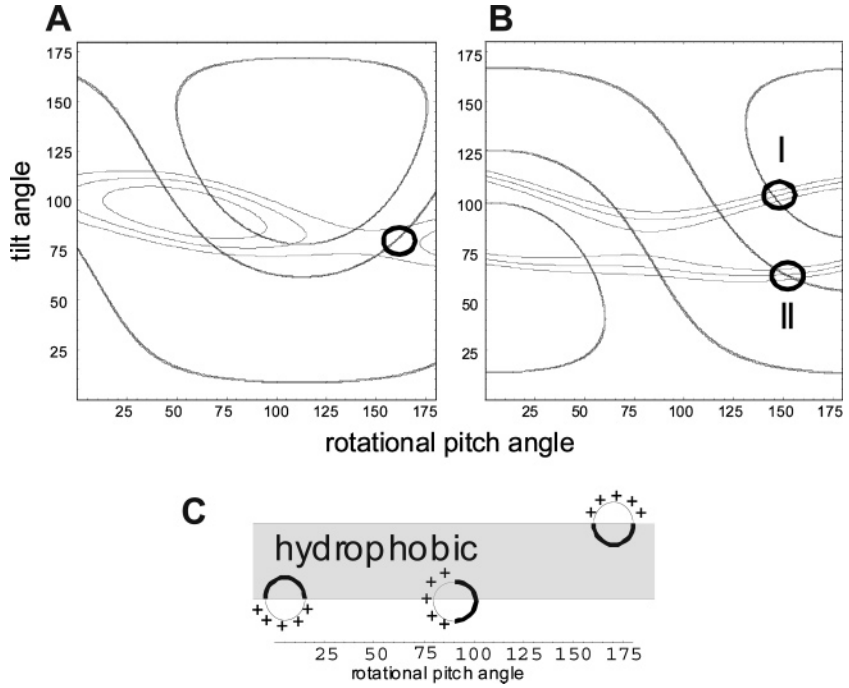

Figure 5. Membrane alignments of KL14 (A) und KL26 (B). For all possible orientations of the peptides, the ^{15}N chemical shift and the deuterium quadrupole splitting are calculated. Peptide alignments that are in agreement with the experimental measurements are shown as hatched (^{15}N) or solid (^{2}H) contours. Several peptide alignments agree with both NMR measurements, but only the circled combinations of tilt and rotational pitch angles are also in agreement with energetic considerations resulting from the amphipathic distribution of residues. (C) The effect of the rotational pitch angle on the relative alignment of an amphipathic helix at a 90° tilt angle is illustrated.

cooled below the gel-to-liquid crystalline phase transition temperature, the ^{2}H NMR solid-state NMR spectra undergo profound alterations (Figure 6). Upon decrease of the temperature, rotational diffusion within the membrane slows down, in particular when the gel state is reached. Therefore, ^{2}H spectra of KL14 in nonoriented DMPC bilayers reveal a powder pattern line shape with ^{2}H quadrupolar splittings of 36 kHz when recorded at 263 K (−10 °C). This is within the range of values observed when powders of (3,3,3)-deuterated alanines have been recorded covering a wide range of temperatures.[57]

The quadrupolar splitting of the transmembrane α-helical sequence hΦ19W[16,59] reconstituted in nonoriented DMPC

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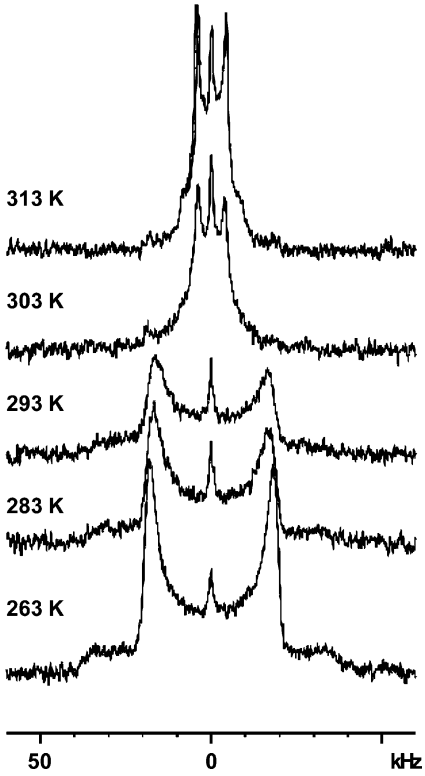

Figure 6. Deuterium NMR spectra of the surface-oriented peptide KL14 labeled with ^{15}N-leucine-10 and (3,3,3-$^{2}H_3$)-alanine-7 after reconstitution into nonoriented DMPC membranes at the temperatures indicated.

membranes increases from 8 kHz to 38 kHz when the sample is cooled to 263 K indicating that this type of investigation is also sensitive to the rotational diffusion rate of transmembrane oriented peptides (Figure 7). However, the quadrupolar splittings in the 38 kHz range are indicative that fast rotation of the methyl group around the $C_\alpha - C_\beta$ bond continues at -10 °C[57] even though efficient molecular diffusion is abolished in gel phase phospholipids (Figures 6 and 7).

When the data shown in Figures 6 and 7 are further analyzed, it becomes evident that not only the line shape but also the total signal intensity changes over the temperature range studied. The integrals are plotted in Figure 8 indicating a minimum close to the phase transition temperature. The overall deuterium signal intensities of the transmembrane and in-plane oriented peptides decrease by up to 90% and 50%, respectively.

Discussion

In this paper model compounds of different geometry and amphipathity were prepared, and their alignment and topology were characterized using solid-state NMR spectroscopic methods. Subsequently, the samples were investigated in more detail at tilted orientations where the membrane normal is oriented perpendicular to the magnetic field direction. The line shapes of ^{15}N and ^{2}H solid-state NMR spectra thus obtained were analyzed to provide information on the rotational diffusion constants of peptides when inserted into oriented lipid bilayers.

The peptides studied represent a transmembrane helical sequence as well as two in-plane oriented peptides of different length. Importantly, the measurements of peptide dynamics can be made with exactly the same samples that have also been used to obtain the constraints used during the determination of peptide topology and conformation. A direct correlation between

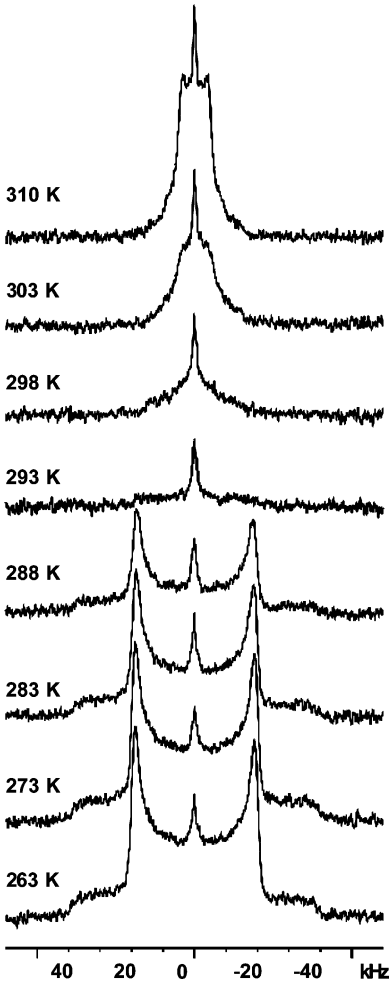

Figure 7. Deuterium NMR spectra of the transmembrane peptide hΦ19W labeled with ^{15}N-leucine-15 and (3,3,3-$^{2}H_3$)-alanine-14 after reconstitution into nonoriented DMPC membranes at the temperatures indicated.

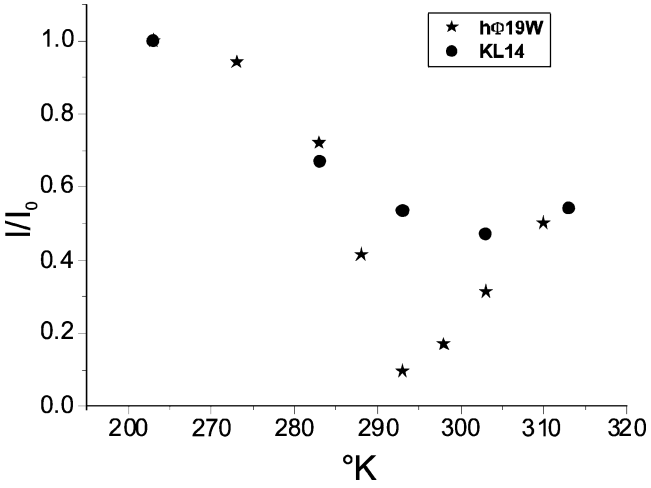

Figure 8. Integrated deuterium signal intensities of the spectra shown in Figures 6 and 7 are shown as a function of temperature. The signal intensities obtained from [(3,3,3-$^{2}H_3$)-alanine-7]-KL14 are shown as circles; those obtained from [(3,3,3-$^{2}H_3$)-alanine-14]- hΦ19W are shown as stars. In each case the signal intensities at 263 K are arbitrarily set to 1.

peptide structure and dynamics can thus be established. Despite the close resemblance of KL14 and KL26 in composition, structure, and alignment, the solid-state NMR spectra of these two peptides exhibit considerable differences at sample orienta-

tions with the membrane normal perpendicular to the magnetic field direction.

An estimate of the approximate dimension of KL14 when inserted into membranes is obtained by taking into account the propagation of an α-helix by 1.5 Å per residue. Thus the peptide exhibits a total length of approximately 20 Å and a helix diameter of about 10−12 Å. The solid-state NMR spectra of the KL14 sample, which are shown in Figures 3A,B and 4A,B, are characterized by efficient motional averaging of the quadrupolar splittings and of the ^{15}N chemical shift anisotropy. This observation is indicative of reorientional correlation times $< 10^{-5}$ s.

In contrast, the KL26 α-helix exhibits an estimated length of approximately 39 Å. The extension of the peptide main chain from 14 to 26 residues results in reorientational correlation times, which are significantly reduced and do not allow for efficient averaging of the orientational dispersion of the peptide within the membrane (Figure 4D). These data indicate that the rotational diffusion around the membrane normal has been reduced sufficiently to cause coalescence of deuterium lines from different alignments of the α-helical peptide. Taking the geometry and alignment of such peptides into consideration, membrane viscosity values of a few poise are obtained, in good agreement with previous investigations using optical or NMR techniques.[22,49,50] It should be noted, however, that the molecular dimensions used for the calculations remain estimates of the real situation. On the other hand, the change in properties observed when extending the length of KL peptides is in good agreement with our theoretical predictions, and these considerations, therefore, provide a reasonable framework to estimate the rotational diffusion rates of other membrane-inserted peptides.

When membrane samples containing in-plane or transmembrane oriented peptides are cooled, significant alterations of the spectral line shapes are observed (Figures 3D,E, 6, and 7). Major changes in the spectral characteristics are observed in particular in the proximity of the liquid crystalline−gel phase transition temperature of DMPC (23 °C). The spectra indicate that when the temperature is decreased the exchange between different orientations is slowed and vanishes when the gel phase is reached. In the proximity of the phase transition temperature these processes occur on intermediate time scales. The minima in signal intensity (Figure 8) correlate with changes in the spectral line shape (Figures 6 and 7) and, therefore, the onset of rotational diffusion in phospholipid bilayers.

A similar loss in signal intensity has been observed previously when deuterium solid-state NMR spectra of DPPC-d62-,[60] [4,4-$^{2}H_2$]-DPPE membranes,[61] or of ^{2}H exchange-labeled peptides of transmembrane orientation have been recorded.[62,63] The T_{2e} relaxation data of membrane-associated peptides and lipids show a minimum close to the phase transition temperature, with values in the 20−60 μs range for peptides[62,63] and 100−300 μs for phospholipids.[64,65] The T_{2e} minimum is thus associated with a modulation of the quadrupolar interactions by slow motions. This in turn results in the loss of phase memory during the two 50 μs interpulse delays used in the ^{2}H-echo solid-state NMR experiments.[64,66]

The molecular diffusion rate within the membrane does not only reflect the viscosity of the membrane environment but also the size of the diffusion unit (eq 3). Therefore, the NMR spectral line shapes at sample orientations perpendicular to the membrane normal can be used to investigate the aggregation state of the polypeptides and allow one to differentiate, for example, between a monomeric and an oligomeric state in the membrane. Whereas fast rotational diffusion is observed for lipids[47] or monomeric transmembrane peptides,[16] oligomers of the size of membrane channels are expected to move in a slow regime when compared to the ^{2}H NMR time scale. In contrast the ^{15}N chemical shift anisotropy at the magnetic field strength used in this study (9.4 T) is only sensitive to changes in diffusion constants when considerably larger aggregates are formed. Our measurements suggest that at room temperature averaging of the ^{15}N chemical shift anisotropy occurs as long as the effective radius of the complex does not exceed 30 Å. This radius corresponds to a circular aggregate of about 25 transmembrane helices. Reduction of rotational averaging on the ^{2}H solid-state NMR time scale already occurs at aggregate sizes encompassing an order of magnitude decreased number of units. Notably, a small "immobilzed" contribution has been observed also in oriented ^{2}H solid-state NMR spectra of hΦ19W.[16]

Whereas in previous work it has been well established that angular restraints for polypeptide structural analysis can be obtained from uniaxially oriented membrane samples, we recently suggested that under conditions of efficient motional averaging around the membrane normal the powder pattern line shapes can be analyzed to extract comparable information.[14,16] An analogous approach was used earlier to extract phospholipid order parameters and thus structural constraints from ^{2}H NMR solid-state NMR spectra.[17−20] The measurements and considerations presented in this paper specify in more detail the size limitations for this condition to be satisfied. At the same time, the paper outlines an approach that can be used to better test the aggregation behavior of polypeptides after they have been reconstituted into oriented membrane samples for structural analysis. Knowledge of the aggregation state is of key importance when correlations between peptide structure and function are desired.

The measurement of angular restraints by solid-state NMR spectroscopy on oriented bilayer samples has become an established method to investigate the structure and the orientation of membrane-associated peptides.[8,14,16] The results shown in this paper demonstrate the utility of this method to also study the polypeptide dynamical properties and thereby peptide aggregation within membranes.

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