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ARTICLE

# Structure, dynamics and topology of membrane polypeptides by oriented $^{2}$H solid-state NMR spectroscopy

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**Abstract** Knowledge of the structure, dynamics and interactions of polypeptides when associated with phospholipid bilayers is key to understanding the functional mechanisms of channels, antibiotics, signal- or translocation peptides. Solid-state NMR spectroscopy on samples uniaxially aligned relative to the magnetic field direction offers means to determine the alignment of polypeptide bonds and domains relative to the bilayer normal. Using this approach the $^{15}$N chemical shift of amide bonds provides a direct indicator of the approximate helical tilt, whereas the $^{2}$H solid-state NMR spectra acquired from peptides labelled with 3,3,3-$^{2}H_3$-alanines contain valuable complimentary information for a more accurate analysis of tilt and rotation pitch angles. The deuterium NMR line shapes are highly sensitive to small variations in the alignment of the $C_\alpha$–$C_\beta$ bond relative to the magnetic field direction and, therefore, also the orientational distribution of helices relative to the membrane normal. When the oriented membrane samples are investigated with their normal perpendicular to the magnetic field direction, the rate of rotational diffusion can be determined in a semi-quantitative manner and thereby the aggregation state of the peptides can be analysed. Here the deuterium NMR approach is first introduced showing results from model amphipathic helices. Thereafter investigations of the viral channel peptides $Vpu_{1–27}$ and Influenza A $M2_{22–46}$ are shown. Whereas the $^{15}$N chemical shift data confirm the transmembrane helix alignments of these hydrophobic sequences, the deuterium spectra indicate considerable mosaic spread in the helix orientations. At least two peptide populations with differing rotational correlation times are apparent in the deuterium spectra of the viral channels suggesting an equilibrium between monomeric peptides and oligomeric channel configurations under conditions where solid-state NMR structural studies of these peptides have previously been performed.

**Keywords** Transmembrane channel protein · Oriented lipid bilayer · Amphipathic α-helix · Membrane protein structure determination · Topology · Angular restraints · Tilt and rotational pitch angle · Vpu · Influenza M2

**Abbreviations**

| | |
|---|---|
| NMR | Nuclear magnetic resonance |
| KL14 | KKLLKKAKKLLKKL |
| POPC | 1-Palmitoyl-2-oleoyl-*sn*-glycero-3 phosphatidylcholine |

## Introduction

Although the high-resolution structural analysis has become routine for small soluble biomolecules, the

Dedicated to Prof. K. Arnold on the occasion of his 65th birthday.

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investigation of membrane proteins remains a major challenge for structural biology. Not only is the quantitative biochemical preparation of membrane proteins difficult, the X-ray or solution NMR techniques also require either the availability of crystalline samples or fast isotropic reorientation, respectively, conditions often difficult to obtain for this class of proteins. Therefore, the structural investigation of membrane proteins by the classical approaches has so far succeeded only in a few exceptional cases (Striebeck and Michel 2006). Furthermore, it should be kept in mind that high-resolution X-ray diffraction techniques provide a static view on the systems investigated and neither solubilized membrane proteins nor crystals thereof represent well their natural bilayer environment. On the other hand, solid-state NMR spectroscopy is an emerging technique for the structural analysis of biomolecules when associated with membranes and a variety of methods have been designed to study peptides or proteins when associated with extended lipid bilayers (reviewed, e.g. in Davis and Auger 1999; Watts 1999; Bechinger et al. 2004).

Although NMR interactions are a priori strongly dependent on the alignment of the molecules under observation relative to the magnetic field direction, fast rotational diffusion in solution results in averaging and, therefore, in such cases only the isotropic values are observed. Similarly, spinning of solid or semi-solid samples around the magic angle results in solid-state NMR spectra that resemble those obtained in solution. However, for static solid samples or samples where averaging is anisotropic the most pronounced features of the spectra can be attributed to the orientational dependence of NMR interactions. For example, during the $^{31}$P solid-state NMR investigations of extended phospholipid vesicles so called powder pattern line shapes are obtained which are composed of the signals of the individual molecular alignments relative to the magnetic field of the spectrometer ($B_0$). This anisotropy of interactions can be used advantageously by investigating samples that are uniaxially aligned relative to $B_0$. Here the molecules express unique sets of chemical shifts, dipolar couplings or quadrupolar interactions in an alignment-dependent manner. These measurements therefore provide valuable information about the relative orientation of bonds and molecules relative to the magnetic field direction, and have been successfully applied in the past to obtain angular constraints from membrane-associated polypeptides. In most cases the orientation-dependent $^{15}$N or $^{13}$C chemical shifts have been analysed but dipolar interactions or quadrupolar splittings have also been investigated (Smith et al. 1994; Cross 1997; Bechinger et al. 2004). The $^{15}$N chemical shift has proven particularly valuable when the alignment of the main axis of α-helical polypeptides needs to be known. The $^{15}$N chemical shift tensor, which describes the anisotropic interactions of the $^{15}$N nucleus with the magnetic field, is characterized by a combination of favourable properties in a manner that the approximate orientation of a helix is directly obtained from the $^{15}$N chemical shift (Bechinger and Sizun 2003). Nitrogen-15 chemical shift measurements in oriented membrane samples have been used as an analytical tool during membrane polypeptide structural (Cross 1997; Aisenbrey et al. 2006a, c) or biophysical investigations (Bechinger 1996; Harzer and Bechinger 2000; Aisenbrey et al. 2006b).

In comparison, only a few studies using the $^{2}$H nucleus have been performed in oriented samples although $^{2}$H NMR spectroscopy is well established during the investigation of membrane-associated lipids (e.g. Trouard et al. 1999; Griffin 1981; Seelig et al. 1987; Marsan et al. 1999), -polypeptides (Lee et al. 1993; Rothgeb and Oldfield 1981; Pauls et al. 1985; Davis 1988; Sharpe and Grant 2000; Sharpe et al. 2002; Ozdirekcan et al. 2005), or -water (Volke et al. 1994; Finer 1973; Mendonca de Moraes and Bechinger 2004). Furthermore, solid-state NMR data obtained from $^{2}$H labelled sites has made important contributions during the structure determination of gramicidin A, in particular its side chain conformation (Prosser et al. 1991, 1994; Cross 1997), or of the retinal moiety in rhodopsin (Grobner et al. 1998).

Whereas the $^{2}$H nucleus has been intensely used to reveal the structural detail of polypeptide side chain angular constraints (Cross 1997), the investigation of backbone labelled sites in many cases has been hampered by the low signal intensities obtained from these atoms. However, the alanine methyl group combines the advantages of a side chain undergoing a high level of motions and thereby exhibiting favourable properties for NMR acquisitions when at the same time it is directly connected to the polypeptide backbone. Recently, we and others have therefore systematically investigated how peptides labelled with $^{2}$H at the methyl group of alanines can be used during the investigation of polypeptides that are reconstituted into oriented phospholipid bilayers (Strandberg et al. 2004; Aisenbrey and Bechinger 2004a, b).

The deuterated alanine methyl group offers several distinct advantages. First, alanine is an abundant amino acid that can often be found in membrane interacting sequences, therefore, many sites can be labelled without the need to modify the sequence. Second, the methyl group is directly attached to the peptide backbone where the $C_\alpha$–$C_\beta$ bond exhibits a well-defined

orientation with respect to the helix axis. Therefore, the alignment of this bond is directly related to the orientation of the helix backbone. Third, the methyl group carries three equivalent deuterons that are in fast exchange at ambient temperatures (Batchelder et al. 1983). The resulting superposition of signal intensities from three sites increases the sensitivity of the $^2$H NMR measurement. Four, the deuterium $T_1$ relaxation times are short and the pulse echo sequences used during the deuterium NMR spectroscopy allow fast repetition times. Although the total intensity of the deuterium NMR signal is affected by the local and global dynamics of the peptide in as much as these have an influence on $^2$H relaxation, this effect is less pronounced than during cross polarization experiments which are typically used in solid-state NMR investigations to enhance the signal intensity of heteronuclei such as $^{15}$N or $^{13}$C. However, these experiments are hampered by a high degree of motions at the labelled sites which would reduce or abolish the dipolar interactions that mediate the magnetization transfer to the low-$\gamma$ nuclei and as a consequence such conditions result in negligible signal intensities (Hallock et al. 2002; Aisenbrey and Bechinger 2004b, 2006c).

## Materials and methods

The peptides were prepared by solid phase peptide synthesis using fmoc chemistry and an automated Millipore 9050 synthesizer. The sequence of KL14 is KKLLKK*A*KK*L*LKKL where position 7 is labelled with (2,2,2-$^2H_3$-alanine) and the leucine-10 amide with $^{15}$N. $Vpu_{1–27}$ was prepared with two isotopic labels: $^2H_3$-Ala18 and $^{15}$N-Ala14. The $M2_{22–46}$ polypeptide was labelled at $^2H_3$-Ala29 and $^{15}$N-Ala30. The primary structures of these sequences are given in Wray et al. (1999) and Nishimura et al. (2002), respectively.

The solid-state NMR samples were prepared in the following manner: 7 mg of the $Vpu_{1–27}$ peptide was co-dissolved with 200 mg of POPC in 2 ml hexafluoroisopropanol. For the preparation of the sample encompassing the $M2_{22–46}$ domain of Influenza A 6 mg of the peptide and 200 mg DMPC were co-dissolved in 2 ml methanol containing 20 µl of $H_2O$. The mixtures were spread on 30 ultra thin cover glasses (9 mm × 22 mm; Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and dried first on air and thereafter in high vacuum. The samples were equilibrated at 93% humidity at room temperature or 37°C in case of POPC and DMPC, respectively. After stacking the glass plates on top of each other the samples were stabilized and sealed by Teflon tape and plastic wrappings. The NMR measurements and the quantitative analysis of data have been performed in an identical or analogous manner to the procedures described in Aisenbrey and Bechinger (2004a, b).

## Results and discussion

The $^2$H spectrum of the amphipathic model peptide KL14, reconstituted in POPC phospholipid bilayers oriented with the membrane normal parallel to the magnetic field direction is shown in Fig. 1a. The deuterium nucleus exhibits a spin 1 and therefore allows for two Zeeman transitions of $\Delta m = 1$ which are perturbed by the quadrupolar interactions (Fig. 1c). Therefore, two intensities, one up- and one downfield from the isotropic deuterium chemical shift, are observed, the distance between these resonances defines the deuterium quadrupole splitting ($\Delta\nu_Q$). For example, the spectrum shown in Fig. 1a exhibits a quadrupolar splitting of 15.3 kHz.

Fast rotational diffusion around the $C_\alpha$–$C_\beta$ bond ensures that the resulting $^2$H tensor is axially symmetric with respect to this vector and the measured splitting $\Delta\nu_Q$ is directly related to the angle $\Theta$ describing the orientation of the $C_\alpha$–$C_\beta$ bond relative to the magnetic field direction (Aisenbrey and Bechinger 2004b): $\Delta\nu_Q = \frac{3}{2}\frac{e^2qQ}{h}\frac{(3\cos^2\Theta-1)}{2}$, where the factor $\frac{e^2qQ}{h}$ is the static quadrupolar coupling constant (Seelig 1977), and amounts to about 50 kHz for the alanyl methyl group undergoing rotation around the $C_\alpha$–$C_\beta$ bond (Batchelder et al. 1983).

Although the measured quadrupolar interaction does not define the alignment of the helix unambiguously it allows one to significantly reduce the number of possibilities. This is demonstrated in Fig. 2 where the solid contours indicate the helix orientations that agree with the measured quadrupolar splitting $\Delta\nu_Q = 15.3 \pm 1$ kHz. Furthermore, the $^{15}$N chemical shift of 87 ppm obtained from position 10 of the same polypeptide helix is a good indicator of the helical tilt angle and thereby provides additional angular restraints (Bechinger and Sizun 2003). The intersections between the two topology plots indicate those peptide alignments that agree with both measurements (Fig. 2, circles). Therefore, by combining both data sets the number of angular pairs is considerably reduced. Notably, of the six remaining possible alignments the energetically most favourable one can be identified by modelling calculations (Aisenbrey and Bechinger 2004b; Aisenbrey et al. 2006c).

In view of the advantages offered by the $^2$H solid-state NMR spectroscopic analysis of oriented samples

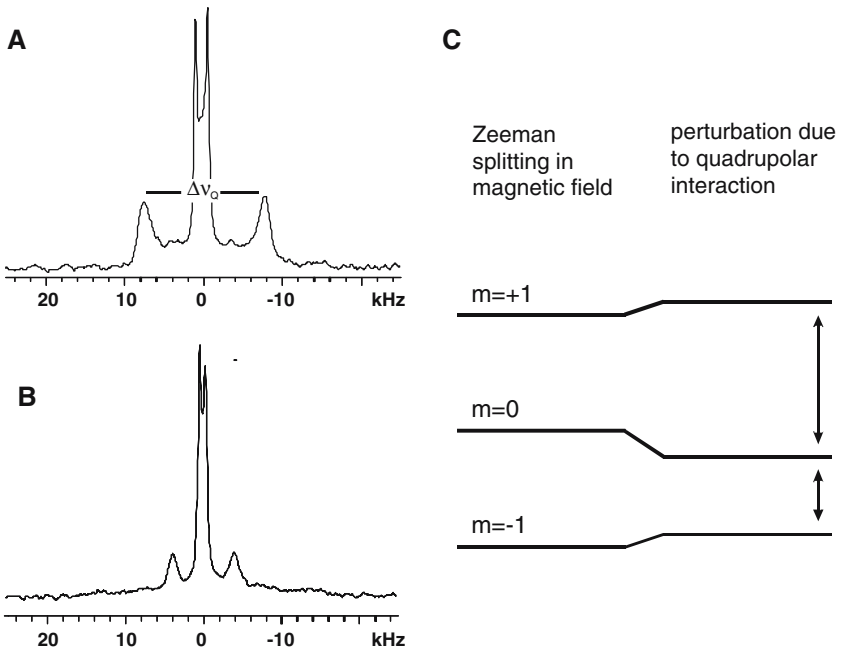


**Fig. 1** $^{2}$H solid-state NMR spectrum of 2 mol% [$^{2}H_3$-Ala7]-KL14 (**a**, **b**) in oriented POPC phospholipid bilayers with the membrane normal parallel (**a**) and perpendicular (**b**) to the magnetic field direction. In *panel a*, the quadrupolar splitting ($\Delta\nu_Q$) of the labelled alanine methyl group is indicated by the *double arrow* whereas the quadrupolar splitting of the residual water deuterons exhibit a much smaller spitting in the center of the spectra (adapted from Finer 1973; Mendonca de Moraes and Bechinger 2004). **c** The spin 1 deuterium nucleus is characterized by three energy levels and thereby exhibits two Zeeman transitions with $\Delta m = 1$. The transitions are modulated by the quadrupolar interaction, therefore the two transitions exhibit different resonance frequencies that are separated by the deuterium quadrupole splitting ($\Delta\nu_Q$)

it seems surprising that relatively few data have been published using this approach. Oriented deuterium solid-state NMR spectroscopy has been applied in only a few cases including during investigations of the effects of hydrophobic mismatch on peptide alignment (Van Der Wel et al. 2002; Strandberg et al. 2004) or when peptides associated with magnetically aligned bicelles were investigated (Glover et al. 2001; Whiles et al. 2001, 2002). In our laboratory we found that oriented $^{2}$H solid-state NMR spectroscopy develops its full capacity when used in combination with a $^{15}$N chemical shift analysis, as taken together they can accurately determine the helix topology and thereby provide much more information than each of the measurements alone (Aisenbrey and Bechinger 2004a, b, 2006c). Whereas the $^{15}$N chemical shift (and, although not shown here, the $^{1}$H–$^{15}$N dipolar interaction) is a good indicator of the approximate tilt angle of the α-helix (Bechinger and Sizun 2003), the rotation angle around the helix long axis (rotational pitch angle) is obtained only in conjunction with additional deuterium NMR investigations. The pitch angle is of particular importance for in-plane oriented interfacial locations of the helix as even small changes in the rotational pitch angles can dramatically change the penetration depth of individual amino acids relative to the membrane surface and thereby the local chemical environment of a given amino acid.

The $^{2}$H line shape of the alanyl methyl group has been shown to be strongly dependent on the alignments of the $C_\alpha$–$C_\beta$ bond relative to the magnetic field direction. Our study on perfectly amphipathic model peptides has revealed significant changes in $\Delta\nu_Q$ by relatively small modifications of the fatty acyl chain composition of phosphatidylcholine bilayers (Aisenbrey and Bechinger 2004b). For example, for the model peptides presented here a 4 kHz change in quadrupolar splitting reflects a change in tilt angle of the peptide helices of as little as 2°. This high sensitivity of these measurements is obtained when the $C_\alpha$–$C_\beta$ bond is oriented close to the magic angle and makes it possible to monitor even small topological differences. However, the pronounced dependence of the quadrupolar splitting on the helix alignments also results in broadened line shapes when the peptide exhibits not a single alignment but rather an orientational distribution. A Gaussian distribution of helix alignments with mosaic spread of, for example, 5° or 10° suffices to broaden the transitions to the extend that the two well-defined transitions melt into a single intensity, an effect

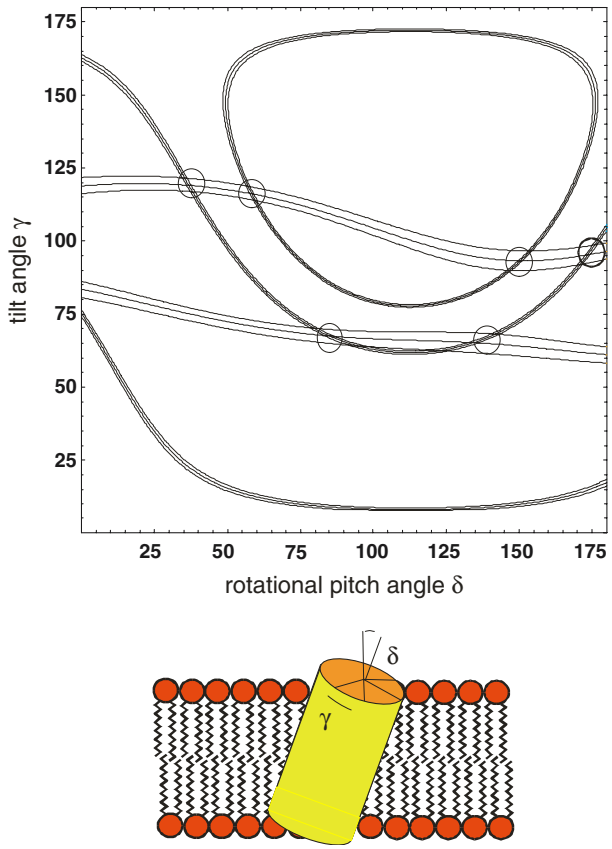


**Fig. 2** Contour plots showing the tilt angle/rotational pitch angular pairs ($\delta/\gamma$) that agree with the $^2$H quadrupolar splitting obtained from [$^2H_3$-Ala7]-KL14 in oriented POPC bilayers (cf. Fig. 1a) and a $^{15}$N chemical shift obtained from the same peptide labelled with $^{15}$N at Leu-10. The *solid lines* indicate the orientation restraints from the deuterium site (15.3 ± 1 kHz), the *hatched lines* those from the $^{15}$N chemical shift position (87 ± 5 ppm). The six *circled regions* of overlap agree with both experimental measurements, the *enhanced circle* represents the region that is energetically the most favourable alignment in the membrane and corresponds to a tilt angle of 95° and a rotational pitch angle of 174° (±4°)

illustrated in Fig. 3 for Θ = 60°. However, the detailed line shape and the effects of superimposing a Gaussian distribution also strongly depends on the average value of Θ (Aisenbrey and Bechinger 2004b), and a detailed analysis requires spectral simulations such as those presented in Fig. 3. It becomes obvious from such spectral simulations that in case of the KL14 peptide the mosaicity of the amphipathic model helix reconstituted into oriented phospholipid bilayers is only about 1° (Figs. 1, 3).

The $^2$H solid-state NMR spectra of the two viral channel peptides $M2_{22–46}$ of Influenza A and $Vpu_{1–27}$ of the human immunodeficiency virus 1 (HIV-1) are shown in Fig. 4. Peptides of the composition used in this work have been shown to exhibit channel activities in phospholipid bilayers (Schubert et al. 1996; Duff and Ashley 1992). The spectra are indicative of considerable mosaic spread of these polypeptides relative to the magnetic field direction. The tilt angles of the $M2_{22–46}$ segment of the Influenza A virus in the absence and presence of the channel blocker amantadine (Nishimura et al. 2002; Bechinger et al. 1999) as well as of the $Vpu_{1–27}$ transmembrane domain of HIV-1 (Wray et al. 1999; Park et al. 2003) have been determined previously in considerable detail using proton-decoupled $^{15}$N solid-state NMR spectroscopy. The Vpu transmembrane domain has also been investigated recently using MAS solid-state NMR spectroscopy (Sharpe et al. 2006). Furthermore, structural investigations with the aim to determine tilt and rotational pitch angles as well as to visualize the aggregation states of the channels have been performed by FTIR spectroscopy and modelling approaches (Kukol and Arkin 1999; Kukol et al. 1999; Zhong et al. 2000; Lopez et al. 2002; Fischer and Sansom 2002).

The deuterium solid-state NMR spectrum of Vpu shows a broad intensity with a shoulder exhibiting a splitting of about 27 kHz (Fig. 4a). The sharp central lines arise from the naturally abundant water deuterons. A small residual splitting of 1.55 kHz is observed also for the solvent molecules as the water interacts with the oriented phospholipid membranes. The proton-decoupled $^{15}$N spectrum of the peptide of the same sample exhibits a resonance at 217 ppm (not shown), thereby confirming the transmembrane alignment of this domain (Wray et al. 1999).

The deuterium spectra of the Influenza M2 peptide exhibit broad spectral line shapes with shoulders corresponding to quadrupolar splittings of about 24 and 36 kHz (Fig. 4c). The features of this signal could be simulated within experimental error using two components with 5° and 10° mosaic spread both adopting helix alignments determined previously using $^{15}$N solid-state NMR investigations (Nishimura et al. 2002). The proton-decoupled $^{15}$N solid-state NMR spectrum of the same sample exhibits a $^{15}$N chemical shift of about 212 ppm (Fig. 4e) indicative of a transmembrane helix alignment (Bechinger et al. 1999).

Peptides and proteins when associated with hydrated liquid crystalline bilayers laterally diffuse and rotate within and along the membrane surface in a highly dynamic fashion. In addition they are free to temporarily and reversibly associate into oligomeric structures (Sansom 1991). Therefore, also in the membrane samples investigated by solid-state NMR spectroscopy there is a high probability that monomeric and oligomeric structures as well as open and closed channel structures co-exists. Clearly such configurational equilibria should be taken into consider-

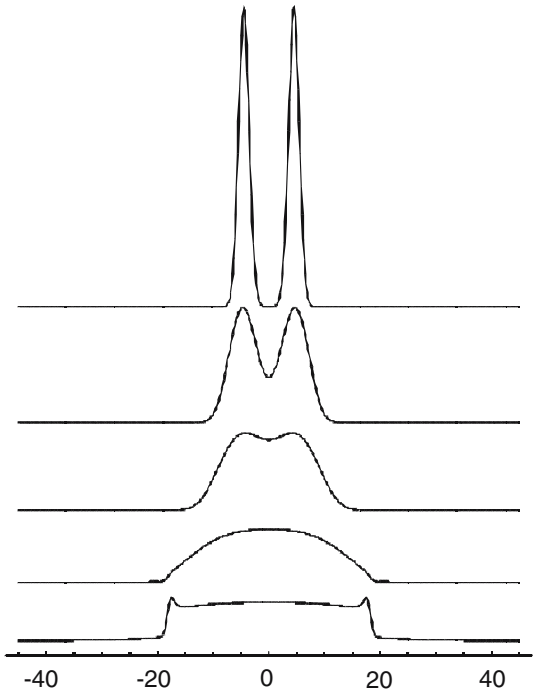


**Fig. 3** Simulations of the deuterium solid-state NMR spectra of $^{2}H_3$-alanine with the $C_\alpha$–$C_\beta$ axis oriented at 60° with respect to the magnetic field direction and exhibiting a Gaussian mosaic spread of 1°, 3°, 5°, 10° or 15° (*top* to *bottom*)

ation when the solid-state NMR data are analysed in terms of structural restraints or of functional models. An unambiguous analysis of this data would require a detailed knowledge of the aggregation state of the polypeptides within the sample, a piece of information not easily available. In previous investigations models of eukaryotic or viral channels have, therefore, been proposed which were based on solid-state NMR data and on the assumption that that samples are characterized by a homogenous aggregation state of the peptides in the oriented lipid bilayers (Nishimura et al. 2002; Park et al. 2003).

Whereas optical methods have been used in the past to study lateral diffusion as well as distances between peptides, these techniques require that the polypeptides carry chromophores either naturally or by modification. These alterations by themselves might have an effect on the oligomerization equilibria and they necessitate the preparation of additional peptide samples. We have therefore evaluated the potential of using the same samples that have provided the $^{15}N$ and $^{2}H$ solid-state NMR data for structural studies also for investigations of the aggregation state within membrane (Figs. 1a, 2, 3a, c).

The solid-state NMR structural analyses described above are performed on static oriented samples where the bilayer normal is aligned parallel to the magnetic field of the NMR spectrometer. With this arrangement lateral or rotational diffusion around the membrane normal does not affect the alignment of the polypeptide relative to the magnetic field direction or the resulting spectra (Fig. 5, $\beta = 0$). Therefore, the resulting solid-state NMR signal is a direct function of the molecular alignment relative to the bilayer normal/

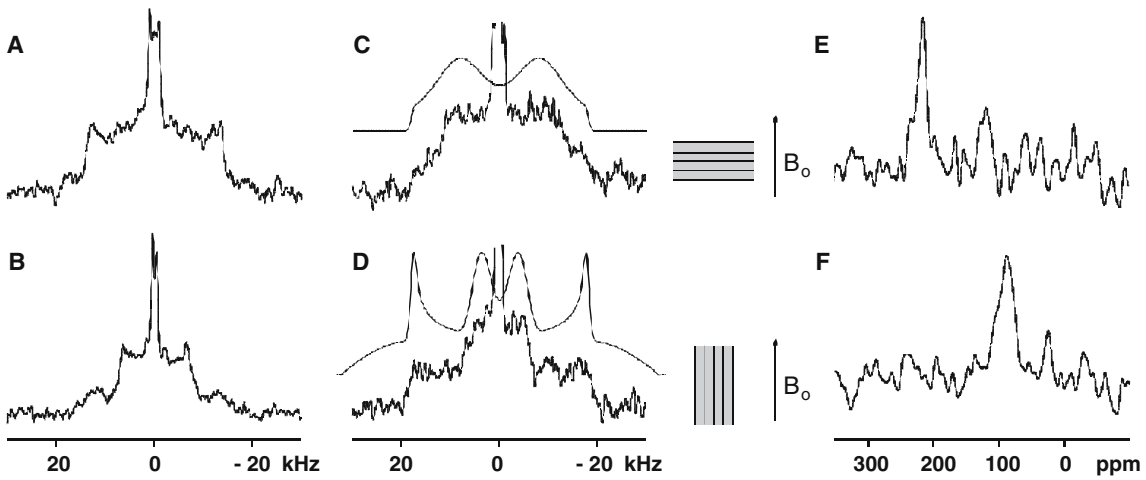


**Fig. 4** $^{2}H$ solid-state NMR spectra of 0.6 mol% of the transmembrane segment [$^{2}H_3$-Ala18; $^{15}N$-Ala14]-$Vpu_{1–27}$ (**a**, **b**) in oriented POPC phospholipid bilayers at room temperature and of 0.75 mol% [$^{2}H_3$-Ala29; $^{15}N$-Ala30]-$M2_{22–46}$ domain of Influenza A reconstituted into oriented DMPC phospholipid bilayers recorded at 310 K (**c**, **d**). The deuterium spectra of the M2 segment were simulated by using two components with $C_\alpha$–$C_\beta$ alignments relative to the membrane normal of 65°. The mosaic spread of the first component is 10° and that of the second 5°, their ratio is 2:1. *Panels e* and *f* show the proton-decoupled $^{15}N$ solid-state NMR spectra of the same samples of (**c**) and (**d**). The membrane normal was either parallel (**a**, **c**, **e**) or perpendicular (**b**, **d**, **f**) to the magnetic field direction $B_0$

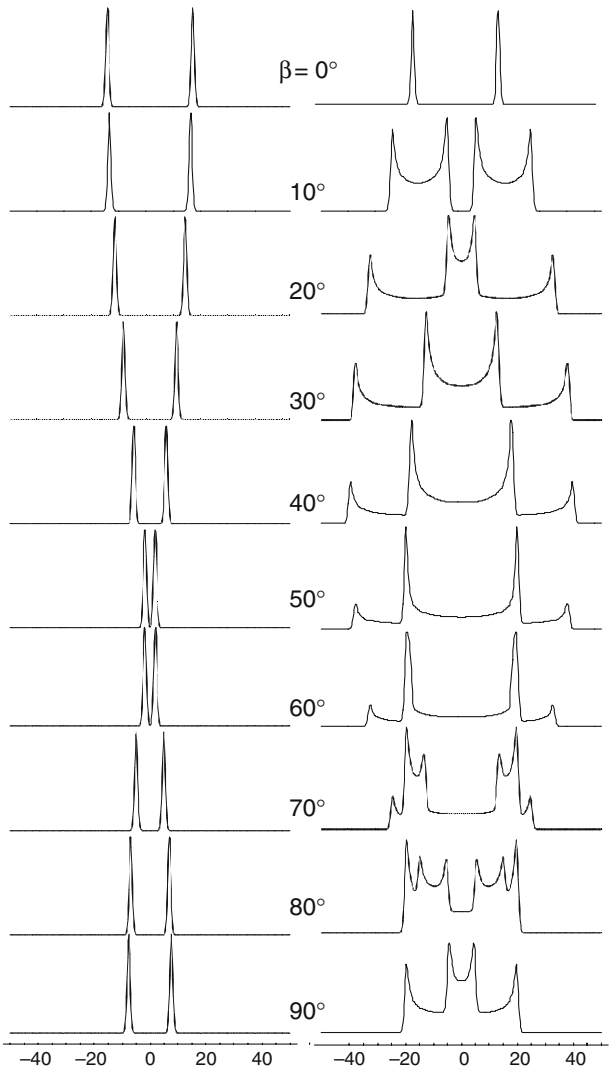


**Fig. 5** $^2$H solid-state NMR spectrum of an oriented membrane sample with semi-angle $\varphi = 40°$ as a function of sample alignment $\beta$ relative to the magnetic field directions under conditions of fast (*left column*) and slow motional averaging around the membrane normal (*right column*). During the simulations a Gaussian line broadening of 500 Hz was applied. Notably, the simulations also indicate that tilting the sample to the magic angle (54.7°) might provide even better means to separate contributions exhibiting fast ($\Delta\nu_Q = 0$) and slow rotation, an approach currently tested by us

$B_0$-vector (Bechinger et al. 2004). Only for high-precision structural analyses averaging of the interaction anisotropies by wobbling, vibrational motions, conformational changes or rotational diffusion needs to be taken into consideration (Lazo et al. 1993; Bechinger and Sizun 2003; Straus et al. 2003).

In contrast, when the sample is tilted, the proteins can adopt a wide variety of alignments. These are symmetrically distributed around the membrane normal, but vary relative to the magnetic field direction. When the sample is frozen and motional averaging can be ignored the resulting signal reflects these different alignments relative to the magnetic field direction and the line shape is a function of the sample tilt angle $\beta$ (Fig. 5).

However, in hydrated liquid crystalline membranes the lipids and peptides often freely diffuse about the membrane normal. Therefore, the $C_\alpha$–$C_\beta$ bond moves on a cone of semi-angle $\varphi$ relative to this direction. In cases where rotational diffusion is fast the averaged quadrupolar splitting is given by Seelig (1977): $\Delta\nu_Q = \frac{3}{2}\frac{e^2qQ}{h}\frac{(3\cos^2\varphi-1)}{2}\frac{(3\cos^2\beta-1)}{2}$ and illustrated in Fig. 5. The comparison of $^2$H solid-state spectra of a given sample once obtained at sample alignments with the membrane normal parallel or perpendicular to the magnetic field direction therefore provides a qualitative indicator of the rotational diffusion rate. In cases where diffusion is fast the quadrupolar splitting of the tilted spectrum ($\beta = 90°$) is scaled by a factor of –1/2 when compared to the parallel alignment ($\beta = 0°$). This is demonstrated in Fig. 1a, b where the $^2$H NMR spectra of the peptide within the same sample at parallel and perpendicular alignments are 15.3 and 8.1 kHz, respectively. In a similar manner the water line splitting decreases from 1.55 to 0.78 kHz (Fig. 1a, b). In contrast, in cases of slow diffusion broadened signal intensities are obtained (Aisenbrey and Bechinger 2004a). Additional motions of the peptide relative to the membrane normal are taken into consideration by calculating the time average of $(3\cos^2\varphi - 1)$.

Whether the signals arising from different orientations are broadened 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 labelled alanine methyl group shift as a function of the molecular alignments within a range ≤80 kHz. In contrast, at a magnetic field strength of 9.4 Tesla (40.5 MHz Larmor frequency) the anisotropy of the $^{15}$N chemical shift of the peptide bond is an order of magnitude reduced. Signal averaging of deuterium lines requires correspondingly higher rotational diffusion rates when compared to the one order of magnitude reduced $^{15}$N chemical shift dispersion. Therefore, the time scale to be tested can be selected by the choice of the NMR measurable and, in case of chemical shift interactions, the magnetic field strength.

The rotational diffusion rate is a function of the membrane viscosity, the size and the shape of the peptide or the aggregation state within the membrane. For an object with surface $S$ in an environment characterized by the viscosity coefficient $\eta$, the rotational diffusion coefficient $D_{pep}$ at absolute temperature $T$ is

given by: $D_{\text{pep}} = \frac{1}{4}\frac{k_B T \times F}{S \times h\eta}$, where $k_B$ is the Boltzmann constant, $F$ the shape factor, and $h$ the thickness within the membrane (Axelrod et al. 1976; Cherry 1979; Wang and Ho 2002). For an ellipse with major and minor axis 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 (Pauls et al. 1985): $D_{\text{pep}} = \tau_c^{-1}$ and thus the anisotropy of the NMR interaction.

The projection of the molecule along the membrane normal $a$ provides a first approximation to model the rotational diffusion rate of membrane-inserted peptides that have been labelled with $^2H_3$-alanine. Thus when assuming $\eta = 5$ poise (Gennis 1989), the limiting radius corresponding to a rotational diffusion at about 80 kHz is of the same order of magnitude as the peptide diameter. As a consequence, the $^2H$ solid-state NMR spectra of $^2H_3$-alanine labelled polypeptides reflect the peptide size as well as their aggregation state in the membrane (Aisenbrey and Bechinger 2004a). This has previously been illustrated by comparing amphipathic peptides aligned parallel to the membrane surface. When the peptide is 14 residues long $\Delta\nu_Q$ exhibits the theoretical –1/2 scaling factor after a 90° tilt of the sample. This result is indicative of fast averaging of this short peptide. In contrast, a broad composite signal is observed upon extension of the peptide length to 26 amino acids indicating that rotational diffusion is slow on the $^2H$ NMR time scale (Aisenbrey and Bechinger 2004a). Furthermore, a sharp change in diffusion rate is observed for all systems investigated when the membranes are cooled below the phospholipid gel-to-liquid crystalline phase transition.

The deuterium NMR signal of the Vpu and M2 polypeptides reconstituted into phospholipid membranes and inserted with the normal perpendicular to the magnetic field direction are shown in Fig. 4b, d, f. Tilting the sample results in a reduction from 27 to 13.4 kHz of most of the deuterium signal intensity of the Vpu transmembrane helical polypeptide indicating that this peptide is predominantly in a monomeric state (Fig. 4b). This result is in agreement with gel electrophoretic analyses of a related sequence. In these experiments the peptides migrate as monomers in the presence of sodium dodecylsulphate but exhibit a monomer–oligomer distribution when the detergent is perfluorooctanoic acid (Park et al. 2003).

Tilting the sample encompassing the M2 peptide results in averaging of about 1/3 of the signal intensity which now exhibits shoulders at about 13 kHz and a broad signal intensity which extends to a quadrupolar splitting to about 38 kHz (Fig. 4d). Simulation of this spectrum by simply summing two contributions, although successful for the spectrum shown in Fig. 4c, represents only some of the features of spectrum 4D. Nevertheless this analysis excludes simple models where a homogenous population of membrane-associated peptides exists. In contrast the spectral features suggest that at least two populations exchange with each other on an intermediate time scale. Notably, conformational equilibria that are a function of the environmental conditions have been observed by EPR spectroscopy when the transmembrane segment of M2 has been investigated (Duong-Ly et al. 2005).

Although the NMR spectra of the viral channel peptides do not reveal well-resolved quadrupolar peak pairs (Fig. 4a–d), thereby contrasting the amphipathic model peptides (Fig. 1a, b), they show that a considerable fraction of the viral peptides undergoes fast rotational averaging. Fast rotational diffusion on the $^2H$ NMR time scale is indicative that the complexes are composed by ≤3 transmembrane helices (Aisenbrey and Bechinger 2004a). As ultracentrifugation experiments in the presence of DPC micelles and the quantitative analysis of thiol-disulphide reactions in the presence of phospholipid bilayers have established the presence of sensitive monomer–tetramer equilibria of this fragment (Salom et al. 2000; Cristian et al. 2003), our NMR data suggests that these averaged signal intensities represent M2 monomers. On the other hand, the signal intensities associated with large quadrupolar splitting Can be attributed to oligomeric structures including the tetrameric (M2) or pentameric (Vpu) aggregates that have been suggested previously to form transmembrane channels (Sansom et al. 1998). Clearly, however, an equilibrium situation exists where monomeric and oligomeric structures are in exchange. The lateral diffusion of helices and their assembly/disassembly into oligomeric transmembrane helical bundles are probably of considerable importance as they form the basis for the opening and closing of membrane channels (Sansom 1991). The distribution of mono- and tetrameric signal intensities is in excellent agreement with the previously measured equilibrium constant of the M2 transmembrane fragment (Cristian et al. 2003). Only a fraction of the peptides exhibits broad signal intensities and slow correlation times. Therefore, in many cases the situation might be more complex and not well represented by a homogenous population of channel aggregates. Notably the interactions, helical tilt angle, the aggregation and channel properties are all functions of the membrane lipid composition as well as the exact composition and length of the polypeptides investigated (Lemmon et al. 1992; Harzer and Bechinger 2000; Park et al. 2003; Duong-Ly et al. 2005). The $^2H$ solid-state NMR

approach provides a means to test in a semi-quantitative manner the aggregation of the polypeptides in lipid bilayers using the identical samples that are also used for the structure determination of these polypeptides by oriented solid-state NMR spectroscopy (Aisenbrey and Bechinger 2004a).

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