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ARTICLE

Determining the depth of insertion of dynamically invisible membrane peptides by gel-phase ^{1}H spin diffusion heteronuclear correlation NMR

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Abstract Solid-state NMR determination of the depth of insertion of membrane peptides and proteins has so far utilized ^{1}H spin diffusion and paramagnetic relaxation enhancement experiments, which are typically conducted in the liquid-crystalline phase of the lipid bilayer. For membrane proteins or peptide assemblies that undergo intermediate-timescale motion in the liquid-crystalline membrane, these approaches are no longer applicable because the protein signals are broadened beyond detection. Here we show that the rigid-solid HETCOR experiment, with an additional spin diffusion period, can be used to determine the depth of proteins in gel-phase lipid membranes, where the proteins are immobilized to give high-intensity solid-state NMR spectra. Demonstration on two membrane peptides with known insertion depths shows that well-inserted peptides give rise to high lipid cross peak intensities and low water cross peaks within a modest spin diffusion mixing time, while surface-bound peptides have higher water than lipid cross peaks. Furthermore, well-inserted membrane peptides have nearly identical ^{1}H cross sections as the lipid chains, indicating equilibration of the peptide and lipid magnetization. Using this approach, we measured the membrane topology of the α-helical fusion peptide of the paramyxovirus, PIV5, in the anionic POPC/POPG membrane, in which the peptide undergoes intermediate-timescale motion at physiological temperature. The gel-phase HETCOR spectra indicate that the α-helical fusion peptide is well inserted into the POPC/POPG bilayer, spanning both leaflets. This insertion motif gives insight into the functional role of the α-helical PIV5 fusion peptide in virus-cell membrane fusion.

Keywords Intermediate motion · HETCOR · Magic-angle spinning · Fusion peptide · Membrane topology · Solid-state NMR

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Introduction

The depth of insertion of membrane peptides and proteins is an important aspect of membrane protein structure determination. A wide range of biophysical techniques such as fluorescence spectroscopy (Chattopadhyay and London 1987; Kleinschmidt and Tamm 1996; Voglino et al. 1999; Zoonens et al. 2008), neutron diffraction (Bradshaw et al. 1998; Chenal et al. 2009), hydrogen–deuterium exchange combined with infrared spectroscopy (Hohlweg et al. 2012), have been used to determine membrane protein depths. Compared to these techniques, NMR spectroscopy has the advantages of providing site-specific depth information without introducing bulky probes that may perturb the structure of the membrane proteins or lipids. One NMR approach is to use paramagnetic relaxation enhancement (PRE) (Solomon 1955) of nuclear spins induced by paramagnetic ions bound to the membrane surface (Buffy et al. 2003; Grobner et al. 1999; Hong and Su 2011; Hong et al. 2012), by dissolved oxygen in the membrane (Al-Abdul-Wahid et al. 2011; Prosser et al. 2000), or by spin labels incorporated into lipid molecules (Esposito et al. 1992; Hilty et al. 2004; Jacob et al. 1999). Depending on the distances of the nuclei from the paramagnetic centers, the T_1 or T_2 relaxation rates of the nuclei are enhanced to varying degrees.

The second NMR approach for depth determination utilizes ^{1}H spin diffusion from lipid and water to the protein (Huster et al. 2002; Kumashiro et al. 1998). In the liquid-crystalline (LC) phase of the membrane where the lipid chains and water are highly dynamic while the protein is immobile, the lipid and water ^{1}H magnetization can be readily selected and allowed to diffuse to the rigid protein (Fig. 1a). The result of this distance-dependent intermolecular spin diffusion is detected as correlation signals between the protein ^{13}C (or ^{15}N) and lipid/water protons in a 2D heteronuclear correlation (HETCOR) experiment (Huster et al. 2002). This LC-phase ^{1}H spin diffusion technique has been applied to a number of membrane peptides and proteins such as bacterial toxins (Gallagher et al. 2004; Huster et al. 2002), antimicrobial peptides (Mani et al. 2006; Su et al. 2011; Tang et al. 2009), and cell-penetrating peptides (Su et al. 2008).

However, the LC-phase ^{1}H spin diffusion technique cannot be applied when the membrane protein of interest undergoes intermediate-timescale motion in the lipid bilayer and causes line broadening and signal loss. This intermediate-exchange broadening results from the intrinsic rotational diffusion of membrane proteins, whose rate depends on the radius of the membrane protein, the viscosity of the lipid bilayer, and the temperature (Saffman and Delbruck 1975). When rotational diffusion occurs on the microsecond timescale in the LC membrane, it interferes with ^{1}H decoupling and polarization transfer processes in the solid-state NMR experiments, thus causing line broadening. This exchange broadening has been reported for various membrane peptides such as the influenza M2 transmembrane

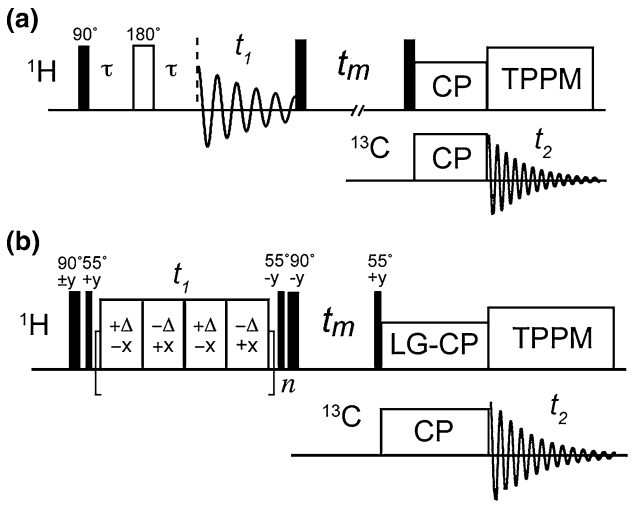

Fig. 1 ^{1}H spin diffusion HETCOR pulse sequences. **a** The LC-phase experiment. A ^{1}H T_2 filter is applied before the t_1 period to suppress the rigid peptide ^{1}H magnetization. Only the lipid and water ^{1}H signals are detected in the t_1 dimension. **b** The gel-phase experiment. The ^{1}H signals of both peptide and lipids are detected in the t_1 dimension

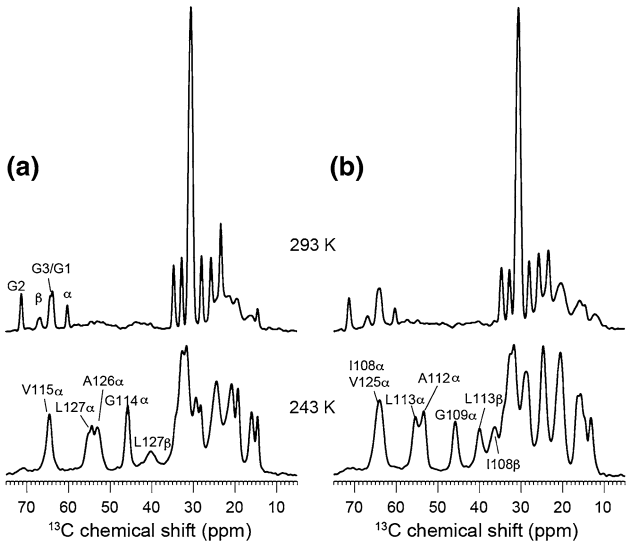

Fig. 2 1D ^{13}C CP-MAS spectra of the PIV5 fusion peptide in POPC/POPG membranes at 293 and 243 K. **a** GVAL-FPK4. **b** IGALV-FPK4. The peptide signals disappear at ambient temperature due to intermediate-timescale motion. In the gel-phase of the membrane, the peptide signals reappear, and the chemical shifts indicate an α-helical conformation

peptide (Cady et al. 2007) and the fusion peptide (FPK4) of the parainfluenza virus 5 (PIV5) (Yao and Hong 2013). In the absence of detectable signals, the depth of insertion cannot be determined using the LC-phase ^{1}H spin diffusion technique.

Figure 2 shows an example of intermediate-exchange broadening of the ^{13}C magic-angle-spinning (MAS) spectra of the PIV5 fusion peptide bound to the POPC/POPG membrane. The ^{13}C signals of nine labeled residues, distributed throughout the peptide, were undetectable at 293 K, but became strong and well resolved at 243 K, in the gel-phase of the membrane. The Cα and Cβ chemical shifts of all labeled residues indicate an α-helical conformation (Yao and Hong 2013). Because of this exchange broadening, it was not possible to determine the membrane topology of the α-helical fusion peptide using the LC-phase ^{1}H spin diffusion experiment. In contrast, in the neutral POPC membrane, FPK4 adopts a β-strand conformation (Yao and Hong 2013) and is immobilized at ambient temperature, giving high intensities. Thus, the depth of insertion of the β-strand fusion peptide could be determined using the ^{1}H spin diffusion technique. The peptide was found to lie on the surface of the POPC membrane, dehydrating the lipid headgroups (Yao and Hong 2013).

Viral fusion proteins such as the PIV5 F protein have long been important structural biology targets because of their essential role in mediating the entry of enveloped viruses into cells (Harrison 2008; Lamb and Jardetzky 2007). Class I viral fusion proteins such as the influenza

virus hemagglutinin (HA), the human immunodeficiency virus (HIV) env, and the paramyxovirus F protein, undergo a series of large conformational changes to drive the merger of the target cell membrane and the virus envelope. During these unfolding and refolding events, an N-terminal fusion peptide, originally enclosed in the interior of the pre-fusion protein globule, becomes exposed and inserts into the target cell membrane. The extended protein intermediate, doubly anchored in the virus envelope and the cell membrane, subsequently folds into a hairpin, in doing so fusing the cell and viral membranes (Yin et al. 2005, 2006). Determining the orientation and depth of insertion of the fusion peptide in lipid membranes is therefore important for elucidating the detailed molecular events during virus-cell fusion.

In this study, we demonstrate a low-temperature gel-phase ^{1}H-homonuclear-decoupled HETCOR experiment for determining the depth of insertion of membrane proteins that undergo intermediate-timescale motion in the LC lipid membrane. We describe the spectral patterns of surface-bound peptides versus well-inserted peptides obtained from this gel-phase spin diffusion HETCOR experiment using structurally known membrane peptides. We then apply this low-temperature spin diffusion experiment to the α-helical PIV5 fusion peptide in the anionic POPC/POPG membrane. Our results not only reveal the qualitative insertion motif of the peptide, but also show that site-specific depth resolution can be obtained from this gel-phase spin diffusion experiment.

Experimental methods

Membrane peptides

The PIV5 fusion peptide was synthesized and purified using Fmoc chemistry by PrimmBiotech (Cambridge, MA, USA). The peptide corresponds to residues 103–129 of the PIV5 F protein, with an amino acid sequence of FAGV-VIGLAALGVATAAQVTAAVALVK. A DIOXA-KKKK tag was appended to the C-terminus of this sequence to increase the solubility of the peptide (Yao and Hong 2013). Two peptides with different ^{13}C, ^{15}N-labeled residues were used in the current study. The first sample contains ^{13}C, ^{15}N-labeled residues at G114, V115, A126, and L127 (GVAL-FPK4), while the second peptide contains labeled I108, G109, A112, L113, and V125 (IGALV-FPK4). The fusion peptide was reconstituted into POPC and POPC/POPG (4:1 mol ratio) membranes using an organic solution mixing protocol. The peptide was dissolved in trifluoroethanol and mixed with lipids in chloroform. The solvents were removed under a stream of nitrogen gas and the mixture was lyophilized. The dry powder was then suspended in 10 mM phosphate buffer (pH = 7.5) with 1 mM EDTA and 1 mM NaN_3, dialyzed for 1 day, then subjected to ultracentrifugation at 55,000 rpm for 4 h at 4 °C. The resulting membrane pellets was transferred to 4-mm MAS rotors. The peptide/lipid molar ratio was 1:20 for these fusion peptide samples.

As a control, we measured the low-temperature HETCOR spectra of the antimicrobial peptide, protegrin-1 (PG-1), bound to POPE/POPG (3:1) membranes at a peptide/lipid molar ratio of 1:12.5. This peptide had been extensively studied (Hong and Su 2011; Tang and Hong 2009) and is known from the LC-phase ^{1}H spin diffusion experiment to insert into the hydrophobic part of the POPE/POPG membrane (Mani et al. 2006). The peptide is ^{13}C-labeled at L5 Cα and V16 ^{13}CO (Tang et al. 2007).

Solid-state NMR spectroscopy

The 1D ^{13}C CP-MAS spectra of FPK4 in POPC/POPG membranes were measured at 293 K and 243 K on a Bruker DSX-400 MHz (9.4 Tesla) spectrometer. All other NMR experiments were conducted on a Bruker Avance II 600 MHz spectrometer at a field strength of 14.1 Tesla using 4 mm MAS probes. Typical radiofrequency field strengths were 50–70 kHz for ^{1}H and 50 kHz for ^{13}C. ^{13}C chemical shift were externally referenced to the ^{13}CO signal of α-Gly at 176.465 ppm on the TMS scale, while the ^{1}H chemical shift was calibrated using *N*-formyl-Met-Leu-Phe-OH, whose ^{1}H chemical shifts have been reported (Li et al. 2010).

The gel-phase ^{1}H spin diffusion HETCOR spectra were measured under 7 kHz MAS using the pulse sequence in Fig. 1b. During the t_1 evolution period, the FSLG sequence (Bielecki et al. 1989) was used to decouple the ^{1}H–^{1}H dipolar interaction. A mixing time after t_1 allowed spin diffusion to occur, followed by Lee-Goldburg cross polarization (CP) (1965) to transfer the ^{1}H magnetization to ^{13}C. The pulse sequence is essentially the rigid-solid HETCOR experiment with an additional spin diffusion period. Applied to LC lipid membranes, this experiment has been used to investigate peptide–water and peptide–lipid intermolecular interactions (Li et al. 2010; Wang et al. 2012).

The temperatures of the gel-phase HETCOR experiments were chosen to be the highest temperature at which the membrane peptides were immobilized. To compare the HETCOR spectra of different samples, we monitored the ^{1}H linewidths of the lipid chain signals, which reflect the spin diffusion coefficient. For FPK4-containing POPC and POPC/POPG membranes, the HETCOR experiments were carried out at 258 K, which is 13 K below the phase transition temperature of these lipid membranes in the absence of peptides. The PG-1-containing POPE/POPG

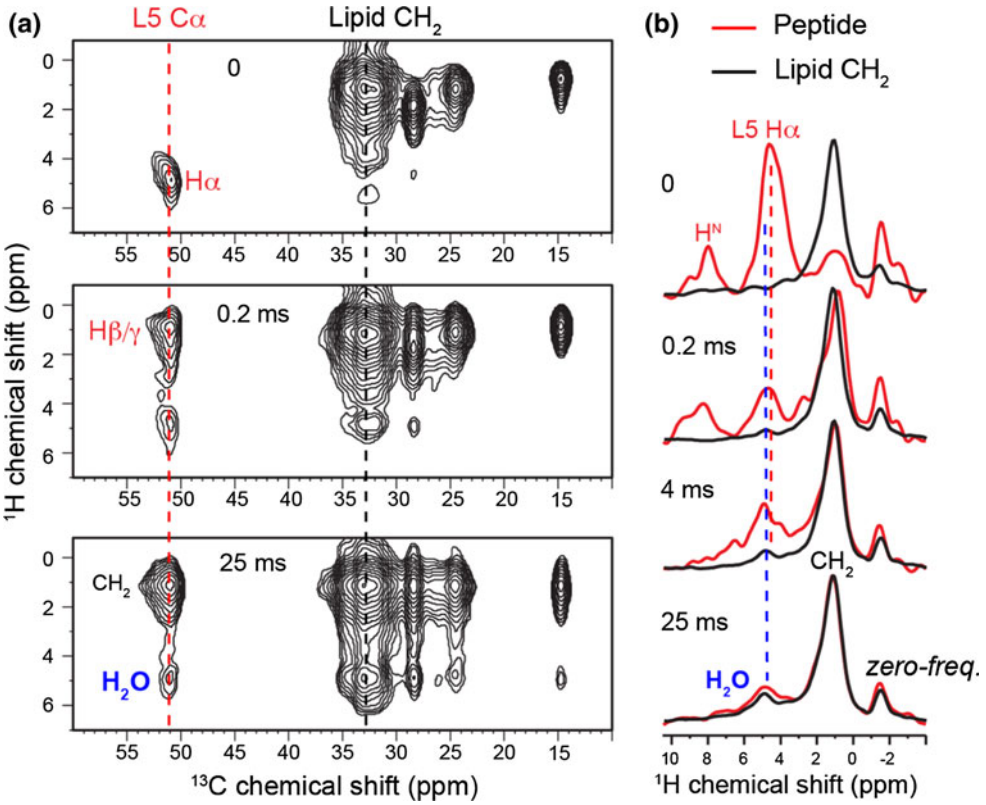

Fig. 3 Depth of insertion of PG-1 in POPE/POPG membranes by the gel-phase spin diffusion HETCOR experiment. **a** 2D spectra with 0, 0.2 and 25 ms mixing times. **b** ^{1}H cross sections of the peptide L5 Cα peak at 51 ppm and the lipid CH_2 peak at 33 ppm at varying mixing times. The peptide and lipid cross sections have very similar intensity patterns by 25 ms, indicating that L5 is well inserted into the middle of the POPE/POPG membrane. The spectra were measured at 253 K under 7 kHz MAS. The 1.5 ppm peak is a zero-frequency artifact

sample was measured at 253 K, at which the lipid ^{1}H linewidths were similar to those of the FPK4 samples (see Fig. 7).

The ^{1}H dimension of the 2D HETCOR spectra was processed using moderate line broadening parameters of LB = −5 and GB = 0.1, which corresponds to a Gaussian full width at half maximum of 0.15 ppm. This line broadening is smaller than the apparent ^{1}H linewidths of these membrane samples under the conditions of the experiments.

Results and discussion

The main difference between the LC-phase and gel-phase ^{1}H spin diffusion HETCOR experiments is that the LC-phase experiment has no ^{1}H homonuclear decoupling during the t_1 evolution period but has a ^{1}H T_2 filter before t_1, while the gel-phase experiment requires ^{1}H homonuclear decoupling during t_1 but no T_2 filter (Fig. 1). These differences match the dynamic properties of the lipid and peptides in the fluid and gel-phases of the lipid membranes. At high temperature, the ^{1}H T_2 filter and the absence of homonuclear decoupling combine to suppress the ^{1}H magnetization of the rigid peptide while retaining the magnetization of the mobile lipid and water, which act as the magnetization source. The fast rotational diffusion of lipids in the LC membrane ensures that moderate MAS frequencies alone are sufficient to give high-resolution ^{1}H spectra (Doherty and Hong 2009; Oldfield et al. 1987). In contrast, at low temperature where both the peptides and lipids are immobilized, homonuclear decoupling is necessary to obtain high-resolution ^{1}H spectra of the peptide and lipids. Under this condition, the peptide and lipid ^{1}H signals need to be distinguished in the ^{1}H dimension in order to differentiate intramolecular versus intermolecular cross peaks. We show that this assignment is possible based on the characteristic chemical shifts of functional groups as well as the mixing time dependence of spectral intensities.

To investigate how low-temperature HETCOR spectra differ between well-inserted and surface-bound membrane peptides, we first demonstrate the experiment on two peptides whose depths of insertion were previously determined using the LC-phase ^{1}H spin diffusion technique. The antimicrobial peptide, PG-1, has been extensively studied before and is known to insert into the bacteria-mimetic anionic membrane, POPE/POPG (Mani et al. 2006; Tang and Hong 2009). In comparison, the PIV5 fusion peptide in the POPC membrane is an example of a surface-bound membrane peptide (Yao and Hong 2013). Figure 3 shows the 253 K ^{1}H–^{13}C HETCOR spectra of POPE/POPG-bound PG-1. For all samples in this study, we used ^{1}H spin diffusion mixing times of 0, 0.2, 4 and 25 ms. In the absence of spin diffusion, only one-bond cross peaks were detected. Specifically, in the L5 Cα cross section, an Hα peak at 4.8 ppm dominates the

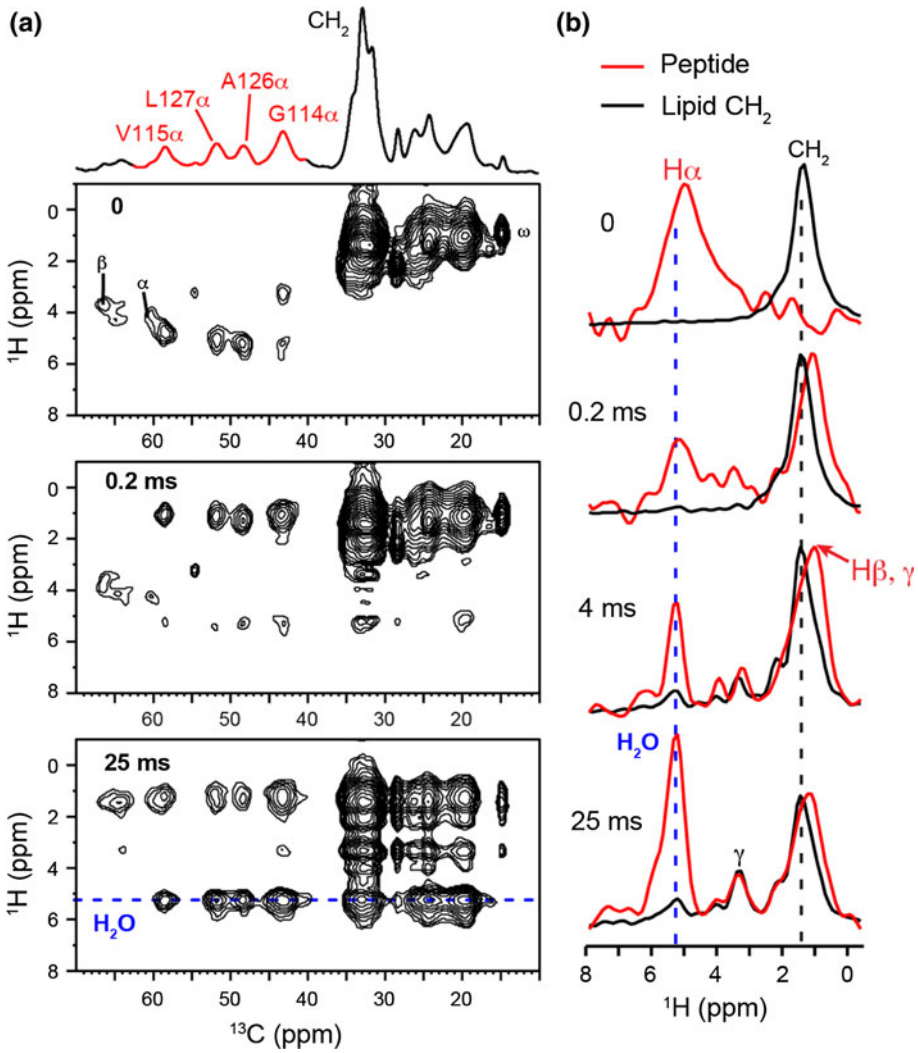

Fig. 4 Depth of insertion of GVAL-FPK4 in the POPC membrane by gel-phase spin diffusion HETCOR. **a** 2D spectra with 0, 0.2 and 25 ms mixing. **b** ^{1}H cross sections of the peptide (*red*) and lipid CH_2 (*black*) peaks at various mixing times. The ^{1}H cross sections of peptide come from the sum of all Cα peaks. The peptide cross section shows much higher water cross peak than the lipid cross section by 25 ms, indicating that FPK4 lies on the surface of the POPC bilayer. The spectra were measured at 258 K under 7 kHz MAS

spectrum, followed by weak H^N and sidechain Hβ/Hγ cross peaks at 8.0 and 0.8 ppm. With a 0.2 ms mixing time, the 0.8-ppm peak becomes the strongest, due to the presence of more sidechain protons than backbone protons. At longer mixing times of 4 and 25 ms, the ^{1}H spectral pattern changed significantly, with a strong ^{1}H peak at 1.1 ppm and a weak signal at 4.9 ppm, and the H^N signal has disappeared. The 1.1-ppm peak can be assigned to lipid CH_2 protons, based on its slightly different chemical shift and its narrower linewidth (1.2 ppm) compared to the L5 Hβ/Hγ linewidths of 1.6 ppm. The assignment of the 4.9-ppm peak to water is based on the similar intensity distribution of the peptide and lipid cross sections at 25 ms mixing and the disappearance of the peptide H^N peak. These observations indicate that spin diffusion has equilibrated the proton reservoir of the peptide and its neighboring lipid and water molecules by 25 ms, and the L5 region of PG-1 is well inserted into the hydrophobic part of the lipid membrane, with shorter distances to the lipid chains than to water. This conclusion is consistent with the LC-phase ^{1}H spin diffusion results, which found PG-1 to be inserted into the hydrophobic center of the POPE/POPG membrane (Mani et al. 2006). Therefore, the signature of an inserted peptide in the gel-phase HETCOR experiment is the similar intensity distribution between the lipid and peptide cross sections within a modest mixing time.

Figure 4 shows the gel-phase HETCOR spectra of the β-strand FPK4 bound to the POPC membrane. While the zero-mixing spectrum is similar to the PG-1 spectrum, by 25 ms, the peptide ^{1}H cross sections have become completely different: they have much higher water intensity than the lipid chain ^{1}H signals, indicating that the β-strand FPK4 resides on the surface of the POPC bilayer, consistent with the high-temperature ^{1}H spin diffusion results (Yao and Hong 2013). In contrast, the lipid cross section at 25 ms is similar to the POPE/POPG cross section in Fig. 3, with the CH_2 self-correlation peak dominating the water signal. The 5.2-ppm peak in the peptide cross sections at 4 and 25 ms can be assigned to water rather than Hα because its linewidth (0.7 ppm) is much narrower than the Hα linewidth (1.8 ppm) in the zero-mixing spectrum. Taken

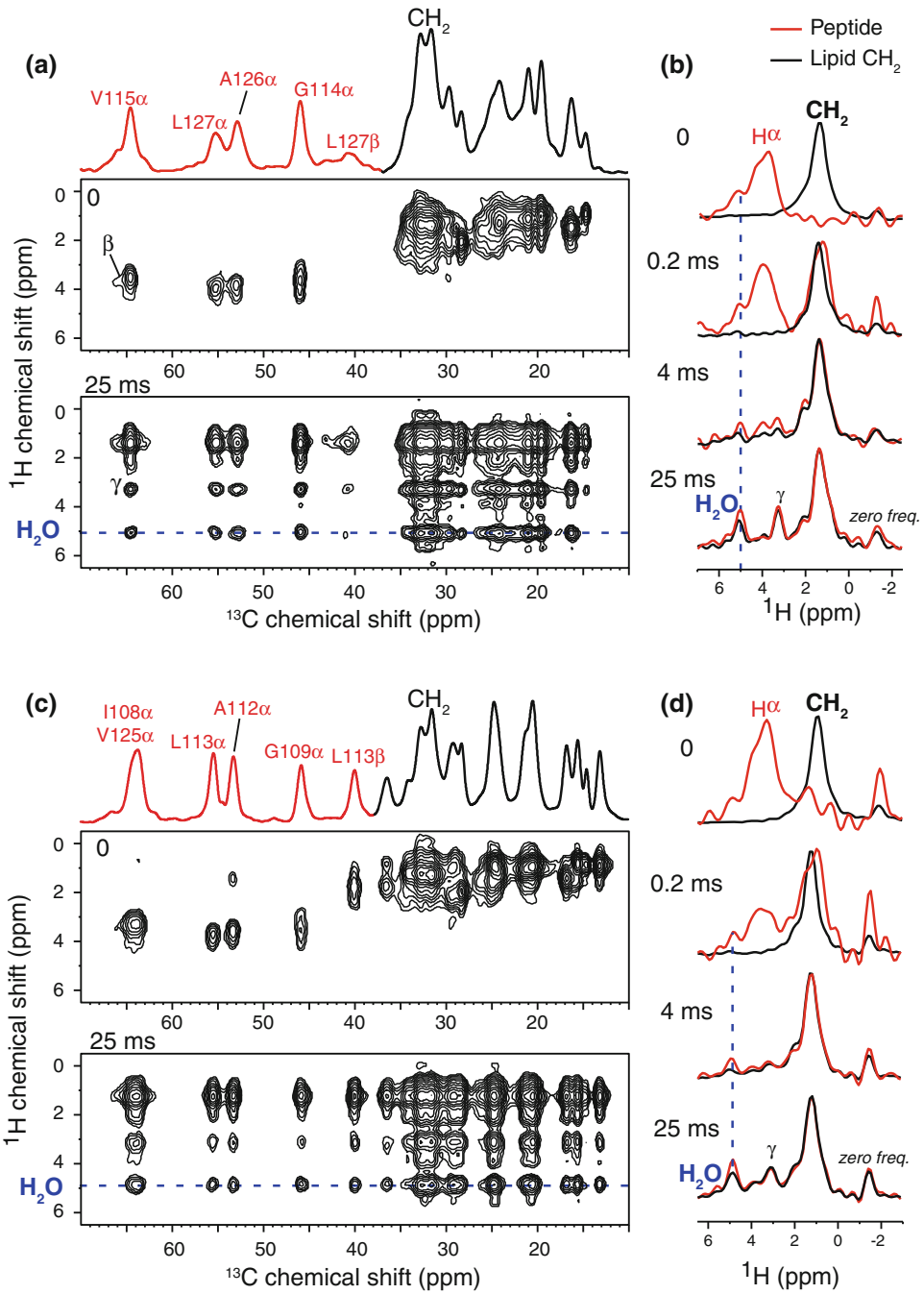

Fig. 5 Depth of insertion of the PIV5 fusion peptide in POPC/POPG membranes by gel-phase ^{1}H spin diffusion HETCOR. **a** 2D spectra of GVAL-FPK4 at 0 and 25 ms mixing. **b** 1D ^{1}H cross sections of the peptide ^{13}C (*red*) and lipid CH_2 (*black*) peaks at various mixing times. The peptide ^{1}H cross sections are the sum of all Cα peaks and the L127 Cβ peak. **c** 2D spectra of IGALV-FPK4 at 0 and 25 ms mixing. **d** 1D ^{1}H cross sections of the peptide (*red*) and lipid CH_2 (*black*) signals as a function of mixing time. The similarity of the peptide and lipid cross sections by 25 ms indicate that the α-helical FPK4 is well inserted into the POPC/POPG membrane. All spectra were measured at 258 K under 7 kHz MAS

together, Figs. 3 and 4 indicate that membrane-inserted and surface-bound peptides have very different ^{1}H intensity distributions: the former has strong lipid CH_2 peaks and weak water cross peaks, while the latter displays the opposite relative intensities.

Using this gel-phase spin diffusion HETCOR experiment, we investigated the depth of insertion of the PIV5 fusion peptide in the anionic POPC/POPG membrane. Fig. 5 shows representative 2D HETCOR spectra of GVAL-FPK4 and IGALV-FPK4 at 258 K. The sum of the peptide Cα cross sections shows similar ^{1}H intensity distributions as the lipid CH_2 cross section by 25 ms, indicating that the α-helical FPK4 is well inserted into the POPC/POPG membrane. Moreover, the relative intensity of the water and lipid cross peaks differs among different residues. ^{13}C cross sections at the water and lipid CH_2 frequencies indicate that residues near the two ends of the peptide, such as I108, G109 and V125, have higher water/lipid intensity ratios than residues in the middle of the peptide such as A112 and L113 (Fig. 6). The difference is particularly clear at short mixing times but is

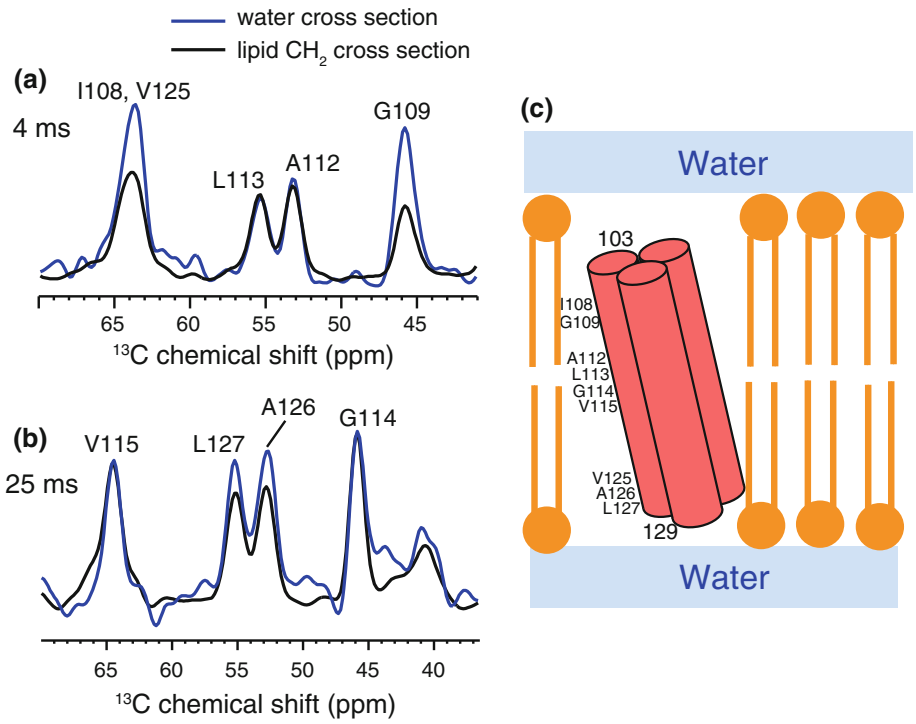

Fig. 6 Depth resolution of the gel-phase HETCOR experiment, shown by the spectra of FPK4 bound to the POPC/POPG membrane. **a** IGALV-FPK4 ^{13}C cross sections at the lipid CH_2 (*black*) and water (*blue*) frequencies from the 4 ms 2D spectrum. The water/lipid intensity ratios are higher for the terminal residues (I108, G109 and V125) than for residues in the middle of the peptide (A112 and L113). **b** GVAL-FPK4 ^{13}C cross sections at the lipid CH_2 and water frequencies from the 25 ms 2D spectrum. The water/lipid intensity ratios are higher for the terminal residues A126 and L127 than for the interior residues G114 and V115. **c** Illustration of the full insertion of FPK4 into the POPC/POPG membrane

also detectable at the longest mixing time of 25 ms. Therefore, the N- and C-termini of FPK4 have closer contacts with water than the middle of the peptide, strongly suggesting that FPK4 spans both leaflets of the bilayer (Fig. 6c). The alternative scenario that the peptide might be highly curved such that the N- and C-termini contact water molecules on the same side of the bilayer can be reasonably ruled out, since the ^{13}C chemical shifts of this peptide indicate a relatively straight α-helix, without significant kinks or bend (Yao and Hong 2013). These data indicate that the gel-phase spin diffusion technique has residue-specific depth resolution, in contrast to the LC-phase experiment. The reason for this difference is that at low temperature, lipids and peptides have similar spin diffusion coefficients, thus the rate of magnetization transfer depends on the actual distances of nuclear spins from the magnetization source, rather than the minimum distance of the peptide from the source spins.

In addition to the water/lipid intensity ratio, the ^{1}H linewidth of the peptide-lipid cross peaks also provides information about the insertion state of the peptide. For the well inserted POPC/POPG-bound FPK4, the peptide Cα to lipid CH_2 cross peak has a ^{1}H linewidth of 0.9 ppm, which is the same as the ^{1}H linewidth of the lipid self-correlation peak (Fig. 5b), supporting complete magnetization exchange between the peptide and lipid chains. The same was observed for PG-1 in the POPE/POPE membrane. In contrast, for the POPC-bound FPK4 (Fig. 4), the peptide cross peak with CH_2 groups resonates at a ^{1}H chemical shift of 1.2 ppm with a linewidth of 1.1 ppm, while the lipid self-correlation peak resonates at 1.4 ppm with a linewidth of 0.8 ppm. These subtle but reproducible differences indicate that peptide sidechain Hβ/Hγ protons make a significant contribution to the 1.2-ppm peak, and the magnetization of the surface-bound FPK4 and the lipid chains are not equilibrated by 25 ms.

The inserted topology of the α-helical PIV5 fusion peptide, together with its intermediate-timescale motion at ambient temperature, gives insight into the structure of FPK4 in the POPC/POPG membrane. If the peptide is monomeric and not significantly tilted, then its dynamics should be much faster than the NMR interactions and fast-averaged spectra with narrow ^{13}C linewidths should be observed. Thus, the fact that the FPK4 spectra are broadened beyond detection between 263 K and 313 K indicates that the α-helical FPK4 is oligomerized and significantly tilted. Since the water-soluble ectodomain of the F protein is trimeric, the fusion peptide domain may also be trimeric in the lipid membrane. But trimerization alone is unlikely to be sufficient for causing intermediate-timescale motion, since even membrane proteins with seven transmembrane helices have been shown to undergo fast motion in fluid membranes (Lewis et al. 1985). Instead, the fusion peptide assembly is likely significantly tilted in the POPC/POPG membrane in order for intermediate dynamics to persist over a wide temperature range (Fig. 6c).

The membrane-spanning topology of the α-helical PIV5 fusion peptide in the POPC/POPG membrane differs dramatically from the surface-bound topology of the β-strand FPK4 in neutral POPC membranes (Yao and Hong 2013). Our recent data correlating membrane curvature and

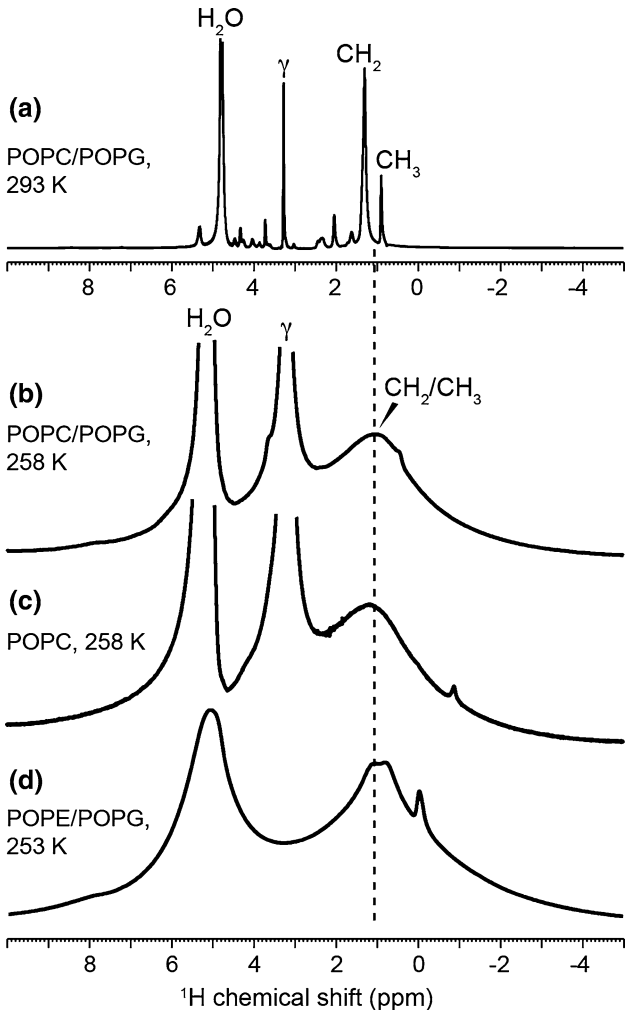

Fig. 7 ^{1}H single-pulse spectra of various lipid membranes. **a** FPK4-containing POPC/POPG membrane at 293 K. **b** FPK4-containing POPC/POPG membrane in the gel-phase at 258 K. **c** FPK4-containing POPC membrane at 258 K. **d** PG-1 containing POPE/POPG membrane at 253 K. The gel-phase spectra in (**b**–**d**) show similar linewidths for the lipid CH_2 peak, indicating comparable spin diffusion coefficients

hydration with FPK4 conformation suggested the β-strand conformation to be more relevant for membrane intermediates of the fusion pathway, while the α-helical conformation is associated with well-hydrated lamellar membranes that may be relevant for the pre-fusion and post-fusion states. Additional experiments will be necessary to test these hypotheses further. Interestingly, solid-state NMR measurements showed that the HIV fusion peptide forms an oligomeric β-sheet in virus-mimetic lipid membranes and spans one leaflet of the lipid bilayer (Qiang et al. 2009). This partially inserted β-sheet peptide also incurs membrane curvature (Gabrys et al. 2010). Information about α-helical viral fusion peptides so far mainly came from solution NMR studies of micelle-bound influenza HA, which found the peptide to form a bent helix that is shallowly inserted into the detergent micelle (Han et al. 2001; Lorieau et al. 2010).

The gel-phase ^{1}H spin diffusion HETCOR experiment is best applied at the highest temperature where the membrane peptides are immobilized. At moderate low temperature, the water molecules remain partly mobile, which would give a distinct and narrow ^{1}H signal that can be readily resolved from the peptide Hα signals. At moderate low temperature, the mixing times required to distinguish well inserted and surface-bound membrane peptides are longer than at extremely low temperatures, thus facilitating the distinction between topologically different membrane peptides. Finally, the use of moderate low temperature avoids the potential hazard of changing the peptide-lipid interactions compared to the physiological situation. To compare the depths of the membrane peptides in different lipid bilayers, the HETCOR experiments should be conducted at similar reduced temperatures from the membrane phase transition temperature so that the lipid chain dynamics are comparable. Figure 7 shows the undecoupled ^{1}H spectra of POPC/POPG, POPC, and POPE/POPG membranes at 253–258 K, where the 2D HETCOR spectra were measured. The CH_2 ^{1}H linewidths are similar, thus allowing the comparison of the relative depths of the PIV5 fusion peptide and PG-1. ^{2}H NMR of chain-deuterated lipids can also be used to verify membrane dynamics at the desired temperatures.

In conclusion, the depth of insertion of membrane peptides and proteins that exhibit intermediate-timescale motion in the LC phase of lipid membranes can now be determined using the gel-phase spin diffusion HETCOR experiment. This gel-phase experiment not only overcomes the exchange-broadening problem of many membrane peptides, but also provides site-specific depth resolution, which is absent from the LC-phase ^{1}H spin diffusion experiment. This gel-phase HETCOR experiment augments the NMR toolbox for determining the membrane topology of peptides and proteins. Further improvement of this technique can be envisioned, for example using advanced ^{1}H decoupling and isotopic dilution methods to enhance the resolution of the ^{1}H dimension to better distinguish lipid and protein signals.

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