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Investigation of the Curvature Induction and Membrane Localization of the Influenza Virus M2 Protein Using Static and Off-Magic-Angle Spinning Solid-State NMR of Oriented Bicelles

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Abstract

A wide variety of membrane proteins induce membrane curvature for function, thus it is important to develop new methods to simultaneously determine membrane curvature and protein binding sites in membranes with multiple curvatures. We introduce solid-state NMR methods based on magnetically oriented bicelles and off-magic-angle spinning (OMAS) to measure membrane curvature and the binding site of proteins in mixed-curvature membranes. We demonstrate these methods on the influenza virus M2 protein, which not only acts as a proton channel but also mediates virus assembly and membrane scission. An M2 peptide encompassing the transmembrane (TM) domain and an amphipathic helix, M2(21-61), was studied and compared with the TM peptide (M2TM). Static ^{31}P NMR spectra of magnetically oriented DMPC/DHPC bicelles exhibit a temperature-independent isotropic chemical shift in the presence of M2(21-61) but not M2TM, indicating that the amphipathic helix confers the peptide with the ability to generate a high-curvature phase. 2D ^{31}P spectra indicate that this high-curvature phase is associated with the DHPC bicelle edges, suggestive of the structure of budding viruses from the host cell. ^{31}P - and ^{13}C -detected ^1H relaxation times of the lipids indicate that the majority of M2(21-61) is bound to the high-curvature phase. Using OMAS experiments, we resolved the ^{31}P signals of lipids with identical headgroups based on their distinct chemical shift anisotropies. Based on this resolution, 2D ^1H - ^{31}P correlation spectra show that the amide protons in M2(21-61) correlate with the DMPC but not the DHPC ^{31}P signal of the bicelle, indicating that a small percentage of M2(21-61) partitions into the planar region of the bicelles. These results show that the M2 amphipathic helix induces high membrane curvature and localizes the protein to this phase, in excellent agreement with the membrane-scission function of the protein. These bicelle-based relaxation and OMAS solid-state NMR techniques are generally applicable to curvature-inducing membrane proteins such as those involved in membrane trafficking, membrane fusion, and cell division.

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Supporting Information Additional NMR spectra are provided. This information is available free of charge via the internet at <http://pubs.acs.org>.

Introduction

Many membrane proteins cause membrane curvature to carry out their functions such as membrane trafficking¹, endocytosis², virus-cell fusion^{3–5} and virus budding^{6, 7}. Membrane curvature has so far been mainly characterized using electron microscopy^{6, 8} and small-angle X-ray scattering (SAXS)⁹. However, these techniques do not have the ability to determine the high-resolution structure of the proteins involved in curvature induction. Solid-state NMR (SSNMR) spectroscopy can in principle simultaneously reveal membrane curvature, protein conformation and protein-lipid interactions^{5, 10–12}. The protein and lipid NMR signals can be readily distinguished by detecting ¹³C and ¹⁵N signals for the former and ³¹P and ²H signals for the latter, and correlation of the two gives information about protein-lipid interactions^{10, 11, 13}. It is therefore of interest to develop new SSNMR techniques to determine membrane curvature, protein structure and protein-lipid interactions.

To investigate curvature-dependent protein-lipid interactions, it is desirable to use a lipid membrane with defined curvatures and lower complexity than the cell membrane and virus envelope, since the full complement of lipids that commonly exists in eukaryotic membranes may obscure the effects of protein interactions with a small subset of lipids to cause curvature. Bicelles present an appealing membrane-mimetic system for elucidating the curvature-inducing propensity of membrane proteins. As bilayered discoidal aggregates formed by mixtures of long-chain and short-chain lipids, bicelles have been widely used in solution and solid-state NMR studies of protein structure^{14–19}. By varying the ratios of long-chain and short-chain lipids, one can produce either weakly aligned isotropic bicelles or strongly aligned anisotropic bicelles to obtain orientational constraints of proteins that are either distributed between bicelles or embedded within bicelles. For SSNMR studies, anisotropic bicelles are usually used. These bicelles are aligned with the planes parallel to the external magnetic field¹⁵ because of their negative anisotropy of magnetic susceptibility. When paramagnetic lanthanide ions²⁰ or lipids with phenyl rings²¹ are added, the bicelle planes can be flipped to be perpendicular to the magnetic field. Compared to mechanically aligned membranes, the magnetically aligned bicelles are easy to prepare, hydrate and stabilize for SSNMR structural studies. The morphology of magnetically oriented bicelles has been extensively studied and variously described as disc shaped²², perforated lamellar bilayers²³, or worm-like micelles²⁴. All these models agree about the coexistence of low- and high-curvature domains in the bicelles, with the planar surface dominated by long-chain lipids whereas round edges composed of short-chain lipids. Moreover, the short-chain lipids can exchange between these two domains²⁵.

The M2 protein of influenza A viruses is a multi-functional protein that acts at several stages of the virus lifecycle. M2 has so far been predominantly studied as a drug-targeted proton channel^{26–30}, which manifests its activity after the virus is endocytosed into the host cell, where the low pH of the endosome opens the channel to acidify the virion and cause virus uncoating³¹. Amantadine and rimantadine inhibit the channel by binding to the transmembrane (TM) pore^{27, 32}. In a later stage of the virus lifecycle, when a newly assembled virus is ready to be released from the host cell, M2 is recruited to the neck of the budding virus and mediates membrane scission. Electron microscopy data indicate that this

second function is carried out by an amphipathic helix (AH) in the cytoplasmic domain, which causes high membrane curvature ⁶: a peptide corresponding to the AH domain is sufficient to bud into giant unilamellar vesicles, and mutations of the AH domain in full-length M2 inhibits vesicle budding in vitro and prevents virus release in vivo. SAXS data further showed that AH-containing M2 peptides incur bicontinuous lipid cubic phases in phosphoethanolamine-rich lipid membranes ⁷, consistent with the curvature-inducing function.

In this work, we explore oriented bicelles as a medium to develop new SSNMR methods for investigating the preferential localization of proteins in membrane domains with distinct curvatures. Using the influenza M2 protein, we show that it is possible to measure the protein binding site in low- versus high-curvature membranes by detecting lipid dynamics in the vicinity of the protein and by 2D ³¹P-¹H correlation experiments. Since magic-angle-spinning (MAS) NMR spectra cannot resolve the ³¹P isotropic chemical shifts of lipids with the same headgroup structure, we introduce off-magic-angle spinning (OMAS) experiments to resolve ³¹P signals based on the motionally averaged ³¹P chemical shift anisotropies (CSAs) of long-chain and short-chain lipids. We find that an AH-containing M2 peptide both induces and partitions into a high-curvature membrane phase, thus providing nanometer-scale structural evidence of the membrane scission function of this protein.

Experimental Section

Membrane sample preparation

Two M2 peptides, M2(22-46) and M2(21-61), were synthesized using Fmoc chemistry and purified by PrimBio (Cambridge, MA). M2(21-61) contains both the TM domain (residues 22-46) and the AH domain (residues 47-61). Uniformly ¹³C, ¹⁵N-labeled residues were incorporated at L26, V27, A30, G34 and I35 in M2(21-61). For simplicity, we interchangeably denote M2(22-46) as M2TM and M2(21-61) as M2TM-AH in this paper.

1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC) were used to prepare most of the oriented bicelle samples in this study. Following the literature ³³, we dissolved DHPC in ~45 μ L of pH 7.5 Tris buffer and pipetted the solution to a dry powder of the peptide. The mixture was vortexed and then added to the desired amount of DMPC powder. This ternary mixture was cooled and heated between 0°C and 42°C 5 – 7 times until the sample became uniform. The final solution was viscous at room temperature but fluid at 4°C. For peptide-free control samples, the DHPC solution was directly added to the DMPC powder and subjected to the same cool/heat cycles. The bicelles samples were transferred to 4 mm MAS rotors for SSNMR experiments. All samples were hydrated to ~65 wt%, and the molar ratio of DMPC : DHPC : peptide was 48 : 16 : 1. d₂₂-DHPC was incorporated into some of the bicelles to investigate the effect of the peptides on lipid chain order. The M2-containing bicelles were stable for about a month, while the peptide-free bicelles were stable for many months. A second bicelle system consisting of DMPC and 1,2-dihexyl-sn-glycero-3-phosphocholine (6-O-PC) was also prepared to assess the generality of curvature induction by M2TM-AH.

Solid-state NMR spectroscopy

SSNMR experiments were conducted on Bruker wide-bore 400 MHz and 600 MHz spectrometers using 4-mm MAS probes. The oriented bicelles were measured under either the static or the spinning condition, and the angle of the rotation axis was varied by $\sim 7^\circ$ around the magic angle. Typical radiofrequency (rf) field strengths were 40–50 kHz for ^{31}P and ^{13}C pulses and ~ 30 kHz for ^1H decoupling. The weak ^1H decoupling field was chosen to avoid overheating and dehydrating the bicelles, which would destroy the orientational alignment. For the same reason, most experiments used long recycle delays of 4.5 – 6.0 s and a slow spinning frequency of 3.5 kHz. All ^{31}P chemical shifts were referenced to the ^{31}P peak of hydroxyapatite at 2.73 ppm while all ^{13}C chemical shifts were externally referenced to the adamantane CH_2 signal at 38.48 ppm on the TMS scale.

Static ^{31}P direct-polarization (DP) spectra were measured as a function of temperature from 283 K to 312 K. To obtain stable alignment, we kept the bicelles in the magnetic field at 312 K for an hour and further stabilized them for 30 min at each temperature before measurements. All reported temperatures were thermocouple values calibrated using the ^{207}Pb signal of lead nitrate. Static 2D ^{31}P - ^{31}P exchange spectra were measured using long mixing times of 0.5 to 1.0 s. Bicelle samples with and without M2TM-AH were measured at 297 K and 306 K, respectively. These temperatures were chosen so that the two samples have the same DHPC ^{31}P chemical shift.

^{31}P and ^{13}C -detected ^1H T_2 relaxation times were measured using a Hahn echo sequence on the ^1H channel, followed by ^1H spin diffusion and cross polarization (CP) to the heteronucleus ³⁴. Increasing the spin diffusion mixing time (t_m) allows us to detect protons further away from the observed ^{31}P site. To exclude the peptide ^1H contribution to the ^{13}C -detected ^1H T_2 data, we additionally carried out an experiment in which a ^{13}C - ^1H dipolar filter of 100 μs was added before the ^1H Hahn-echo period. This dipolar dephasing period suppresses the ^1H magnetization of the rigid peptide while retaining the ^1H magnetization of the mobile lipids and water. The lipid and water ^1H T_2 relaxation times were also measured by direct ^1H detection at 302 K. ^2H quadrupolar echo experiments were conducted on d_{22} -DHPC-containing bicelles to investigate DHPC dynamics in different membranes. A ^2H rf field strength of 50 kHz and a recycle delay of 0.8 s were used for these quadrupolar echo experiments.

OMAS experiments were conducted to resolve the ^{31}P chemical shifts of different membrane domains and to obtain high-sensitivity and high-resolution 2D ^1H - ^{31}P correlation spectra. A spinning frequency of 3.5 kHz was used. This frequency was small enough to avoid perturbing the bicelle morphology but high enough to avoid ^{31}P spinning sidebands ³⁵. The 2D ^1H - ^{31}P correlation spectra ¹³ were measured with MREV-8 ^1H homonuclear decoupling during the t_1 evolution period. The ^1H 105° pulse length in the MREV-8 pulse train ³⁶ was 6 μs . The ^1H chemical shift was calibrated using N-formyl-Met-Leu-Phe-OH (MLF), whose ^1H chemical shifts have been reported ³⁷.

Results

Temperature-dependent spectra of peptide-free DMPC/DHPC bicelles

^{31}P NMR is a useful probe of membrane morphology and has been extensively used for studying bicelles. The magnetic alignment of bicelles leads to well-resolved ^{31}P anisotropic chemical shifts under the static condition. From 300 and 312 K, the peptide-free DMPC/DHPC bicelles exhibit two ^{31}P chemical shifts (Fig. 1a). The upfield signal at about -10 ppm can be assigned to DMPC while the downfield peak can be assigned to DHPC. With increasing temperature, both ^{31}P signals move upfield, but the DHPC chemical shift change (from -2.8 ppm to -4.8 ppm) is larger, making it approach the DMPC signal at high temperature. The DMPC versus DHPC chemical shift difference is 6.4 ppm at 300 K and decreases to 5.6 ppm at 312 K. The larger upfield movement of the DHPC ^{31}P signal results from increased exchange of DHPC between the planar and curved regions of bicelles at higher temperature^{25, 38}.

At 283 – 291 K, below the phase transition temperature of DMPC, the ^{31}P spectra exhibit a single peak at the isotropic chemical shift of -0.9 ppm, indicating the formation of a micellar phase or isotropic bicelles^{25, 39}. At the intermediate temperature of 294 K, a residual power pattern is observed in addition to the isotropic peak, indicating the coexistence of lamellar and isotropic phases. At 297 K, the ^{31}P chemical shifts are discontinuous from the high-temperature values: both DMPC and DHPC peaks moved significantly upfield, to -13.4 ppm and -9.7 ppm, respectively. The DMPC chemical shift is similar to that of glass-plate aligned membranes, suggesting that at this temperature the bicelles are either aligned closer to the 90° angle from the magnetic field or have less internal motion and hence higher order parameters. At this temperature, the DHPC ^{31}P peak is only 3.7 ppm downfield from the DMPC peak, indicating that DHPC undergoes significant exchange between the edge and planar regions of the bicelles.

The M2 amphipathic helix generates high membrane curvature

The addition of M2TM did not change the qualitative trend of the ^{31}P NMR spectra. Two ^{31}P peaks are observed that move upfield with increasing temperatures (Fig. 1b). Thus, M2TM does not perturb the bicelle morphology. Above 300 K, the ^{31}P chemical shifts are about 3 ppm upfield for M2TM-containing bicelles than for peptide-free bicelles. This may be caused by reduced motional amplitudes and thus larger ^{31}P CSAs of the lipids in the presence of M2TM. Between 294 and 297 K, the chemical shift discontinuity and the lamellar phase disappeared, supporting the notion that M2TM stabilizes the bicelles. Both peptide-free and peptide-bound ^{31}P spectra in Fig. 1a, b exhibit DMPC : DHPC ^{31}P area ratios of $3.0 \pm 0.3 : 1$, which is the same, within experimental uncertainty, as the molar ratio of the lipids in the bicelles. But the M2TM-containing samples show lower height and broader linewidths for the DHPC peak, suggesting that M2TM may have slowed down DHPC motion.

Interestingly, binding of the AH-containing M2(21-61) peptide caused qualitatively different ^{31}P spectra. In addition to the two anisotropic ^{31}P chemical shifts, a temperature-independent ^{31}P peak at the isotropic frequency of -0.8 ppm is observed from 294 to 312 K

(Fig. 1c). In general, for non-spinning membrane samples, the ^{31}P CSA is determined by both the chemical structure of the headgroup and lipid reorientational motions. Immobilized lipid headgroups at low temperature give a rigid-limit ^{31}P chemical shift span of ~ 190 ppm⁴⁰. Fast uniaxial rotational diffusion and wobble of the molecular axis in liquid-crystalline lamellar bilayers average this CSA to ~ 45 ppm, and the maximum intensity occurs at the upfield edge of the power pattern. This uniaxial ^{31}P chemical shift lineshape is further averaged when lipid lateral diffusion and whole-body tumbling are sufficiently fast on the NMR timescale, which occur when the radius of curvature of the membrane is small. Micelles, small vesicles and bicontinuous cubic phases typically have radii of curvature of 5–30 nm, and possess sufficiently high symmetry such that lateral diffusion and tumbling average the ^{31}P CSA to the isotropic value. Thus, the isotropic ^{31}P peak in Fig. 1c indicates that M2(21-61) induces a high-curvature membrane phase, which can be micelles, small vesicles, or bicontinuous cubic phases. The temperature independence of this peak frequency is consistent with the symmetry of these phases.

The ^{31}P isotropic peak represents 10–20% of the total ^{31}P intensities (Fig. 1c). The lipid composition of the high-curvature phase can be estimated from the ^{31}P peak intensities and the 3 : 1 total molar ratio of DMPC/DHPC. The bicelle DMPC and DHPC peaks in Fig. 1c have intensity ratios of $3.0 \pm 0.1 : 1$, thus the high-curvature phase has a similar lipid composition (3 : 1) as the average composition of this sample. Other bicelle samples prepared in this study had ^{31}P intensity ratios of 2.2 : 1 to 3.5 : 1 (data not shown), suggesting that the composition of the high-curvature phase can vary from predominantly DMPC to $\sim 50\%$ DMPC. Since M2TM does not generate this peak, the amphipathic helix is responsible for causing this high-curvature phase. This isotropic peak was also observed in DMPC/6-O-PC bicelles with bound M2TM-AH (Fig. S1), indicating that curvature induction is an inherent property of the AH-containing M2 and is independent of the exact composition of the bicelle.

To confirm that the M2(21-61)-induced isotropic ^{31}P peak indeed results from the high curvature of the membrane rather than an accidental magic-angle orientation of the lipid headgroups, we measured the ^2H spectra of d_{22} -DHPC, which report lipid chain dynamics. Both bicelles without and with M2TM-AH were measured. Fig. 2 shows that the peptide-free bicelles do not exhibit any zero-frequency peak while the M2TM-AH bound bicelles do, thus the isotropic ^{31}P peak reflects the curvature of the entire membrane. Consistent with the ^{31}P spectra, the ^2H quadrupolar splittings are slightly larger in the M2-containing bicelles than in the peptide-free bicelles, indicating that either the lipid order parameters are larger or the bicelles align closer to 90° from the magnetic field.

To investigate whether the high-curvature membrane phase is in spatial contact with the bicelles, we measured 2D ^{31}P - ^{31}P exchange spectra of the M2(21-61)-containing bicelle. At mixing times of 0.5 – 1.0 s, the spectra exhibit a cross peak between the isotropic ^{31}P peak and the DHPC peak (Fig. 3a, b), indicating that the high-curvature phase is in physical contact with the round edges of the bicelle. At 1.0 s, the cross peak accounts for 20–30% of the diagonal intensity of the high-curvature phase. No cross peak is observed between DHPC and DMPC. Lipid lateral diffusion over the nanometer dimension of the bicelles occurs on the microsecond timescale, which is sufficiently fast to average out the ^{31}P

CSA⁴¹ but too short for the millisecond timescale of 2D exchange NMR experiments, thus the lack of an DMPC/DHPC cross peak in the 2D spectra is understandable. In a recent study of lipid lateral diffusion using ³¹P exchange NMR⁴², 200 mM sucrose was added to the membrane to slow down the diffusion rate and to enable the detection of lipid reorientations on the millisecond timescale. Similar strategies may be applicable for studying exchange between the edge and planar regions of bicelles, if small-molecule additives that do not affect bicelle alignment can be found.

¹³C spectra of M2-containing DMPC/DHPC bicelles

Any indirect detection methods to determine the site of protein binding in the mixed-curvature oriented bicelles require the ability to resolve the peptide ¹³C signals under the static condition. While ¹³C MAS spectra readily provide this site resolution in terms of isotropic chemical shifts, the resolution of static ¹³C spectra of membrane peptides with uniformly ¹³C-labeled residues can be much lower due to ¹³C-¹³C dipolar coupling. We thus measured the static and MAS ¹³C spectra of DMPC/DHPC bicelles with and without M2TM-AH to identify and assign the peptide signals. Comparison of the static and MAS spectra allows us to distinguish anisotropic and isotropic chemical shifts, while comparison of peptide-bound and peptide-free bicelles allows us to identify the peptide ¹³C signals. Temperature variation provides additional clues for resonance assignment by changing the membrane phase and alignment.

Many lipid signals such as glycerol G2 and G1/G3 are detected in the ¹³C spectra (Fig. 4). Their chemical shifts change between 283 K, where the bicelles are in an isotropic phase, and 300 K or higher, where the bicelles are oriented. Thus the high-temperature chemical shifts are anisotropic while the low-temperature shifts are isotropic. This is confirmed by the fact that the 283 K static spectrum matches the MAS spectrum (albeit with broader linewidths) while the static high-temperature spectra differ from the MAS spectra. Two peaks are observed for some of the lipid functional groups in the static spectra, which result from the presence of two different headgroup conformations in phosphocholine and the known conformational differences between the *sn*-1 and *sn*-2 carboxyl carbons⁴³. In addition to these lipid signals, the static ¹³C spectra also exhibit peptide signals such as I35/V27 C α (65.3 ppm), L26 C β (42.2 ppm), V27 C γ (18.9 ppm) and I35 C δ 1 (10.9 ppm). These anisotropic chemical shifts do not deviate significantly from the isotropic chemical shifts, indicating that whole-body reorientation of the bicelle significantly scales the ¹³C CSAs. The L26 C β and I35 C δ 1 signals are particularly unambiguous and well resolved, thus they are used as probes of M2 partitioning in mixed-curvature membranes. To verify that the TM domain retains the same α -helical conformation in bicelles as in multilamellar liposomes, we measured a double-quantum filtered ¹³C spectrum of the sample, where the natural-abundance lipid ¹³C signals are suppressed (Fig. S2). The spectrum shows the same α -helical chemical shifts for the labeled residues as reported before for multilamellar liposome samples^{32, 44}.

^1H T_2 relaxation times indicate preferential localization of M2TM-AH in the high-curvature membrane

With resolved lipid and peptide ^{13}C signals in the static spectra of the oriented bicelles, we can measure the ^1H T_2 relaxation times of lipids with ^{31}P and ^{13}C detection (Fig. 5a). ^{31}P detection allows us to investigate the dynamics of DMPC, DHPC, and the high-curvature lipids, while ^{13}C detection allows us to detect the dynamics of lipids in the vicinity of the peptide, thus revealing the membrane environment in which M2 resides ¹³.

Fig. 5 shows ^{31}P -detected ^1H T_2 relaxation data and representative ^{31}P spectra. We measured the ^1H T_2 at 300 K using 10 ms and 100 ms mixing, to probe the dynamics of lipids in small and large domains. Among the three resolved membrane phases, DMPC undergoes the fastest relaxation with a ^1H T_2 of 0.1–0.2 ms, while the high-curvature phase has the slowest relaxation: apart from an initial decay of 0.1–0.2 ms, 40–50% of the intensity exhibits a T_2 of 5–6 ms (Fig. 5d, e). The bicelle-edge DHPC lipids exhibit an intermediate ^1H T_2 of ~0.5 ms. The slow relaxation of the high-curvature phase is understandable, because the lipids in this phase undergo not only uniaxial rotational diffusion around the local membrane normal but also isotropic tumbling and lateral diffusion over the highly curved membrane surface. Increasing the mixing time from 10 ms to 100 ms did not change the relaxation rates of DMPC and DHPC but increased the relaxation rates of the high-curvature phase, and the initial decay now overlaps with the initial DHPC decay. This convergence is in good agreement with the observed ^{31}P cross peak between DHPC and the high-curvature lipids, further supporting the conclusion that the high-curvature phase is associated with the bicelle-edge DHPC lipids.

Increasing the temperature to 312 K brought interesting counter-directional changes to the relaxation rates of DHPC and the high-curvature lipids: the high-curvature lipids relax more slowly, whereas the DHPC relaxation rates increased. The former indicates faster reorientations of the high-curvature lipids at high temperature, while the latter can be attributed to increased diffusion of DHPC between the planar and edge regions of the bicelle. The upfield ^{31}P chemical shift of –7.6 ppm at 312 K, compared to the value of –4.8 ppm at 300 K, is consistent with this increased exchange.

To determine whether the indirectly measured ^1H relaxation times of lipids differ from the directly measured values, we measured the T_2 of bicelles containing M2TM-AH by direct ^1H detection (Fig. 6a). The acyl-chain CH_2 and CH_3 signals and the headgroup H_γ peak all exhibit biexponential decays, with a fast component of 0.4–0.7 ms and a slow component of ~10 ms for the chain protons and ~50 ms for the headgroup trimethylamine. For acyl-chain protons, we assign the short T_2 component to the oriented bicelle phase and the long T_2 to the dynamic high-curvature phase. The long-component T_2 values are larger than the T_2 's measured by ^{31}P detection, likely because direct detection favors the detection of highly dynamic protons while indirect detection by ^1H - ^{31}P CP preferentially enhances the signals of the more immobile protons. Compared to the lipid ^1H T_2 's, the water T_2 is much longer, ~93 ms, consistent with the presence of bulk water in these highly hydrated (65 wt %) samples.

Switching to ^{13}C detection for the ^1H T_2 measurement allowed us to probe the dynamics of lipids in the vicinity of M2TM-AH. At 302 K and with a short spin diffusion time of 10 ms, fast T_2 relaxation times of 50–110 μs were observed, indicating that only the relatively immobile peptide protons were detected (Fig. 7e). Increasing the mixing time to 100 ms dramatically slowed down the T_2 relaxation: both I35 and L26 sidechains now exhibit biexponential decays with a slow component of 7 and 15 ms, respectively (Fig. 7f). The initial fast decay is dominant for the L26 $\text{C}\beta$ signal and accounts for ~30% of the I35 $\text{C}\delta 1$ signal. To verify that this initial fast decay results from peptide protons instead of reflecting an immobile pool of lipids, we added a ^{13}C - ^1H dipolar filter of 100 μs before the echo period (Figs. 7a, S3) to suppress the signals of the rigid peptide. Since the lipids are not ^{13}C labeled, the dipolar filter does not impact the lipid signals. Fig. 7g shows that this dipolar filter removed most of the fast initial decay and dramatically increased the L26 $\text{C}\beta$ intensity, thus confirming that the lipids near the peptide have long ^1H T_2 relaxation times of ~7 ms. This T_2 value is closest to the ^{31}P -detected ^1H T_2 of the high-curvature lipids, but an order of magnitude longer than the relaxation times of bicelle lipids. Therefore, these ^{13}C - and ^{31}P -detected ^1H T_2 data indicate that M2TM-AH is preferentially bound to the high-curvature domain.

OMAS 2D ^1H - ^{31}P correlation NMR for detecting M2(21-61) partitioning in bicelles

We measured 2D ^{31}P - ^1H correlation spectra to detect lipid ^{31}P – protein H^{N} correlation signals directly. Although the ^{31}P signals of the three membrane components are well resolved under the static condition, the collective protein H^{N} signals are expected to be much broader than the lipid ^1H signals under the static oriented condition, because membrane-bound proteins usually exhibit larger orientational disorder and slower motional rates than lipids, thus giving rise to larger residual ^1H - ^1H dipolar couplings. Therefore, we chose to spin the bicelle to obtain the necessary sensitivity and resolution. Since all three lipid components have the same ^{31}P isotropic chemical shift, they are unresolved in the ^{31}P MAS spectrum (Fig. 8a), making it necessary to conduct OMAS experiments. Deviating from the magic angle by a small degree already retrieved sufficient ^{31}P CSAs and site resolution, and retained reasonable ^1H linewidth simultaneously. In the spectrum shown in Fig. 8b, all three lipid signals are resolved and readily assigned based on their relative intensities and the effective ^{31}P CSAs. The most downfield and highest peak can be assigned to DMPC while the most upfield and weakest peak can be assigned to the high-curvature membrane domain. Changing the sample axis to the other side of the magic angle caused the opposite frequency changes, with DMPC giving the most upfield chemical shift (Fig. 8c).

The precise angles (θ) of the rotation axis from the magnetic field can be determined from the chemical shift differences among the ^{31}P MAS, OMAS and static spectra. The static spectrum in Fig. 8d is equivalent to a $\theta = 0^\circ$ OMAS spectrum with a scaling factor $(3\cos^2\theta - 1)/2$ of 1. The OMAS spectrum of Fig. 8b has the opposite intensity distribution from the static spectrum, indicating that the rotation axis is tilted by an angle larger than 54.7° to cause a negative scaling factor to the ^{31}P CSA. The DMPC ^{31}P chemical shift is 1.0 ppm larger than the isotropic chemical shift in Fig. 8b but 10.6 ppm smaller than the isotropic frequency in the static spectrum (Fig. 8d). Thus the scaling factor is $1/(-10.6) = -0.094$,

which corresponds to a rotation angle of 58.7° . Similar arguments indicate a rotation angle of 52.5° for the spectrum in Fig. 8c.

Fig. 9 shows the 2D ^1H - ^{31}P correlation spectra DMPC/DHPC bicelles containing M2TM-AH under 58.7° OMAS. Without ^1H spin diffusion, the DMPC and DHPC ^{31}P signals only correlate with lipid protons such as glycerol G2 (5.4 ppm) and G3 and headgroup α/β (4.2 ppm) (Fig. 9b). These anisotropic ^1H chemical shifts do not deviate significantly from the isotropic values measured in the MAS spectrum (Fig. 9d, e) because of the small motionally averaged ^1H CSAs and the small deviation of the rotation angle from the magic angle. With 0.5 ms ^1H spin diffusion, ^{31}P – lipid chain ^1H cross peaks were observed. Importantly, broad H^{N} signals at 6 – 9 ppm were detected to correlate with the DMPC ^{31}P peak but not the DHPC peak (Fig. 9c, f), indicating that some M2TM-AH partitions into the planar region of the bicelles. The amide protons that correlate with the DMPC ^{31}P most likely result from the surface-bound amphipathic helix rather than the TM domain ⁴⁵.

Taken together, the 1D ^{31}P and ^2H spectra and the 2D ^{31}P - ^{31}P exchange spectra show that the amphipathic helix of M2 generates a high-curvature membrane domain that is associated with the DHPC edges of bicelles, while ^1H relaxation data and 2D ^1H - ^{31}P correlation spectra indicate that the protein predominantly binds the high-curvature domain, followed by a small fraction that partitions into the planar region of the bicelle. These results are depicted in Fig. 10, where the high-curvature phase is represented as micelles, although bicontinuous cubic phase cannot be ruled out.

Discussion

So far, bicelles have been mainly used to determine the high-resolution structures of membrane proteins by solution and solid-state NMR spectroscopy ^{46–49}. The data shown here represent a novel application of bicelles as a medium for characterizing protein-induced membrane curvature. The static ^{31}P spectra distinguish the lipid signals in the planar and edge regions of the bicelle versus lipids in high-curvature phases. By comparing lipid ^{31}P -detected and protein ^{13}C -detected lipid ^1H relaxation times, we can deduce the dynamic and curvature environment of the protein. By correlating lipid ^{31}P and protein ^1H chemical shifts, we can further define the protein binding sites.

The fact that M2(21-61) and not M2(22-46) generates the isotropic ^{31}P chemical shift and a zero-frequency ^2H peak in the static spectra indicates that the amphipathic helix is solely responsible for incurring membrane curvature. The high-curvature phase can be micelles, small vesicles, or bicontinuous cubic phases, all of which possess the symmetry to give an isotropic NMR peak. Recent synchrotron SAXS data indicate that both full-length M2 and an M2 peptide encompassing the TM and AH domains generate bicontinuous cubic phases ⁷. Full-length M2 required >60 mol% phosphatidylethanolamine (PE) in the membrane to induce the cubic phase, and the SAXS spectra index to an Ia3d gyroid phase with lattice parameters of ~20 nm. The TM - AH peptide generated a mixture of Pn3m and Im3m phases in a wider range of lipid compositions. In PE-free DOPC/DOPS membranes, a Pn3m double-diamond phase was observed with lattice parameters of 21–29 nm. These bicontinuous cubic phases have the common characteristics of possessing negative Gaussian

curvature, which is the type of curvature present in the neck of a budding virus. Thus their generation by the AH-containing M2 protein is consistent with the membrane scission function of M2⁶. Although it is tempting to conclude, on the basis of these SAXS data, that the isotropic ³¹P and ²H peaks detected here also results from a cubic phase, several factors argue for a micellar interpretation. DMPC and DHPC lipids have significantly stronger positive intrinsic curvature than the unsaturated DOPC, DOPS and DOPE lipids used in the SAXS study, thus a micellar phase with positive curvature is possible. From the biological standpoint, while the bicontinuous cubic phase captures the membrane geometry at the neck of the budding virus⁶, the micelle morphology captures the membrane curvature of the virus away from the budding neck. Finally, the exchange peak between DHPC and the isotropic peak in the 2D ³¹P-³¹P correlation spectra indicates that the high-curvature phase is partly associated with the bicelle edges (Fig. 3). Since bicontinuous phases have much larger overall dimensions due to their periodically repeating nature^{50, 51}, they are less likely to form significant contacts with other membrane phases. Thus, the ³¹P cross peak in the 2D spectra is also in favor of a micellar interpretation of the isotropic peak in the ³¹P and ²H NMR spectra.

Since the high-curvature phase generated by M2TM-AH represents only a small fraction (10–20%) of all lipids in our samples, preferential M2 binding to this domain may appear counter-intuitive. However, consideration of equilibrium indicates that this preferential binding is to be expected, because if the protein only resides in the low-curvature membrane while inducing high-curvature domains, the lamellar domain will be eventually depleted. Therefore, the small fraction of the high-curvature domain must be enriched with the protein at equilibrium. Given the preference of M2TM-AH for the high-curvature phase, a natural question is why a small amount of M2 binds the planar DMPC region rather than the round DHPC edges of the bicelle. We attribute this result to the unfavorable geometry of the bicelle edges. For the amphipathic helix to lie on the round caps formed by DHPC, the TM helix must lie perpendicular to the interior DMPC chains. This orientation is not only sterically unfavorable but also prevents the exposure of the N-terminus of the TM helix to water, which is seen in all high-resolution structures of M2^{27, 30, 45, 52}. In contrast, M2 binding to the planar DMPC region of the bicelle satisfies hydrophobic interactions of the TM helix with the membrane as well as polar interactions of the terminal residues with water, and is thus energetically favorable. Based on the ¹³C-detected ¹H T₂ relaxation data (Fig. 7f), we estimate that ~70% of M2TM-AH binds the high-curvature domain while ~30% binds the lamellar domain.

Several mechanisms exist with which proteins can induce high membrane curvature. First, amphipathic helices in proteins can insert asymmetrically into one leaflet of the membrane to promote local curvature. An example is the fusion peptide of the influenza hemagglutinin^{4, 53}. Analogously, integral membrane proteins or protein assemblies with intrinsic curvature can induce curvature by inserting into the membrane. Second, peripheral membrane proteins can brace the lipid bilayer like a scaffold to deform them into nonlamellar morphologies. For example, the dynamin family of proteins can bend the membrane into tubules to mediate vesicle invagination, membrane scission and cell division¹. Third, arginine-rich peptides such as antimicrobial peptides and cell-penetrating peptides can induce membrane curvature by electrostatic interactions between the cationic

residues and lipid phosphates^{12, 40, 54–57}. The M2 amphipathic helix, which lies on the membrane surface⁴⁵, likely induces membrane curvature through the scaffolding mechanism.

The fact that M2TM-AH generates this high-curvature phase in the simple membrane composition of DMPC/DHPC, without cholesterol, is noteworthy. A previous study of M2TM-AH in a virus-mimetic membrane containing phosphocholine, phosphoethanolamine, sphingomyelin, and cholesterol, also detected a ³¹P isotropic peak¹³. Combined with the recent SAXS results, which were obtained in cholesterol-free membranes⁷, these biophysical data together show that M2TM-AH can generate curvature in a variety of membrane compositions, and cholesterol is not a necessary condition for curvature induction. However, the exact type of curvature may differ among different membrane compositions, since the lipid components may affect the depth of the amphipathic helix, the clustering of the M2 tetramers, and the orientation of the TM and amphipathic helices^{45, 58–60}. Additional experiment will be necessary to determine the types of membrane curvatures induced by M2 in various lipid environments.

The 2D OMAS correlation NMR experiment shown here is analogous to variable-angle spinning, switched-angle spinning, and dynamic-angle spinning experiments pioneered by Terao, Pines and Fung^{61–65}. Most of these techniques focused on separating anisotropic interactions such as dipolar couplings and quadrupolar couplings in the indirect dimension by isotropic chemical shifts in the direct dimension. The current experiment differs in using anisotropic chemical shifts to resolve different membrane curvatures rather than chemical functional groups. Interestingly, the rotation angle determined from our ³¹P spectra (Fig. 8) under 3.5 kHz spinning indicates a different bicelle alignment from that predicted by the classical theory of variable-angle spinning of liquid crystals⁶⁶. The classical theory states that when the spinning frequency is larger than a critical frequency of several hundred hertz, liquid crystals do not have time to align with the magnetic field and adopt an orientation that minimizes the time-averaged potential energy. For liquid crystals with a negative anisotropy of magnetic susceptibility, χ , which is the case for bicelles, the bilayer directors are predicted to be distributed in a plane perpendicular to the rotation axis ($\beta = 90^\circ$) when the rotation angle θ is smaller than the magic angle, but the directors would be parallel to the rotation axis ($\beta = 0^\circ$) when $\theta > 54.7^\circ$ ^{67, 68}. The overall scaling factor, $(3\cos^2\theta - 1)/2 \cdot (3\cos^2\beta - 1)/2$, is thus negative under both conditions, indicating that the anisotropic ³¹P chemical shift should be *smaller* than the isotropic shift at all rotation angles. This was observed for spinning frequencies less than 1 kHz^{35, 69}. But under our spinning frequency of 3.5 kHz, the spectrum in Fig. 8b shows ³¹P chemical shifts that are *larger* (i.e. downfield) from the isotropic chemical shift, which translates to a positive overall scaling factor. This indicates that the directors remain perpendicular to the rotation axis even when $\theta > 54.7^\circ$, in contrast to the classical theory. This bicelle orientation under fast spinning has also been reported by Meier and coworkers³⁵, and suggests that under sufficiently fast spinning, the directors do not have time to change their alignment axis from the non-spinning condition and instead remain perpendicular to the rotation axis.

Relatively high amide H^N chemical shift resolution of ~0.4 ppm on bicelle-bound membrane proteins has recently been shown to be achievable under the static condition, without

spinning, by using optimized ^1H - ^1H homonuclear decoupling sequences and by correlating H^{N} chemical shifts with ^{15}N chemical shifts^{70, 71}. The OMAS ^1H - ^{31}P correlation technique is complementary to that approach. Since H^{N} - ^{31}P cross peaks are intermolecular by design, all protein amide H^{N} signals are observed together in the same ^{31}P cross section, thus residue-specific H^{N} resolution is not necessary. In addition, surface-bound membrane protein segments such as the M2 amphipathic helix may be less uniformly oriented than transmembrane segments, thus the OMAS approach, which is independent of the degree of alignment, is more robust for these difficult-to-orient membrane proteins.

Conclusion

We presented two bicelle-based SSNMR methods for determining membrane curvature and the binding site of proteins in mixed-curvature membranes. We demonstrated these methods on the influenza M2 protein, which mediates membrane scission during virus budding in addition to serving as a proton channel for virus uncoating. Our data show that the amphipathic helix in M2 confers the ability to cause high membrane curvature, and directs the protein to this high-curvature domain, while a small fraction of the protein resides in the planar region of the bicelle. The M2-induced high-curvature phase is associated with the round edges of the bicelles, reminiscent of the membrane geometry and deformation in a budding virus. The indirect detection of lipid ^1H relaxation NMR experiment and the 2D OMAS heteronuclear correlation technique are generally applicable to a wide range of membrane proteins to determine their binding sites in complex membranes with multiple curvatures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Variable-temperature ^{31}P spectra of static DMPC/DHPC (3 : 1) bicelles with and without M2. (a) Bicelles without protein. (b) Bicelle with M2TM. (c) Bicelle with M2TM-AH. M2TM-AH creates a temperature-independent isotropic ^{31}P peak, indicating the formation of a high-curvature membrane phase. The other ^{31}P chemical shifts are anisotropic and change with temperature.

Figure 2.

Static ^2H spectra of d_{22} -DHPC in DMPC/DHPC bicelles. (a) Bicelles without the peptide.

(b) Bicelles with M2TM-AH. The peptide-bound membrane shows a zero-frequency peak, indicating the presence of an isotropic membrane domain or a bicontinuous cubic phase.

Figure 3.

2D ^{31}P - ^{31}P exchange spectra of static DMPC/DHPC bicelles. (a) With M2TM-AH and 0.5 s spin diffusion. (b) With M2TM-AH and 1.0 s spin diffusion. (c) Without M2 and with 0.5 s spin diffusion. The spectra were measured at 297 K for (a, b) and 306 K for (c) to obtain the same DHPC ^{31}P chemical shift. Cross peaks between the isotropic peak and the DHPC peak (dotted circle) are observed for the protein-bound bicelles in (a) and (b).

Figure 4.

1D ^{13}C CP spectra of DMPC/DHPC bicelles with and without M2TM-AH under static and MAS conditions. (a) ^{13}C CP spectrum of peptide-bound bicelles at 283 K under MAS, showing resolved protein (red) and lipid (blue) signals at isotropic ^{13}C chemical shifts. (b) Static ^{13}C spectrum of peptide-bound bicelles at 283 K. The bicelle is in the isotropic phase at this temperature, thus the chemical shifts are the same as in the MAS spectrum but the linewidths are broader. (c) ^{13}C static spectrum of oriented bicelles without M2 at 300 K. The lipid chemical shifts differ from those in the MAS spectrum in (a) due to the presence of CSA under the oriented condition. (d) Static ^{13}C spectrum of M2-bound oriented bicelles at 302 K. The peptide ^{13}C chemical shifts are slightly different from the isotropic shifts in (a) and (b) due to the presence of CSA. Blue and red dashed lines guide the eye for chemical shift changes under different experimental conditions.

Figure 5.

^{31}P -detected ^1H T_2 relaxation data of DMPC/DHPC bicelles containing M2TM-AH under the static condition. (a) Pulse sequence of the indirectly detected ^1H T_2 experiment. (b) Representative ^{31}P DP and CP spectra with ^1H spin diffusion mixing at 300 K. (c) Representative ^{31}P DP and CP spectra with ^1H spin diffusion at 312 K. The ^{31}P chemical shifts differ between 300 K and 312 K for DMPC and DHPC peaks due to temperature-dependent exchange of DHPC between the planar and edge regions of the bicelle. (d) ^1H T_2 relaxation decays at 300 K after 10 ms ^1H spin diffusion. (e) ^1H T_2 relaxation decays at 300 K after 100 ms ^1H spin diffusion. (f) ^1H T_2 relaxation decays at 312 K after 10 ms spin diffusion. The DMPC, DHPC and high-curvature phase data are shown in black, blue and red, respectively.

Figure 6.

Directly detected ^1H T_2 relaxation data of DMPC/DHPC bicelles containing M2TM-AH under the static condition at 302 K. (a) Representative ^1H spectra with different echo delays. (b) ^1H T_2 relaxation curves with single or double exponential fits. The decay constants and the corresponding fractions are listed. The lipid relaxation times range from 0.4 to 50 ms, whereas the water ^1H T_2 relaxation time is much longer, 93 ms.

Figure 7.

^{13}C -detected ^1H T_2 relaxation data of M2TM-AH in oriented bicelles at 302 K under the static condition. (a) Pulse sequence of the dipolar-dephased ^1H T_2 relaxation experiment used to obtain the data in (g). (b) Representative ^{13}C spectra without dipolar dephasing, measured with 10 ms ^1H spin diffusion. (c) Representative ^{13}C spectra without dipolar dephasing, measured with 100 ms ^1H spin diffusion. (d) Representative ^{13}C spectra with dipolar dephasing and 100 ms ^1H spin diffusion. The echo delay is 0 for all the spectra in (b–d). (e) ^{13}C -detected ^1H T_2 relaxation curves with 10 ms spin diffusion. The schematic illustration below indicates that ^1H magnetization transfer is mostly within the peptide at this mixing time. (f) ^{13}C -detected ^1H T_2 relaxation curves after 100 ms spin diffusion. At this mixing time, lipid ^1H magnetization from the surrounding environment is transferred to the peptide, thus giving much slower relaxation. A fast initial decay is especially pronounced for the L26 Cb signal. (g) ^1H T_2 relaxation curves measured with 100 μs C-H dipolar dephasing and 100 ms spin diffusion, to selectively detect the T_2 relaxation of lipids near the peptide. The disappearance of the fast initial decay of the L26 Cb data verifies that the origin of the fast initial decay is the rigid peptide protons.

Figure 8.

1D ^{31}P spectra of bicelles containing M2TM-AH under 3.5 kHz spinning at 300 K. (a) ^{31}P DP MAS spectrum. (b) ^{31}P DP and CP spectra under OMAS at 58.7° . Three ^{31}P peaks are resolved. (c) ^{31}P DP and CP spectra under OMAS at 52.5° . (d) Comparative static ^{31}P spectrum, showing three well resolved peaks.

Figure 9.

2D ^{31}P - ^1H correlation spectra with ^1H homonuclear decoupling, measured at 306 K under OMAS at 58.7°. (a) Pulse sequence for the homonuclear-decoupled HETCOR experiment. (b) 2D spectrum without ^1H spin diffusion. (c) 2D spectrum with 0.5 ms ^1H spin diffusion. (d) 1D ^1H MAS spectrum. (e) 1D ^1H OMAS spectrum at 58.7°. (f) ^1H cross sections of DMPC with 0.5 ms (top) and 0 ms (bottom) spin diffusion, extracted from the 2D spectra in (b) and (c).

Figure 10.

Schematic of M2TM-AH binding to membranes with mixed curvatures. The peptide creates a high-curvature phase that is partially associated with the round caps of bicelles. Depicted here are micelles, but bicontinuous cubic phases cannot be ruled out. Most of the peptide binds to this high-curvature phase, while a small fraction resides in the planar region of the bicelle due to its stabilization of the hydrophobic transmembrane helix. For simplicity, only two out of four molecules of the tetrameric protein is represented.