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Solid-state NMR spectroscopy of a membrane protein in biphenyl phospholipid bicelles with the bilayer normal parallel to the magnetic field

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ABSTRACT

Bicelles composed of the long-chain biphenyl phospholipid TBBPC (1-tetradecanoyl-2-(4-(4-biphenyl)butanoyl)-sn-glycero-3-PC) and the short-chain phospholipid DHPC align with their bilayer normals parallel to the direction of the magnetic field. In contrast, in typical bicelles the long-chain phospholipid is DMPC or DPPC, and the bilayers align with their normals perpendicular to the field. Samples of the membrane-bound form of the major coat protein of Pf1 bacteriophage in TBBPC bicelles are stable for several months, align magnetically over a wide range of temperatures, and yield well-resolved solid-state NMR spectra similar to those obtained from samples aligned mechanically on glass plates or in DMPC bicelle samples "flipped" with lanthanide ions so that their bilayer normals are parallel to the field. The order parameter of the TBBPC bicelle sample decreases from approximately 0.9 to 0.8 upon increasing the temperature from 20 °C to 60 °C. Since the frequency spans of the chemical shift and dipolar coupling interactions are twice as large as those obtained from proteins in DMPC bicelles without lanthanide ions, TBBPC bicelles provide an opportunity for structural studies with higher spectral resolution of the metal-binding membrane proteins without the risk of chemical or spectroscopic interference from the added lanthanide ions. In addition, the large temperature range of these samples is advantageous for the studies of membrane proteins that are unstable at elevated temperatures and for experiments requiring measurements as a function of temperature.

1. Introduction

Bicelles provide a fully hydrated, planar phospholipid bilayer environment for membrane proteins that ensures that they are in their functional, native conformations for structural studies by NMR spectroscopy and other biophysical methods [1]. Bicelles self-organize from an appropriate mixture of long-chain (e.g. DMPC) and short-chain (e.g. DHPC) phospholipids in aqueous solution. The principal characteristics of bicelles are determined by q, the molar ratio of long-chain to short-chain phospholipids, the temperature, and the lipid concentration. Small isotropic bicelles ($q < 1.0$) share many properties with micelles, reorient rapidly in solution, and are often used in solution-state NMR studies of membrane proteins [2,3]. In contrast, large anisotropic bicelles ($q > 2.0$) share many properties with lipid bilayers, immobilize membrane proteins on relevant NMR timescales, and align in the magnetic field of the NMR spectrometer at temperatures above the gel to liquid crystalline transition temperature of the long-chain phospholipids; they can be used to align membrane proteins for solid-state NMR experiments [4,5]. Bicelles are also widely used as media to induce weak alignment of soluble proteins for solution-state NMR experiments [6].

Sample alignment has an important role in both solution-state NMR and solid-state NMR studies of proteins, since it provides access to valuable orientational constraints for the calculation of atomic-resolution structures. We have determined the structures of several membrane-associated polypeptides in magnetically aligned bicelles by solid-state NMR spectroscopy [7–9]. Extension of the approach to larger and more complex membrane proteins has been hampered by limitations of the available bicelle-forming phospholipids, especially the requirement of relatively high sample temperatures (>40 °C) for alignment of the most commonly used long-chain phospholipids and the need to add paramagnetic lanthanide ions to the samples to "flip" the direction of the alignment so that the bilayer normal's are parallel to the magnetic field. There are advantages to studying protein-containing bicelles that align with their bilayer normals parallel to the field, since the spectral ranges of the chemical shift and dipolar coupling interactions are twice as large as those in samples aligned perpendicular. This property is very desirable because of the potential for obtaining higher resolution in the

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spectra of parallel samples. Although this alignment can be obtained with conventional bicelle samples, it requires the addition of lanthanide ions, and there is always concern about the effects of the paramagnetic metals on both the protein and phospholipid components of the samples.

Recently, it has been shown that 1-tetradecanoyl-2-(4-(4-biphenyl)butanoyl)-sn-glycero-3-PC (TBBPC), which contains a biphenyl group in one of the acyl chains (Fig. 1B), forms bicelles that are stably aligned in a magnetic field over a wide range of temperatures (10–75 °C) when mixed with an appropriate amount of the short-chain lipid DHPC [10]. Since the biphenyl group has a large positive magnetic anisotropy [11], TBBPC bicelles align with their bilayer normals parallel to the applied magnetic field without the addition of paramagnetic ions. Thus, TBBPC bicelles address both of the major limitations of the standard DMPC bicelles, and provide a phospholipid bilayer environment conducive to structural studies of membrane proteins that are not stable at high temperatures, that require a parallel alignment for adequate spectral resolution, or that are adversely affected by the presence of lanthanide ions.

In this article, we demonstrate the application of TBBPC bicelles in solid-state NMR studies using the membrane-bound form of the major coat protein of Pf1 bacteriophage, a well-characterized membrane protein with a single transmembrane helix [12–14], as an example. Uniformly ^{15}N labeled coat protein was incorporated into TBBPC bicelles, and the solid-state NMR spectra from the protein in a magnetically aligned TBBPC bicelle sample were compared to those from a mechanically aligned DOPC/DOPG bilayer sample and from a "flipped" DMPC bicelle sample. The temperature dependence of the spectra from protein-containing TBBPC bicelles was also characterized.

2. Results and discussion

2.1. Pf1 coat protein in TBBPC bicelles with their bilayers normal parallel to the magnetic field

A uniaxially ordered sample aligned with its single axis of symmetry parallel to the direction of the applied magnetic field yields NMR spectra characterized by single line resonances in all frequency dimensions. Because the observed resonance frequencies depend on the orientation of their respective molecular sites relative to the axis of alignment (and the field), they provide powerful orientational constraints for the characterization of secondary structure and input for the calculation of the three-dimensional structures of proteins [15].

^{15}N NMR spectra of Pf1 coat protein in three types of phospholipid bilayers aligned with their normals parallel to the direction of the magnetic field are compared in Fig. 1. The two phospholipids used in each of the samples are shown on top of the spectra. Aligned samples of Pf1 coat protein-containing bilayers were prepared in two ways; mechanically on glass plates (Fig. 1A) and magnetically in two different types of bicelles (Fig. 1B and C). The resulting spectra are similar because all three samples have their bilayer normals parallel to the field. In the case of DMPC bicelles (Fig. 1C), this required the addition of lanthanide ions to "flip" the bicelles from the perpendicular to parallel alignment. In all three samples, the narrow resonance linewidths observed in both one- and two-dimensional spectra are representative of those of well-aligned proteins.

The one-dimensional NMR spectra in Fig. 1 are well-resolved with many individual resonances distinguishable within the 70–220 ppm span of the ^{15}N amide chemical shift interaction, how-

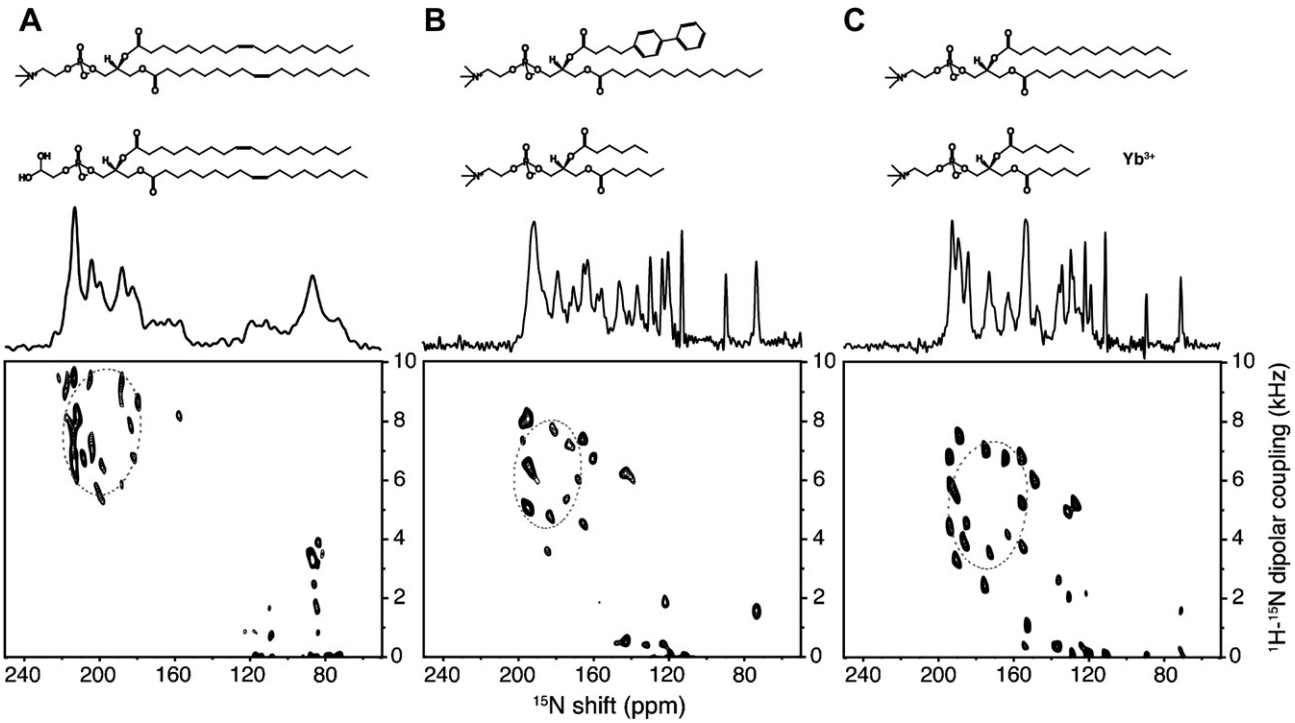

Fig. 1. One-dimensional ^{15}N chemical shift and two-dimensional ^{15}N chemical shift/^{1}H–^{15}N dipolar coupling SLF solid-state NMR spectra of uniformly ^{15}N labeled Pf1 coat protein aligned with its bilayer normal parallel with respect to the applied magnetic field in various lipid bilayer environments represented on top. (A) DOPC/DOPG (9:1, w/w) bilayers aligned mechanically on glass plates at 23 °C. (B) TBBPC:DHPC bicelles (q = 8, 20% lipid (w/v)) at 40 °C. (C) DMPC:DHPC bicelles (q = 3.2, 28% lipid (w/v)) flipped with 3 mM $YbCl_3$ at 40 °C. The molar ratios of lipid bilayers to protein concentration (L/P) are 88 (A), 50 (B), and 54 (C), respectively. Superimposed on the experimental data are the ideal PISA wheels for an α-helix with uniform dihedral angles (phi = −61° and psi = −45°) tilted at 20° with δ = 1.0 (A), 21° with δ = 0.83 (B), and 27° with δ = 0.8 (C), respectively.

ever, most of the signals are within the range associated with tilted transmembrane helices (160–220 ppm). Nearly complete spectral resolution can be observed in the two-dimensional separated local field (SLF) spectra where each amide site contributes a correlation resonance characterized by unique ^{1}H–^{15}N dipolar coupling and ^{15}N chemical shift frequencies, and the characteristic PISA Wheel patterns corresponding to helical wheel projections are formed by the resonances from residues in the transmembrane helix [16,17].

The resonance linewidths in the spectra from the magnetically aligned bicelle samples (Fig. 1B and C) are narrower than those from the mechanically aligned bilayer sample on glass plates (Fig. 1A). Also, the frequency ranges of the resonances of the spectra in Fig. 1B and C are somewhat smaller than that in Fig. 1A because membrane proteins (and phospholipids) in magnetically aligned bicelles have an overall order parameter (0.8–0.9), slightly reduced [18] compared to that of a rigid lattice (1.0) observed in mechanically aligned bilayers.

The alignment of protein-containing bicelles is a consequence of the interaction of the magnetic field and the magnetic susceptibility anisotropy of the liquid crystalline phospholipid bilayers. The proteins do not appear to play a role in the mechanism of alignment, but rather are aligned passively by the lipid matrix in which they are embedded. The negative sign of the anisotropy tensor of long-chain dialkanoylphospholipids (e.g. DMPC or DPPC) results in bicelles that align with their normals perpendicular to the direction of the magnetic field. The orientation of the bilayer normals can be "flipped" from perpendicular to parallel to the field by introducing a large positive magnetic susceptibility to the sample. The spectra in Fig. 1B and C illustrate two approaches used to align the bicelles with their bilayer normals parallel to the magnetic field, yielding similar high-resolution NMR spectra that differ only because of slightly different order parameters and helix tilt angles that most likely reflect differences in the dynamics and thicknesses of the bilayers formed by TBBPC and DMPC, although it is not possible to rule out the influence of the biphenyl group itself. Similarly, a larger order parameter (0.85) and smaller tilt angle of the transmembrane helix have been observed in bicelles with C16 long-chain lipids (DPPC) compared to C14 long-chain lipids (DMPC) [7].

2.2. Effect of temperature on the order parameter of TBBPC bicelles

The magnetically alignable phase of TBBPC bicelles exists over a much larger range of temperatures and a narrower range of q values than DMPC bicelles [10]. The one-dimensional ^{15}N NMR spectra of uniformly ^{15}N labeled Pf1 coat protein in TBBPC bicelles shown in Fig. 2 demonstrate that the samples are highly aligned, and there is no evidence of powder patterns indicative of unoriented material, between 20 °C and 60 °C; however, the linewidths of peaks from residues in the transmembrane helix (140–200 ppm) appear to be somewhat broader at the lowest temperature (Fig. 2C). Significantly, the sample was stable and gave reproducible results over a period of several months.

There are two sets of spectra in Fig. 2. The spectra in the left column (Fig. 2A–C) were obtained by cross-polarization with a relatively long mix time (1 ms), which ensures that signals are present from amide ^{15}N sites with both strong and weak heteronuclear dipolar couplings. The spectra in the right column (Fig. 2D–F) were obtained with a short mix time (60 μs), which discriminates towards signals from sites with strong dipolar couplings, and thus enables the experimental results to focus on signals from residues in the transmembrane helix. A systematic downfield (toward higher frequency) change in the chemical shifts was observed for the resonances from residues in the transmembrane helix with decreasing temperature; in contrast, signals from residues between the amphipathic surface helix and the transmembrane helix (110–130 ppm) were not significantly affected, as highlighted in Fig. 2A–C.

For membrane proteins with a single transmembrane helix, the tilt angle of the helix can be readily determined from the one-dimensional chemical shift spectrum of a uniformly ^{15}N labeled sample. This is most clearly demonstrated in spectra obtained with a short mix time where only signals from the transmembrane helix are present. With increasing tilt angle, not only does the average chemical shift frequency of the spectrum move upfield, but also the frequency breadth of the wheel-like pattern associated with the transmembrane helix decreases [19]. In the case of the TBBPC bicelle sample, the average value of the chemical shift frequencies moves downfield with decreasing temperature, while the frequency breadth of the pattern remains unchanged. Measurements obtained from the spectra in Fig. 2 are plotted in Fig. 3 as a function

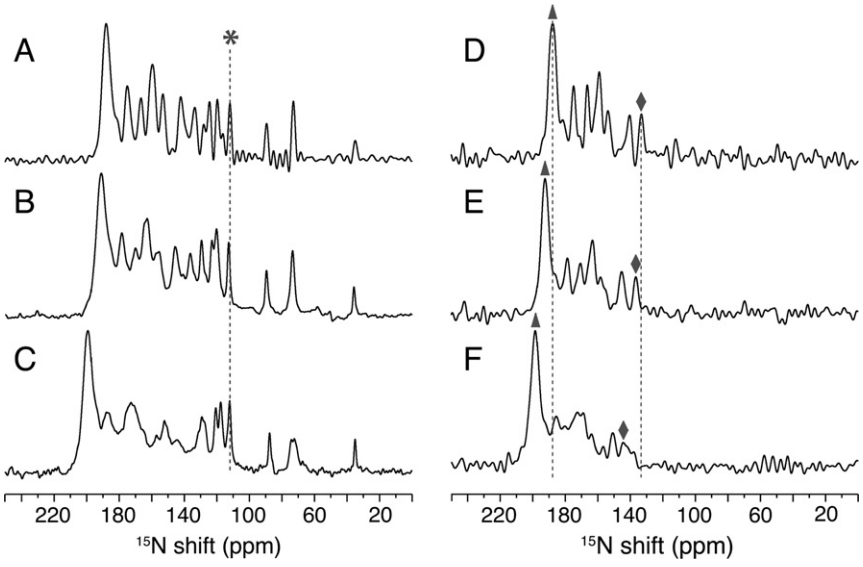

Fig. 2. One-dimensional ^{15}N chemical shift solid-state NMR spectra of uniformly ^{15}N labeled Pf1 coat protein in TBBPC:DHPC bicelles (q = 8, L/P = 50, 20% lipid (w/v)) at 60 °C (A and D), 40 °C (B and E), and 20 °C (C and F). The spectra were obtained by cross-polarization using 1 ms mix time (A, B, and C) and 60 μs mix time (D, E, and F). The resonances with the largest (▲) and smallest (♦) chemical shift frequencies from the transmembrane helix and Gly17 (*) are represented.

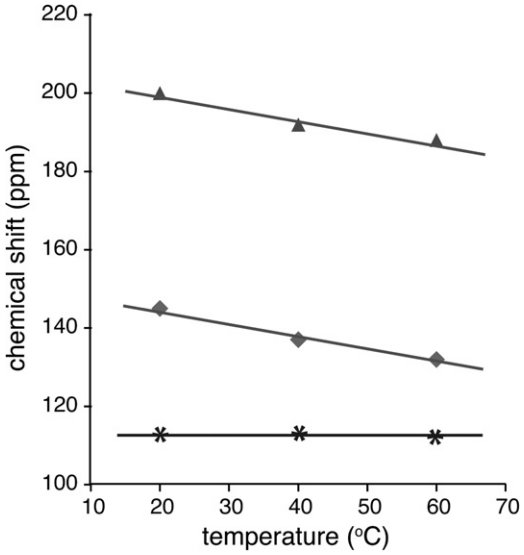

Fig. 3. Plot of chemical shifts from the spectra in Fig. 2 as a function of temperature. The experimental values are included by the symbols defined in Fig. 2. Straight lines are drawn through the data points.

of temperature. These results suggest that the tilt angle of the helix is unchanged but that the order parameter of the transmembrane helix in TBBPC bicelles increases from 0.8 to 0.9 upon decreasing the temperature from 60 °C to 20 °C. Deuterium NMR spectra (data not shown) obtained from TBBPC-d_{27} bicelles also showed an increase of the order parameter for the C–D bonds of the labeled lipid with decreasing temperature [10].

2.3. Manipulation of the bicelle orientation

Among the most useful characteristics of bicelle samples for NMR studies of membrane proteins is the ability to "flip" the orientation between perpendicular and parallel alignments of the bilayer normal relative to the direction of the magnetic field. This enables the spectral range of the frequencies to be changed by a factor of two. Most obviously, this improves the resolution available in the chemical shift dimension, since in general the linewidths do not increase proportionately. Also, the comparison of spectra obtained with perpendicular and parallel alignments enables the identification of isotropic resonances, since they are the only signals that do not change frequencies, as well as the assignment of resonances to protein sites whose isotropic frequencies have been identified in other experiments [20]. At high magnetic fields, the performance of the pulse sequences that effect homonuclear proton decoupling are often limited by the bandwidth of the radiofrequency irradiation; in this situation, the overall resolution available from many two-dimensional experiments is actually improved by operating with half the spectral range in bicelles with their normals perpendicular to the field.

Consequently, it is valuable to have the flexibility to choose the perpendicular or parallel alignment for specific experiments, and the demonstration that the orientation of bicelles could be "flipped" 90° by addition of lanthanide ions was a landmark in the development of these systems for studies of membranes. The ions associate with the phospholipids head groups of the lipids in the bicelles [21], effectively changing their overall magnetic susceptibility [21,22]. Yb^{3+} ions with large positive magnetic susceptibilities flip the DMPC bicelles (Fig. 4C), while Dy^{3+} ions with large negative magnetic susceptibilities flip the TBBPC bicelles (Fig. 4B). Although no significant line broadening was observed with the addition of lanthanide ions Yb^{3+} up to a final concentration of 3 mM for the signals from the transmembrane region of Pf1 coat protein (Fig. 4C), the presence of lanthanide ions are always of concern as a perturbation on the spectra or structure of the proteins. The comparison of the spectra in Fig. 4A and 4B demonstrate that it is possible to "backflip" the TBBPC bicelles from the parallel to perpendicular alignments with lanthanides, while illustrating perturbations in the form of broad signals in Fig. 4B that result from the significant effects on chemical shift and line width broadening associated with Dy^{3+} ions [23].

3. Conclusions

The two major differences between TBBPC bicelles and typical DMPC bicelles have the potential to facilitate structure determination of challenging membrane proteins. First, the parallel alignment of TBBPC bicelles does not require the presence of lanthanide ions, which can interfere with the spectroscopic experiments and alter the native structure of the protein. Thus, TBBPC bicelles may be essential for studies of metal-binding membrane proteins, and the twice as large chemical shift range for parallel bicelles may be especially helpful for studies of membrane surface-associated proteins, which typically have narrow chemical shift dispersions due to their helical axes being aligned almost parallel to the membrane plane. Second, the large temperature range of TBBPC bicelles, especially below 25 °C, will be very useful for studies of temperature-sensitive membrane proteins [24–26].

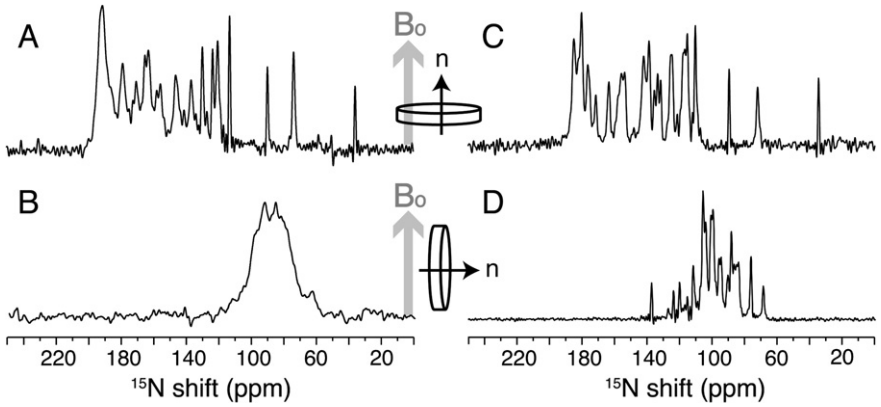

Fig. 4. One-dimensional ^{15}N chemical shift solid-state NMR spectra of uniformly ^{15}N labeled Pf1 coat protein aligned in bicelles. (A) TBBPC:DHPC bicelles. (B) TBBPC:DHPC bicelles with 5 mM $DyCl_3$. (C) DMPC:DHPC bicelles with 3 mM $YbCl_3$. (D) DMPC:DHPC bicelles. The conditions of bicelle samples are the same as for Fig. 1. Schematic diagrams of parallel and perpendicular bicelles are represented.

The advantages of TBBPC bicelles will contribute to structure determination of membrane proteins by NMR by providing greater flexibility in sample conditions, in particular the parallel alignment without lanthanides and range of temperatures. The continuing development of bicelle systems means that a broader range of membrane proteins can be reconstituted and immobilized in phospholipid bilayers under conditions where they retain their native conformations and biological activities [27,28].

4. Materials and methods

4.1. Sample preparation

Uniformly ^{15}N labeled Pf1 bacteriophage was prepared as described previously [29]. The Pf1 major coat protein was purified by reverse phase HPLC (Waters Delta 600 Chromatography System) on a Delta-Pak C18 column (15 m, 300 Å, $7.8 \times 300\ nm^2$, Waters, Milford, MA). A concentrated sample of the bacteriophage Pf1 (~50 mg/ml) was dissolved in the HPLC injection solvent (50% trifluoroethanol, 49.9% H_2O, 0.1% trifluoroacetic acid) at a concentration of 2 mg/ml. The DNA precipitate was removed by filtration with a 0.2 μm hydrophobic fluoropore (PTFE) membrane (Millipore Corp. Bedford, MA) and the resulting clear solution was injected onto a column equilibrated with 90% buffer A (90% H_2O, 10% acetonitrile, 0.1% trifluoroacetic acid) and 10% buffer B (90% acetonitrile, 10% H_2O, 0.1% trifluoroacetic acid). Elution was carried out with a 10 min wash with 90% buffer A and 10% buffer B, followed by a linear gradient to 90% buffer B over 50 min, at a flow rate of 3 ml/min with the absorbance monitored at 280 nm. Fractions containing pure Pf1 protein were pooled. Organic solvents and trifluoroacetic acid were removed by flowing nitrogen gas, and then the sample was lyophilized.

The mechanically oriented sample of uniformly ^{15}N labeled coat protein in hydrated lipid bilayers was prepared as described previously [30]. The phospholipids used to prepare bilayer samples were 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DOPG) from Avanti Polar Lipids, Inc. (Alabaster, AL). Seventy-five milligrams of lipid mixture (DOPC:DOPG, 9:1, w/w) was dissolved in 300 μl chloroform and mixed with 5 mg of Pf1 coat protein dissolved in 300 μl trifluoroethanol. The protein/lipid mixture was spread onto 20 glass slides and dried completely using a vacuum pump. The stacked plates were hydrated to 95% of relative humidity in a chamber saturated with potassium sulfate, pH 7.0 at 40 °C, and then sealed with a polymer wrap.

The bicelle samples were prepared using a modified version of the method described previously [7]. Uniformly ^{15}N labeled Pf1 coat protein (3–5 mg) was dissolved in 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC). Then this solution was added to the long-chain lipids, either 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) or 1-tetradecanoyl-2-(4-(4-biphenyl)butanoyl)-sn-glycero-3-PC (TBBPC) [10], followed by extensive vortexing and freeze/heating cycles (liquid nitrogens/45 °C). The lipid compositions were arranged so that the samples had $q = 3.2$ and a lipid concentration of 28% (w/v) for DMPC bicelles, and $q = 8$ and a lipid concentration of 20% (w/v) for TBBPC bicelles in 160 μl volume, at pH 6.7. A final concentration of 3 mM $YbCl_3$ was added to "flip" the DMPC bicelle samples from the perpendicular to the parallel orientation, and 5 mM $DyCl_3$ was added to "backflip" the TBBPC bicelles from the parallel to the perpendicular orientation.

4.2. Solid-state NMR spectroscopy

The solid-state NMR experiments were performed on 750 MHz Bruker Avance spectrometer equipped with a home-built double resonance probe. The one-dimensional ^{15}N chemical shift spectra were recorded with cross-polarization using contact times of 60 μs or 1 ms, recycle delay of 7 s, and a 90° pulse width of 5.1 μs. Transients (2048–4096) were accumulated, and an exponential line broadening of 50–100 Hz was applied to all spectra. The two-dimensional separated local field experiments were performed as described previously [7,30]. The number of t1 increments varied between 48 and 64. Signal averaging was performed with 128–256 transients for each t1 increments. The ^{15}N chemical shift was externally referenced to ^{15}N ammonium sulfate at 26.8 ppm. The data were zero-filled in both t2 and t1 dimensions, yielding a 1024×1024 real matrix. A 30° phase-shifted sine bell multiplication followed by 100 Hz of exponential multiplication was applied in t1. The NMR data were processed using the program NMRPipe/NMRDraw [31].

Acknowledgments

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