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# High-Resolution NMR Spectroscopy of Membrane Proteins in Aligned Bicelles

Anna A. De Angelis, Alexander A. Nevzorov, Sang Ho Park, Stanley C. Howell,
Anthony A. Mrse, and Stanley J. Opella*

*Department of Chemistry and Biochemistry, University of California San Diego, 9500 Gilman Drive,
La Jolla, California 92093-0307*

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The ideal sample for structure determination of membrane proteins can be envisaged as a mixture of lipids, salts, and water that, upon addition to purified polypeptide, self-assembles into bilayers that both immobilize the protein in its active, native conformation and align it magnetically. This is remarkably close to the description of bicelles;[1] however, their use in structural studies of expressed membrane proteins has been limited by several factors. Previous studies of lipid assemblies aligned perpendicular to the magnetic field[2] have been restricted to synthetic peptides where motional averaging was essential to obtain resolution in samples with more than one labeled site. In this paper, we demonstrate that magnetically aligned bicelles can be used for structure determination of uniformly ^{15}N-labeled membrane proteins by solid-state NMR spectroscopy; this is possible because the proteins undergo rotational diffusion about the direction of the bilayer normal that is rapid on the $10^4$ Hz time scales of the spin interactions at backbone amide sites.[3]

Bicelles consist of long-chain phospholipids that form planar bilayers and short-chain lipids that "cap" the rim of the bilayer and are characterized by $q$, the ratio of long-chain to short-chain lipids. Small "isotropic" bicelles ($q < 0.5$) reorient rapidly in solution.[1e] By contrast, large bicelles ($q > 2.5$) align as shown in Figure 1E, with the bilayer normal perpendicular to the direction of the applied magnetic field. Liquid crystalline bicelles have been variously described as disks,[1a−g] perforated lamellae,[1h] and wormlike micelles,[1i] all of which are consistent with the experimental spectra in Figures 1 and 2 and are effectively modeled by a disk undergoing rotational diffusion about an axis perpendicular to the magnetic field (Figure 1E). A seminal paper demonstrating that the disks could be "flipped" 90° by the addition of lanthanide ions[4] elicited substantial interest because these samples are compatible with structure determination by solid-state NMR of aligned samples, which is predicated on the molecules being immobile and uniaxially aligned parallel to the magnetic field.[5] While it is possible to obtain high-resolution solid-state NMR spectra of membrane proteins aligned in this way,[1c] there are undesirable spectroscopic complications and sample instabilities due to the presence of the paramagnetic ions.

Nonhydrolyzable ether-linked lipids 1,2-*O*-ditetradecyl-*sn*-glycero-3-phosphocholine (14-O-PC) and 1,2-*O*-dihexyl-*sn*-glycero-3-phosphocholine (6-O-PC), previously shown to form a lyotropic liquid crystalline phase under similar conditions as bicelles consisting of the phospholipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC),[6] contribute to sample stability. The resulting $q = 3.2$ bicelles have near-perfect uniaxial alignment and a molecular order parameter of 0.8, reflecting a small degree of high-frequency "wobble" of the disks. The 160 $\mu$L samples have 2.8 mg of protein and 28% (w/v) lipids (Avanti). "Flipped" bicelles contain 3 mM $YbCl_3 \cdot 6H_2O$ (Sigma). Proteins were incorporated in bicelles, as described previously for DMPC/DHPC bicelles.[1c]
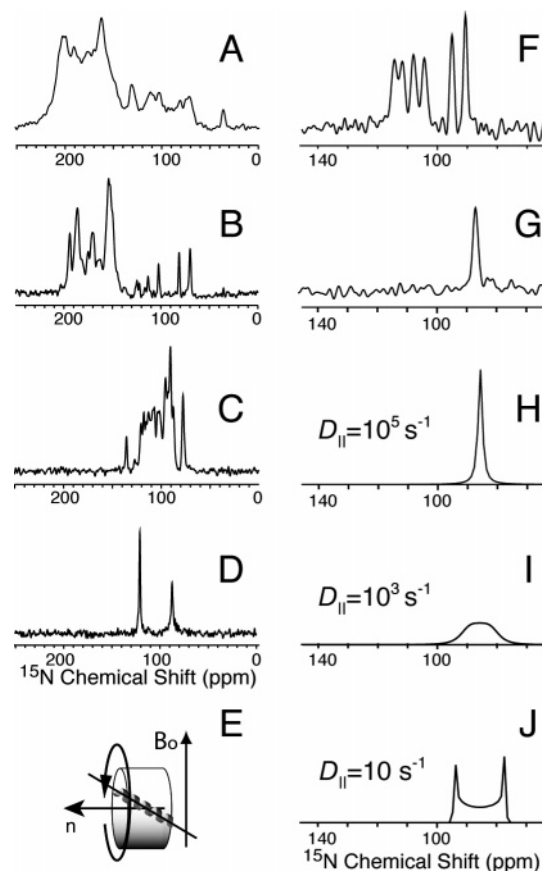


**Figure 1.** ^{15}N solid-state NMR spectra of the transmembrane domain of Vpu. (A) Uniformly labeled protein in bilayers on glass plates, (B) in "flipped" bicelles, and (C) in "unflipped" bicelles. (D) [15]N Trp-labeled protein in "unflipped" bicelles (one residue). (E) Diagram of an "unflipped" bicelle containing a transmembrane helix. (F) Val (six residues) and Leu (one residue) selectively ^{15}N-labeled proteins in "unflipped" bicelles. (H−J) Simulated ^{15}N NMR spectra for a single NH bond in "unflipped" bicelles undergoing axial diffusion about the bilayer normal **n** on different time scales. The experimental spectra were obtained at 313 K using a Bruker Avance console, a 16.4 T Magnex magnet with an ^{1}H resonance frequency of 700 MHz, and a home-built probe with a 5 mm ID double-tuned solenoid coil. Acquisition parameters were 54 kHz [1]H $B_1$ field, 1K scans, 1 ms cross-polarization mixing time, 7 s recycle delay, and 10 ms acquisition time using SPINAL-16 for heteronuclear decoupling.[14]

The solid-state ^{15}N NMR spectra (Figure 1A−C) of a 36-residue polypeptide[7] aligned in bilayers on glass plates with normals parallel to the magnetic field, parallel "flipped" bicelles, and perpendicular "unflipped" bicelles contain narrow single-line resonances. Significantly, there is no evidence of residual "powder pattern" line shapes in any of the spectra. By comparison with simulated spectra (Figure 1H−J), the rotational diffusion coefficient about the bicelle axis of alignment **n** is $D_\| \geq 1 \times 10^5$ s$^{-1}$.
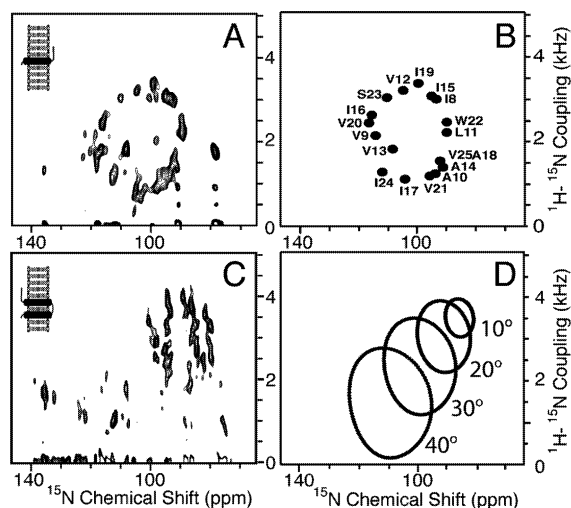
**Figure 2.** Two-dimensional $^1H-^{15}N$ dipolar coupling and $^{15}N$ chemical shift PISEMA spectra of uniformly $^{15}N$-labeled membrane proteins in "unflipped" bicelles. (A) Transmembrane domain of Vpu (37 residues). (B) Resonance assignments for the spectrum in A. (C) MerF-t (60 residues). (D) Simulated PISA wheel patterns in "unflipped" bicelles undergoing rapid rotational diffusion (Figure 1E) for an α-helix with uniform dihedral angles $(\phi, \psi) = (-61°, -45°)$ tilted with respect to the bilayer normal. Parameters for PISEMA were 54 kHz $^1H$ $B_1$ field, 128 scans, 1 ms cross-polarization mixing time, 7 s recycle delay, 128 $t_1$ increments, and 10 ms acquisition time.

The resonance line widths (1−2 ppm) for the magnetically aligned bicelle samples are narrower than those typically observed for mechanically aligned bilayers or peptide single crystals. The dramatic differences in chemical shift frequencies between "flipped" (Figure 1B) and "unflipped" (Figure 1C) bicelles demonstrate that the orientations of individual peptide planes relative to the magnetic field are reflected in the spectra. PISEMA (polarization inversion spin exchange at the magic angle)[8] yields high-resolution separated local field spectra where each resonance is characterized by orientation-dependent heteronuclear $^1H-^{15}N$ dipolar coupling and $^{15}N$ chemical shift frequencies. Helices result in characteristic PISA (polarity index slant angle) wheel patterns of resonances that reflect their tilt and polarity in the bilayers.[9] The magnitudes of the chemical shift and dipolar coupling frequencies measured from these spectra can be plotted as a function of residue number, generating sinusoidal waves with a period of 3.6 for an α-helix.[10] Furthermore, atomic-resolution structures of the proteins can be calculated[11a] or obtained by "structural fitting"[11b] to the orientation-dependent frequencies.

Panels A and C of Figure 2 contain experimental PISEMA spectra of uniformly $^{15}N$-labeled membrane proteins in "unflipped" bicelles. Comparison of the wheel-like pattern of resonances in Figure 2A to the simulated PISA wheels in Figure 2D shows that the transmembrane helix of Vpu has a tilt angle of approximately 30° in bicelles; this differs from the 13° tilt observed in bilayers,[7] reflecting the influence of different lipid chain lengths in the samples. As indicated in Figure 2B, nearly all of the resonances from residues in the transmembrane helix have been assigned using the "shotgun" approach[11a] that takes advantage of the structural mapping present in spectra of both uniformly and selectively labeled helical proteins. MerF-t is a 60-residue protein that corresponds to the core of the bacterial mercuric ion transporter MerF,[12] and its spectrum (Figure 2C) displays resolved resonances from essentially all residues in the two transmembrane helices as overlapping PISA wheel patterns as well as in the interhelical loop and terminal regions; there is little or no isotropic resonance intensity in the spectrum

that would be associated with mobile residues. Both of the transmembrane helices in MerF-t have tilt angles of approximately 20°.

The ability to obtain spectra like those in Figure 2 means that bicelles can be used as samples for structure determination of membrane proteins. Significantly, there is no reason that this method is limited to membrane proteins with only one or two transmembrane helices. Although the spectra of larger proteins will be more crowded, the resonances will not be broader or weaker, and there are many multidimensional solid-state NMR experiments that can be used to resolve and assign resonances that overlap in two-dimensional spectra.[13] Among the advantages of magnetically aligned bicelles are their ease of preparation, the use of a sealed sample tube that ensures sample stability (>1 year), and the placement of the sample inside a solenoid coil for optimal probe performance; in contrast, the widely used glass plate samples are marginally sealed in plastic films, have a significant fraction of the active volume wasted on glass, and require the use of "flat coil" probes. However, the most important feature of these samples is that the membrane proteins are functional[15] in fully hydrated lipid bilayers under physiological conditions.

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