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Bicelles enable magic angle spinning solid-state NMR structural studies on a large soluble domain containing membrane protein, cytochrome b5**

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Various important functional roles, related to a number of diseases, played by membrane proteins can be better understood by solving their high-resolution structures and dynamics. While structural studies on membrane proteins have been a great challenge to most biophysical techniques, recent NMR studies have overcome some of the difficulties for several proteins.¹ However, structural studies of membrane proteins still remain as a great challenge mainly because of the difficulty in finding a well-behaved model membrane sample. The use of multilamellar vesicles containing a transmembrane protein could enable the application of solid-state NMR techniques but they are not suitable for membrane proteins containing large soluble domains as they may not fold well to result in high-resolution spectra; whereas obtaining high-resolution spectra is a mandatory first step in solving the protein structure using NMR. In this study, we demonstrate that bicelles² are suitable to overcome these difficulties and enable the use of MAS (magic angle spinning) solid-state NMR experiments for the structural studies on a large soluble domain containing membrane anchored protein, cytochrome b₅ (cyt b₅). Cyt b₅ participates in and modifies the

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catalytic activity of cytochrome *P450*, and its high-resolution structure is not known.³ Cyt b5 is also involved as an electron transfer component in a number of oxidative reactions in biological tissues which includes the biosynthesis of fatty acids and steroids.³

Isotropic ¹⁵N chemical shift spectra of uniformly-¹⁵N labeled full-length 16.7 kDa rabbit cyt b₅ embedded in DMPC:DHPC bicelles and DMPC MLVs are given in Figure 1. RampCP⁴ and RINEPT⁵ sequences were used to transfer magnetization from ¹H to ¹⁵N under 5 kHz MAS. RampCP experiments were carried out on MLVs and bicelles with various contact times to optimize the sensitivity (see the Supporting Information). Amide-¹⁵N spectral lines appear in the 105–130 ppm range of all spectra in Fig. 1. Overall, the resolution of spectra of bicelles (Fig. 1a,b) is better than that of MLVs (Fig. 1c–f). In particular, RINEPT provided the best resolution in bicelles (Fig. 1b) and well-hydrated MLVs (Fig. 1d) as compared to the RampCP sequence. The sensitivity was considerably reduced when MLVs were less hydrated (Fig. 1e,f); in fact, almost no peaks were observed in the RINEPT spectrum (Fig. 1f). While the peaks from side chains of Arg (84 ppm and 72 ppm) are observed from RampCP experiments on both MLVs and bicelles, amino peaks from Lys (32 ppm) is observed only from the RampCP experiment on bicelles. These peaks do not appear from RINEPT experiments.

2D experiments that correlate the isotropic chemical shifts of ¹H and ¹⁵N nuclei of bicelles containing a uniformly ¹⁵N-labeled cyt-b5 were performed to test if the resolution of 1D ¹⁵N spectra (Fig. 1) can be amplified by spreading the resonances. High-resolution of spectral lines in both frequency dimensions is evident from a representative 2D spectrum given in Fig. 2. However, some resonances still overlap in the spectrum. These results suggest that the resonance assignment can be accomplished if this data is suitably combined with more MAS experimental data from cyt-b5 double-labeled with ¹³C&¹⁵N isotopes.

Isotropic ¹³C chemical shift spectra of uniformly-¹³C and ¹⁵N double-labeled cyt-b₅ embedded in bicelles were also optimized for better sensitivity and resolution under MAS. Spectra obtained using RINEPT, RampCP and NOE approaches are given in Fig. 3. While NOE and RINEPT sequences provide high-resolution spectral lines in the aliphatic (20–80 ppm) and aromatic (30–150 ppm) regions, NOE is the only scheme that provides high sensitivity for carbonyl spectral lines (180–200 ppm). While RampCP spectrum shows a weak signal in the carbonyl region of the spectrum, the overall sensitivity and resolution are poor as compared to NOE and INEPT schemes. Acyl chains of DMPC and DHPC produce strong signals in RINEPT while they are considerably reduced in (b) and (c).

2D chemical shift correlations of ¹³C nuclei through-bond ¹³C-¹³C couplings using the CTUC⁶ sequence and via the ¹³C-¹³C dipolar couplings using the DARR (or RAD mixing) sequence⁷ are given in Figs. 4(a) and 4(b) respectively. Both the sequences provide spectra with remarkable resolution and sensitivity, which demonstrate the recently developed MAS techniques⁸ can be applied to solve the structure of cyt b5 embedded in bicelles. A significant number of resonances in the 2D spectra (Fig. 4) were assigned to specific amino acid sequences in cyt b5. More experiments are needed to accomplish the complete resonance assignment are in progress.

Since the soluble catalytic heme domain and the linker region that connects the heme domain and the transmembrane (TM) region of cyt b5 are expected to be relatively more mobile than the TM region in NMR time scale, it was essential to optimize the 2D correlation experiments with various mixing sequences on bicelles. Other 2D correlation experiments based on homonuclear ¹³C-¹³C dipolar recovery sequences and using a 2D UC2QF (uniform-sign cross-peak double-quantum-filtered correlation spectroscopy) COSY sequence⁶ that employs a double quantum filter were also carried out on the same sample.

While these 2D methods also provided cross peak patterns among ^{13}C nuclei, overall, 2D CTUC COSY and DARR sequences provided better resolution and cross peak patterns (Figure 4) that will be more suitable for structural studies on bicelles under MAS. The difference in the performance of these 2D correlation sequences and resultant spectra may be attributed to the dynamics of cyt b5 that significantly reduces the dipolar couplings. These data suggest that a significant portion of the protein, most likely the soluble domain of cyt b5, is highly mobile in NMR time scale, which is in excellent agreement with our previous study on static aligned bicelles.³ It is likely that the resonances in RINEPT and CTUC spectra could originate from the mobile regions of the protein like the soluble domain, while spectra obtained from dipolar coupling based experiments like rampCP and DARR could be mainly due to residues in relatively immobile regions of the protein. This is further confirmed by experiments at various temperatures. Our experimental results suggest that cooling bicelles below the room temperature enhanced the sensitivity of the RampCP experiment and reduced the RINEPT sensitivity, while the reverse was observed at 35°C.

Magnetically aligned bicelles are commonly used in solid-state NMR studies of membrane-associated peptides, proteins and drugs.^{2, 9, 10} They are also commonly used as alignment media to measure residual anisotropic interactions such as dipolar couplings using solution NMR experiments.¹¹ Variable angle sample spinning (VASS) studies showed that it is possible to measure ^1H - ^{15}N dipolar couplings in field-aligned bicelles^{12, 13} and also the ability to determine the molecular orientation and conformation of phosphatidylinositides.¹⁴ ^1H spectral line widths of lipid components of bicelles under MAS¹⁵ and mosaic spread of bicelles under sample rotation have been reported.¹⁶

Our experimental results presented here suggest that bicelles are well suited to study membrane-associated cyt b5 than the commonly used MLVs using MAS experiments at a physiologically relevant temperature without having to freeze the sample even with a concentration of the protein as low as 268 nmol. It should also be noted that solution NMR experiments could also be useful to study the full-length protein embedded in suitable detergent micelles or near-isotropic bicelles. While the mobile soluble domain of cyt b5 could result in high-resolution spectral lines, we expect that it would be difficult to study the structure of the rigid transmembrane domain in micelles. Nevertheless, such solution NMR studies will be helpful to augment solid-state NMR studies on bicelles.

Since our static experiments on magnetically aligned bicelles suggested that the overall order parameter for DMPC:DHPC ratio is 3.5:1 (a q ratio of 3.5) is 0.88,³ the difference in the size of bicelles and MLVs is not the main reason for the high-resolution in the MAS spectra. Instead, the observed high-resolution spectral lines could be due to the presence of bulk water in bicelles that may retain the dynamic folding of the catalytic soluble heme-containing domain of cyt b5. In addition, the experimental results suggest that the variation in the dynamics of different regions of a membrane protein could be utilized for optimizing resolution of spectra that are needed to solve the structure of the protein and protein-protein or protein-ligand complexes in membranes; importantly the dynamics of the protein can also be measured. Therefore, we expect that the use of bicelles will have wide applications in the structural studies of membrane proteins, particularly to those proteins that contain a large soluble domain, using the recently developed MAS NMR methods.^{8, 17} Interestingly, as demonstrated in this study, the use of a single bicelles sample for static aligned and MAS solid-state NMR experiments would be of great importance; and we believe that the use of VASS experiments to measure residual dipolar couplings from the protein would be highly valuable.

Experimental Section

All NMR experiments were performed on a Chemagnetics/Varian 400 MHz using a triple resonance MAS probe at 37°C. Final 2D spectra presented in Figures 2 and 4 were obtained from a Bruker 900 MHz at Lansing and a Bruker 600 MHz at NHMFL respectively. Details on the preparation of samples for NMR experiments are given in the Supporting Information.

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Figure 1.

^{15}N isotropic chemical shift spectra of 3.5:1 DMPC:DHPC (1,2-Dimyristoyl-*sn*-Glycero-3-Phosphocholine: 1,2-Diheptanoyl-*sn*-Glycero-3-Phosphocholine) bicelles (a and b), well-hydrated DMPC MLVs (multilamellar vesicles) (c and d) and DMPC MLVs with less hydration (e and f) containing a 268 nmol of ^{15}N -labeled rabbit cyt b₅ (protein:DMPC ratio is 1:220) under a 5kHz MAS at room temperature. The intensity and resolution of spectra obtained using two different preparation schemes, namely RINEPT (refocused insensitive nuclei enhanced by polarization transfer) (b, d and f) and RampCP (ramped-cross-polarization) (a, c and e), are different. In the RINEPT sequence, 2.66 and 1.5 ms were used in the first (before the pair of 90° pulses) and second (after the pair of 90° pulses) delays, respectively. A 2 ms contact time was used in RampCP experiments. Observation of isotropic ^{31}P chemical shifts (data not shown) from bicelles under MAS suggests that the

magnetic alignment of the bicelle sample was lost and therefore the spectral resolution in ^{15}N MAS spectra was rendered by the sample spinning.

Figure 2.

2D $^1\text{H}/^{15}\text{N}$ chemical shift correlation spectrum of DMPC:DHPC bicelles containing ^{15}N -labeled rabbit cyt b_5 under 5kHz MAS. The pulse sequence consisted of a 90° pulse to prepare ^1H transverse magnetization, a 180° pulse during t_1 to refocus ^1H - ^{15}N dipolar couplings, a RINEPT sequence to transfer ^1H magnetization to ^{15}N , and acquisition of ^{15}N magnetization under ^1H decoupling. 128 t_1 increments with 64 scans and a recycle delay of 2s were used.

Figure 3. ^{13}C chemical shift spectra of DMPC:DHPC bicelles containing ^{13}C & ^{15}N -labeled rabbit cyt b_5 under 5kHz MAS obtained using (a) RINEPT, (b) RampCP and (c) NOE (nuclear Overhauser effect) sequences. The peaks from DMPC and DHPC molecules are marked as *. Use of deuterated DMPC and DHPC would significantly suppress these signals and further improve the resolution of RINEPT and NOE spectra.

Figure 4.

2D ^{13}C isotropic chemical shift correlation spectra of DMPC:DHPC bicelles containing ^{13}C & ^{15}N -labeled rabbit cyt b_5 under 5kHz MAS obtained using the (CTUC) (constant-time uniform-sign cross-peak) COSY (correlation spectroscopy) (a) and DARR (dipolar-assisted rotational resonance) or RAD (radiofrequency-assisted diffusion)-mixing (b) sequences. Cross peaks correspond to ^{13}C nuclei that are scalar coupled and dipolar coupled appear in the CTUC (a) and DARR (b) spectra, respectively. Cross peaks correspond to specific amino acids are labeled in the spectrum (a).