

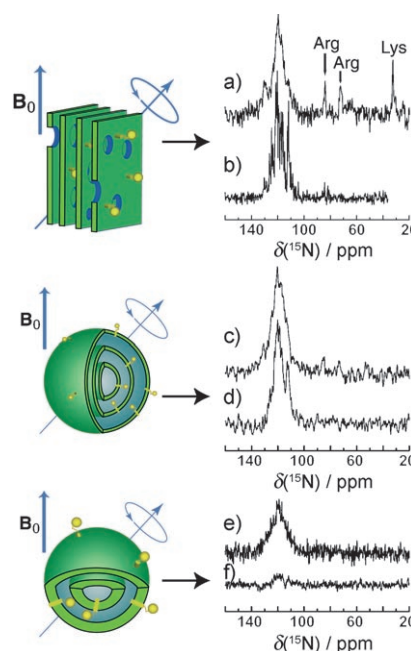
# Bicelle-Enabled Structural Studies on a Membrane-Associated Cytochrome $b_5$ by Solid-State MAS NMR Spectroscopy\*\*

Jiadi Xu, Ulrich H. N. Dürr, Sang-Choul Im, Zhehong Gan, Lucy Waskell, and Ayyalusamy Ramamoorthy\*

Various important functional roles played by membrane proteins that are related to a number of diseases, will be better understood once their high-resolution structures and dynamics are revealed. Although structural studies on membrane proteins have been a great challenge to most biophysical techniques, recent NMR spectroscopic studies were able to overcome many of the difficulties of structural elucidations for a number of proteins.<sup>[1]</sup> However, structural studies of membrane proteins still remain a great challenge, mainly because of the difficulty in finding a well-behaved model membrane. The use of multi-lamellar vesicles containing a transmembrane protein could enable the application of solid-state NMR spectroscopic techniques, but they are not usually suitable, as membrane proteins containing large soluble domains may not fold well to result in high-resolution spectra. Obtaining high-resolution spectra is a mandatory first step in solving the protein structure using NMR spectroscopy. In this study we demonstrate that bicelles<sup>[2]</sup> are suitable to overcome these difficulties and enable the use of MAS (magic-angle-spinning) solid-state NMR spectroscopic experiments for the structural studies on a large soluble domain containing membrane-bound protein such as cytochrome  $b_5$  (cyt $b_5$ ). Cyt $b_5$  modifies the catalytic activity and takes part in the catalytic reactions of cytochrome P450, however, its high-resolution structure remains unknown.<sup>[3]</sup> In addition, cyt $b_5$  is involved as an electron transfer component in a number of oxidative reactions in biological tissues which include the biosynthesis of fatty acids and steroids.<sup>[3]</sup>

Isotropic  $^{15}\text{N}$  chemical shift spectra of a uniformly  $^{15}\text{N}$ -labeled full-length 16.7 kDa rabbit cyt $b_5$  embedded in bicelles consisting of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine

(DHPC) and multilamellar vesicles (MLVs) using DMPC are given in Figure 1. RAMP-CP (ramped cross polarization)<sup>[4]</sup> and RINEPT (refocused intensive nuclei enhanced by polarization transfer)<sup>[5]</sup> sequences were used to transfer



**Figure 1.**  $^{15}\text{N}$  isotropic chemical shift spectra of 3.5:1 DMPC/DHPC bicelles (a and b), well-hydrated DMPC MLVs (multilamellar vesicles) (c and d) and DMPC MLVs of less hydration (e and f) containing a 268 nmol of  $^{15}\text{N}$ -labeled rabbit cyt $b_5$  (protein/DMPC ratio 1:220) with 5 kHz MAS at room temperature. RINEPT (b, d, and f) and RAMP-CP (a, c, and e) yielded different intensities and spectral resolution. Observation of isotropic  $^{31}\text{P}$  chemical shifts (data not shown) from bicelles under MAS suggests that the magnetic alignment of the bicelle was lost and therefore the spectral resolution in  $^{15}\text{N}$  MAS spectra was rendered by the sample spinning.

[\*] Dr. J. Xu, Dr. U. H. N. Dürr, Prof. A. Ramamoorthy  
Biophysics and Department of Chemistry, University of Michigan  
930 North University Avenue, Ann Arbor, MI 48109-1055 (USA)  
Fax: (+1) 734-615-3790  
E-mail: ramamoor@umich.edu  
Homepage: <http://www.umich.edu/~ramslab/>  
Dr. S.-C. Im, Dr. L. Waskell  
Department of Anesthesiology, University of Michigan, and  
VA Medical Center (USA)  
Dr. Z. Gan  
National High Magnetic Field Lab, Tallahassee (USA)

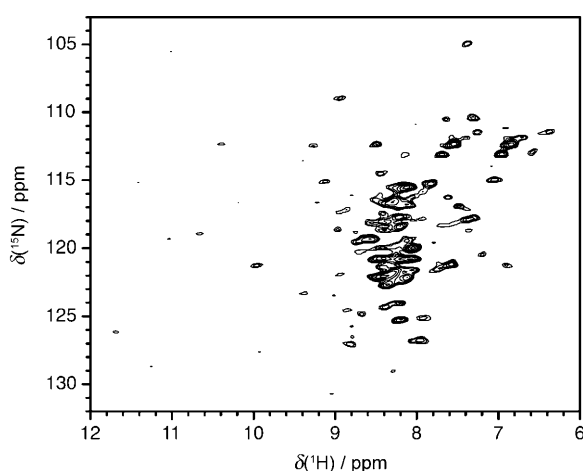
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magnetization from  $^1\text{H}$  to  $^{15}\text{N}$  under 5 kHz MAS. RAMP-CP experiments were carried out on multilamellar vesicles (MLVs) and bicelles with various contact times to optimize the sensitivity of the measurements (see the Supporting Information). Amide  $^{15}\text{N}$  spectral lines appear in the 105–130 ppm range for all spectra in Figure 1. Overall, the resolution of spectra of bicelles (Figure 1a,b) is better than that of MLVs (Figure 1c–f). In particular, RINEPT provided the best resolution in bicelles (Figure 1b) and well-hydrated MLVs (Figure 1d) as compared to the RAMP-CP sequence. The sensitivity was considerably reduced when MLVs were

less hydrated (Figure 1 e,f); in fact, almost no peaks were observed in the RINEPT spectrum (Figure 1 f). Although the peaks from side chains of Arg ( $\delta = 84$  ppm and 72 ppm) are observed from RAMP-CP experiments on both MLVs and bicelles, such peaks from Lys ( $\delta = 32$  ppm) are observed only in the spectra from the RAMP-CP experiment on bicelles. These peaks do not appear in the RINEPT experiments.

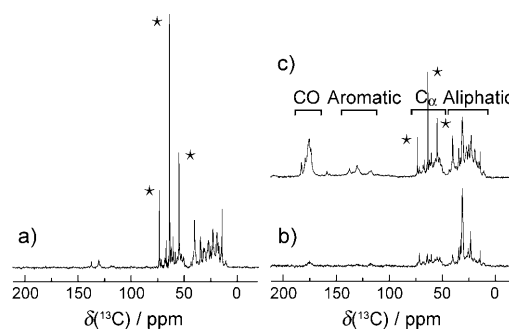
2D experiments that correlate the isotropic chemical shifts of  $^1\text{H}$  and  $^{15}\text{N}$  nuclei of bicelles containing a uniformly  $^{15}\text{N}$ -labeled cytb<sub>5</sub> were performed to ascertain whether the resolution of 1D  $^{15}\text{N}$  spectra (Figure 1) can be amplified by spreading the resonances. The high resolution spectral lines in both frequency dimensions is evident from a representative 2D spectrum given in Figure 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 results from cytb<sub>5</sub> double-labeled with  $^{13}\text{C}$  and  $^{15}\text{N}$  isotopes.



**Figure 2.** 2D  $^1\text{H}/^{15}\text{N}$  correlation spectrum of DMPC/DHPC bicelles containing  $^{15}\text{N}$ -labeled rabbit cytb<sub>5</sub> under 5 kHz MAS.

Isotropic  $^{13}\text{C}$  chemical shift spectra of uniformly  $^{13}\text{C}$  and  $^{15}\text{N}$  double-labeled cytb<sub>5</sub> embedded in bicelles were also optimized for better sensitivity and resolution under MAS. Spectra obtained using RINEPT, RAMP-CP, and NOE approaches are given in Figure 3. Although NOE and RINEPT sequences provide high-resolution spectral lines in the aliphatic ( $\delta = 20$ –80 ppm) and aromatic ( $\delta = 30$ –150 ppm) regions, NOE is the only technique that provides high sensitivity for carbonyl spectral lines ( $\delta = 180$ –200 ppm). Whereas RAMP-CP spectrum shows a weak signal in the carbonyl region of the spectrum, the overall sensitivity and resolution are poor compared to NOE and RINEPT techniques. Acyl chains of DMPC and DHPC produce strong signals in RINEPT, however, they are considerably decreased in RAMP-CP (Figure 3 b) and NOE spectra (Figure 3 c).

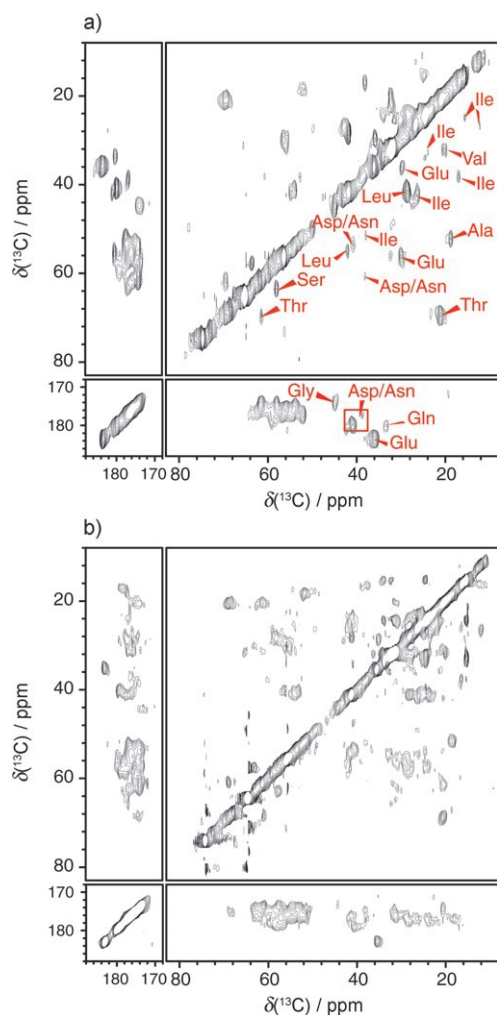
2D chemical shift correlations of  $^{13}\text{C}$  nuclei through-bond  $^{13}\text{C}$ – $^{13}\text{C}$  couplings using the constant-time uniform-sign cross-peak (CTUC)<sup>[6]</sup> sequence and with the  $^{13}\text{C}$ – $^{13}\text{C}$  dipolar couplings using the dipolar-assisted rotational resonance (DARR) or radio-frequency field-assisted diffusion (RAD)



**Figure 3.**  $^{13}\text{C}$  chemical shift spectra of DMPC/DHPC bicelles containing  $^{13}\text{C}$  and  $^{15}\text{N}$ -labeled rabbit cytb<sub>5</sub> under 5 kHz MAS obtained using (a) RINEPT, (b) RAMP-CP, and (c) NOE 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.

mixing sequence<sup>[7]</sup> are given in Figure 4 a and b, respectively. Both sequences provide spectra with remarkable resolution and sensitivity, which demonstrate that the recently developed MAS techniques<sup>[8]</sup> can be applied to solve the structure of cytb<sub>5</sub> embedded in bicelles. A significant number of resonances in the 2D spectra (Figure 4) were assigned to specific amino acid sequences in cytb<sub>5</sub>. Further experiments are in progress to accomplish the complete resonance assignment.

As the soluble catalytic heme domain and the linker region that connects the heme domain and the transmembrane (TM) region of cytb<sub>5</sub> are expected to be more mobile than the TM region on the NMR timescale, it was essential to optimize the 2D correlation experiments with various mixing sequences on bicelles. Other 2D correlation experiments based on homonuclear  $^{13}\text{C}$ – $^{13}\text{C}$  dipolar recovery sequences and a 2D UC2QF COSY (uniform-sign cross-peak double-quantum-filtered correlation spectroscopy) sequence<sup>[6]</sup> that employs a double quantum filter were also carried out on the same sample. Although these 2D methods also provided cross-peak patterns among  $^{13}\text{C}$  nuclei, overall, 2D CTUC COSY and DARR sequences provided better resolution and cross-peak patterns (Figure 4) that are 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 cytb<sub>5</sub> that significantly reduces the dipolar couplings. These results suggest that a significant portion of the protein, most likely the soluble domain of cytb<sub>5</sub>, is highly mobile in NMR time scale, which is in excellent agreement with our previous study on statically aligned bicelles.<sup>[3]</sup> It is likely that the resonances in RINEPT and CTUC spectra could originate from the mobile regions of the protein, such as the soluble domain, whereas spectra obtained from dipolar coupling experiments, such as RAMP-CP and DARR, could mainly be due to residues in relatively immobile regions of the protein. This is further confirmed by experiments at varying temperatures. Our experimental results suggest that cooling bicelles below room temperature enhance the sensitivity of the RAMP-CP experiment and reduce the RINEPT sensitivity, whereas the reverse is observed at 35 °C.



**Figure 4.** 2D  $^{13}\text{C}$  isotropic correlation spectra of DMPC/DHPC bicelles containing  $^{13}\text{C}$  and  $^{15}\text{N}$ -labeled rabbit cytb<sub>5</sub> under 5 kHz MAS obtained using the a) CTUC COSY and b) DARR or RAD mixing sequences. Cross-peaks correspond to  $^{13}\text{C}$  nuclei that are scalar coupled and dipolar coupled appear in the a) CTUC COSY and (b) DARR spectra, respectively. Cross-peaks corresponding to specific amino acids are labeled in spectrum (a).

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

The experimental results presented herein suggest that bicelles are well suited to study membrane-associated cytb<sub>5</sub> than the commonly used MLVs using MAS experiments at a physiologically relevant temperature without having to freeze the sample and at a concentration of the protein as low as 268 nmol L<sup>-1</sup>. It should also be noted that solution NMR

spectroscopic experiments could also be useful to study the full-length protein embedded in suitable detergent micelles or near-isotropic bicelles. Whereas the mobile soluble domain of cytb<sub>5</sub> 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 spectroscopic studies will be helpful to augment solid-state NMR spectroscopy studies on bicelles.

As our static experiments on magnetically aligned bicelles suggested that the overall order parameter for a DMPC/DHPC ratio of 3.5:1 is 0.88,<sup>[3]</sup> 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 cytb<sub>5</sub>. In addition, the experimental results suggest that the variation in the dynamics of different regions of a membrane protein could be utilized for optimizing the resolution of the 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 wider applications in the structural studies of membrane proteins, particularly to those proteins that contain a large soluble domain, using the recently developed MAS NMR spectroscopic methods.<sup>[8,17]</sup> Interestingly, as demonstrated in this study, the use of a single bicelle for statically aligned and solid-state MAS NMR spectroscopic 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 spectroscopic experiments were performed on a Chemagnetics/Varian 400 MHz using a triple-resonance MAS probe at 37 °C. RINEPT sequence: 2.66 ms (delay before the first 90° pulse) and 1.5 ms (second delay before the second 90° pulse). RAMP-CP sequence: a 2 ms contact time was used in the experiments. Final 2D spectra presented in Figure 2 and 4 were obtained from a Bruker 900 MHz at Lansing and a Bruker 600 MHz at NIMFL, respectively. Pulse sequence for the 2D  $^1\text{H}/^{15}\text{N}$  correlation spectrum: 90° pulse to prepare  $^1\text{H}$  transverse magnetization, 180° pulse during  $t_1$  to refocus  $^1\text{H}$ - $^{15}\text{N}$  dipolar couplings, a RINEPT sequence to transfer  $^1\text{H}$  magnetization to  $^{15}\text{N}$ , and acquisition of  $^{15}\text{N}$  magnetization under  $^1\text{H}$  decoupling. 128  $t_1$  increments with 64 scans and a recycle delay of 2 s were used. Details on the preparation of samples for NMR spectroscopic experiments are given in the Supporting Information.

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