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Probing the Transmembrane Structure and Topology of Microsomal Cytochrome-P450 by Solid-State NMR on Temperature-Resistant Bicelles

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Though the importance of high-resolution structure and dynamics of membrane proteins has been well recognized, optimizing sample conditions to retain the native-like folding and function of membrane proteins for Nuclear Magnetic Resonance (NMR) or X-ray measurements has been a major challenge. While bicelles have been shown to stabilize the function of membrane proteins and are increasingly utilized as model membranes, the loss of their magnetic-alignment at low temperatures makes them unsuitable to study heat-sensitive membrane proteins like cytochrome-P450 and protein-protein complexes. In this study, we report temperature resistant bicelles that can magnetically-align for a broad range of temperatures and demonstrate their advantages in the structural studies of full-length microsomal cytochrome-P450 and cytochrome-b5 by solid-state NMR spectroscopy. Our results reveal that the N-terminal region of rabbit cytochromeP450B4, that is usually cleaved off to obtain crystal structures, is helical and has a transmembrane orientation with $\sim 17^\circ$ tilt from the lipid bilayer normal.

Biological cell membranes are vital boundaries that separate the intracellular elements from the extracellular environments, and the membrane proteins in such borders are fundamental regulators of a number of essential cellular and physiological phenomena in life, including signal transductions, electron transport chains, and photosynthesis. Furthermore, membrane-associated proteins comprise more than 30% of the human genome and 50% of known drug targets. In order to understand the roles of these proteins in biological activities, and to develop medical treatments of related diseases, it is critical to establish biophysical methods to investigate the functional form of membrane protein structures at atomic-level^{1–10}. However, membrane protein structure determination is an extremely challenging task due to the lack of stability of the protein outside the native membrane environment. For this reason, development of novel methods to stabilize the native structure of membrane proteins is essential for driving high-resolution structural studies using biophysical techniques such as nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography^{11–22}. Bicelles are increasingly used as model membranes for the studies of biomolecules with various biophysical methods, including solid-state NMR, solution NMR, X-ray crystallography, EPR (Electron Paramagnetic Resonance), CD (Circular Dichroism), fluorescence, IR (Infra Red), Raman, UV-Vis spectroscopy, ITC (Isothermal Titration Calorimetry), DSC (Differential Scanning Calorimetry), and microscopy, as their planar domain provides an excellent environment for the study of membrane-associated proteins in transparent fluid solutions, which prevent light scattering^{11,12,14,15,23–27}. Bicelles are typically made from a mixture of long-chain phospholipid and short-chain phospholipid/detergent (e.g. DHPC (1,2-dihexanoyl-sn-glycero-3-phosphocholine), CHAPSO (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), DPC (dodecylphosphocholine), or Triton X-100). The size of bicelles can be controlled by the lipid to detergent molar ratio, called q ratio ($=[\text{lipid}]/[\text{detergent}]$), and also by the hydration level. Large bicelles (q ratio > 2.5), that spontaneously align in a magnetic field above the phase transition temperature, are highly valuable to measure distance and orientational constraints from embedded membrane proteins using static solid-state NMR experiments. Similarly, anisotropic NMR interactions, such as residual dipolar couplings (RDCs) and residual chemical shift anisotropy of soluble proteins, can be obtained using solution NMR experiments on bicelles with low concentrations. Small and fast-tumbling bicelles



(q ratio < 1.5) are an excellent reconstitution medium for solution NMR studies of membrane proteins. Recent studies have shown that bicelles retaining the function of proteins are also useful to study membrane-bound protein-protein complexes. While many useful bicelle compositions have been reported in the literature, bicelles composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) are most frequently used. These DMPC/DHPC bicelles have a narrow temperature range, between 25 and 45 °C, for magnetic alignment and have been well utilized in static solid-state NMR experiments on membrane proteins under this temperature range. This high temperature requirement of magnetically-aligned bicelles is problematic for heat-sensitive biomolecules, such as cytochromes P450; in addition, solid-state NMR experiments employ high power RF pulses that can induce sample heating. Therefore, there is considerable interest in developing fluid model membranes that can align at a low temperature. Several methods to improve the stability of bicelles, and to extend the ranges of alignment temperatures, using unsaturated/modified lipids or chemical additives have been reported^{28–33}. Amongst these NMR studies, the lowest temperature at which aligned bicelles used to study proteins is 25 °C³⁴. In this study, we report the experimental conditions for the preparation of *temperature resistant bicelles* and demonstrate their use in the structural studies of a full-length mammalian microsomal cytochrome P450 2B4.

Cytochromes P450, which metabolize approximately 75% of the pharmaceuticals in clinical use today, are monooxygenases that activate the stable carbon hydrogen bond of alkanes, commonly referred to as Mother Nature's blowtorch^{35–42}. The cytochromes P450 family is found in all kingdoms of life and involved in a wide variety of enzymatic reactions in living organisms, such as drug metabolism, and the synthesis of steroids and lipids. Microsomal cytochrome P450 2B4 has the molecular weight of 55.7 kDa and consists of a catalytic heme-containing soluble domain and a hydrophobic transmembrane domain. The transmembrane region of microsomal cytochromes P450 is essential for their functions because the lack of this membrane anchor results in a decrease to only 40% of all enzymatic activities. What the role of the transmembrane structure for enzymatic mechanisms is and how the membrane-associated region of the enzyme interacts with lipid bilayers are key questions to be addressed in order to explain how cytochrome P450s take hydrophobic compounds into their reaction centers. Despite its importance, the atomic-level transmembrane structure of microsomal cytochrome P450 has not been revealed experimentally, since its first identification in 1962⁴³. Major difficulties in the structure determination of full-length microsomal cytochrome P450 include: (i) optimization of conditions to satisfy its thermal stability on both a larger soluble domain and a relatively smaller hydrophobic transmembrane domain simultaneously for crystallization, (ii) challenges related to the colossal molecular weight for solution NMR spectroscopy, and (iii) its thermal instability during biophysical experiments including solid-state NMR spectroscopy. In this study, we report the first study on the transmembrane structure and topology of the functional form of a heat sensitive cytochrome P450, using a newly developed temperature resistant bicelle compositions in a low temperature environment by means of solid-state NMR spectroscopy.

Results

Magnetic-alignment of DLPC/DHPC bicelles for a wide range of temperatures. The stability of the perforated lamellar phases of bicelles for a wide range of temperatures can be accomplished by optimizing various conditions. Most important factors are: (1) use of lipids having a low phase transition temperature such as DLPC (1,2-dilauroyl-*sn*-glycero-3-phosphocholine, 12:0 PC, main phase transition temperature is -1 °C), DDPC (1,2-didecanoyl-*sn*-glycero-3-phosphocholine, 10:0 PC, main phase transition temperature is

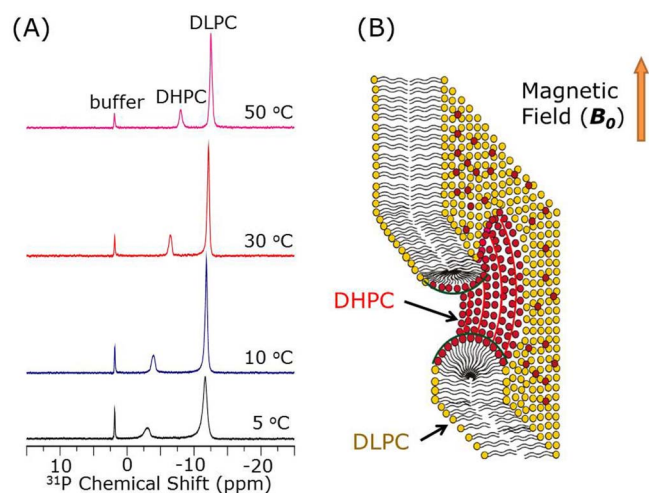


Figure 1 | (A) Proton-decoupled³¹P NMR spectra indicate that DLPC/DHPC ($q = [\text{DLPC}]/[\text{DHPC}] = 4.0$) bicelles, are magnetically-aligned over a wide range of temperatures. Spectra acquired at other temperatures and for DLPC/DHPC bicelles with various other q ratios are given in the Supporting Information (Figure S1). The peaks around -12, -4 and 0 ppm arise from DLPC, DHPC and phosphate buffer, respectively. (B) Schematic representation of perforated lamellar phase DLPC/DHPC bicelles consisting of both planar and curvature toroidal-pore regions. Long (DLPC, yellow) and short (DHPC, red) chain phospholipids are mostly found in the planar and toroidal-pore regions, respectively.

-20 °C), or unsaturated lipids, (2) addition of a small amount of charged stabilizer chemicals, such as cholesterol, hexadecyl trimethyl ammonium bromide (CTAB), or cholesterol 3-sulfate, (3) careful selection of a detergent, (4) the appropriate ratio of lipid/detergent mixture, and (5) the level of hydration. Based on the optimization of these conditions, we found out that a combination of DLPC and DHPC provides thermally stable bicelles as shown in Figure 1. This innovation of temperature resistant bicelles can be advantageous for NMR measurements at both high and low temperatures. Particularly, it would be beneficial for the investigation of residual dipolar coupling measurements from soluble proteins by solution NMR spectroscopy, and structure/topology/dynamics studies of heat-sensitive membrane-associated proteins by solid-state NMR spectroscopy. Phosphorus-31 NMR spectra of DLPC/DHPC bicelles are shown in Figure 1. The observed anisotropic chemical shift peaks for DLPC and DMPC indicate the magnetic-alignment of bicelles even at a significantly low temperature. Remarkably, these bicelles are stable enough to hold their perforated lamellar phase and also magnetic-alignment at low temperatures, lower than that of any previously reported bicelles.

Magnetic-alignment of temperature resistant bicelles containing cytochrome-b5. Though our results in Figure 1 demonstrate that DLPC/DHPC bicelles can be aligned for a wide range of temperatures, it is important to examine the quality of alignment of these bicelles when a membrane protein is incorporated so that solid-state NMR experiments can be used to measure the structural constraints from the embedded protein. We have previously used DMPC/DHPC bicelles to investigate the structure, dynamics and topology of cytochrome b5. When compared to cytochrome P450, cytochrome b5 is relatively easy to handle and less expensive to produce from *E.coli* in a large quantity. Therefore, we used cytochrome b5 to examine the suitability of the temperature resistant DLPC/DHPC bicelles for solid-state NMR measurements. First, we obtained³¹P chemical shift spectra to determine the magnetic-alignment of bicelles containing cyt-b5. As shown in Figure 2(B), the observation of narrow³¹P peaks for DLPC and DHPC (in addition to the



phosphate buffer peak) indicate that the samples were aligned for a range of temperatures. Then, we performed simple one-dimensional ramped-cross-polarization (CP) experiments to obtain ^{15}N chemical shift spectra of U- ^{15}N -cyt-b5 incorporated into DLPC/DHPC bicelles. Spectra shown in Figure 2(C) confirm the alignment of cyt-b5 even at 5°C. As reported in our previous study, resolution of aligned ^{15}N spectra can be further improved by utilizing spectral editing techniques based on the dynamic differences between the rigid transmembrane and highly mobile heme-containing soluble domains of cyt-b5^{20–22}. Since the transmembrane domain of cyt-b5 can be easily cleaved off (by sample mishandling, heat or impurities), the intact form of the full-length protein was confirmed by running the sample on a gel page after NMR measurements. Overall, our results demonstrate that DLPC/DHPC bicelles are quite stable for solid-state NMR measurements and magnetically-align for a wide range of temperatures. Therefore, these DLPC/DHPC bicelles can be used to study heat sensitive proteins like cytochrome P450.

Solid-state NMR reveals the structural topology of the transmembrane domain of rabbit cytochrome P450 2B4. Though many crystal structures of the soluble domain of cyt-P450 have been reported in the literature, high-resolution structure of the full-length protein is unknown. The N-terminal 60-residues segment containing the hydrophobic domain is usually cleaved off to obtain a single crystal for structural studies by X-ray crystallography^{44–47}. In addition, the full-length protein is quite unstable and highly sensitive to heat. Therefore, the thermally stable magnetic-alignment of DLPC/DHPC bicelles is a breakthrough to investigate the structure of a heat-sensitive full-length membrane-bound protein like cytochrome P450 2B4 in fluid lamellar phase lipid bilayers. To optimize the experimental conditions, we first confirmed the functional folding of the catalytic site of cytochrome P450 2B4 by performing carbon monoxide assays and also tested the stability of the protein in these bicelles for various temperatures. As shown in Figure 3(A), these assays indicated that the temperature resistant bicelles retained the stable native structural folding of cytochrome P450 in a biologically active state (P450 instead of P420) under NMR sample conditions. Using this functional form, the transmembrane

structure and topology of cytochrome P450 2B4 are then determined using solid-state NMR experiments on magnetically-aligned DLPC/DHPC bicelles containing a uniformly- ^{15}N -labeled-P450.

Experimental conditions were optimized to observe the ^{15}N spectrum of a uniformly- ^{15}N -labeled-P450 (Figure 3B). The contact time in the CP experiment was optimized to observe the signal mainly originating from the relatively rigid regions of cytochrome P450; otherwise, there are too many residues in the soluble domain of P450 and it is very difficult to observe the relatively short transmembrane region of the protein. As a result, peaks from amino acid residues in the mobile soluble domain of P450 are largely suppressed to reveal peaks from the transmembrane region of the protein. The 2D HIMSSELF (Heteronuclear Isotropic Mixing leading to Spin Exchange via the Local Field) or HERSELF (HEteronuclear Rotating-frame Spin Exchange via the Local Field) spectrum correlating the ^{15}N chemical shift and ^1H - ^{15}N dipolar coupling is shown in Figure 3 (C). Though the resonances are not spanned like an ideal circle, they do not have the same chemical shift and NH dipolar coupling frequencies, suggesting that the transmembrane helix is definitely tilted away from the bilayer normal. Simulation of the resonances associated with large NH dipolar coupling values was carried out to determine the tilt angle of the transmembrane helix. A comparison of the experimental and simulated results suggest that the transmembrane helix is $17(\pm 3)^\circ$ tilted away from the lipid bilayer normal. The distortion of the resonances from the circular pattern could indicate that the transmembrane region could be a distorted helix. Indeed, the presence of three Gly residues (Gly14, Gly22 and Gly28) in the hydrophobic N-terminal domain (see Figure 3(D, E)) suggests that the transmembrane region of P450 is most likely a distorted helix. Interestingly, the transmembrane helical domains of cytochrome P450 and cytochrome b₅ reconstituted in bicelles have very similar tilt angles. Such a topology of the TM helices could aid in the direct interaction within the hydrophobic lipid bilayer to stabilize the protein-protein interactions that are essential for an efficient transfer of electron from b₅ to P450. More experimental studies are needed to fully understand the protein-protein interactions that drive the formation of a productive cytochromes-b₅-P450 complex.

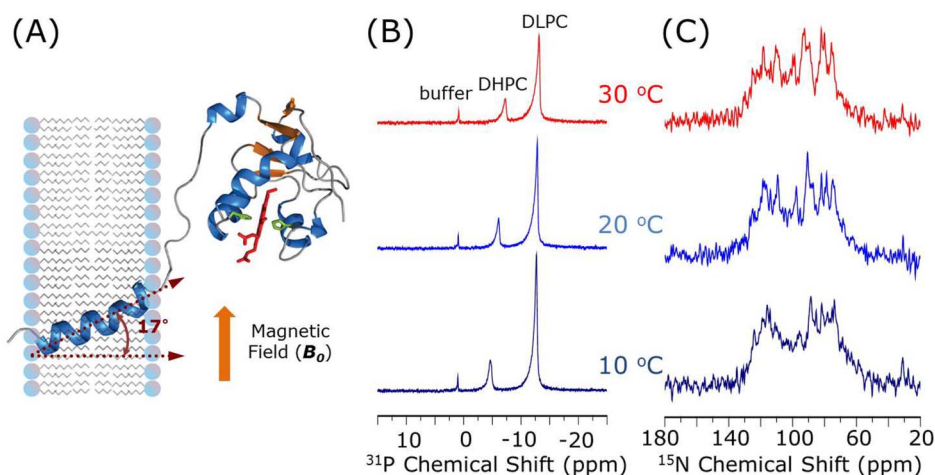


Figure 2 | Low temperature magnetic alignment properties of temperature resistant DLPC/DHPC bicelles ($q = [\text{DLPC}]/[\text{DHPC}] = 4.0$) containing a uniformly labeled ^{15}N cytochrome b₅. (A) A representation of lipid bilayers containing cytochrome b₅. Cytochrome b₅, a 15.7 kDa membrane-associated protein, has been shown to incorporate into lipid bilayers with its C-terminal transmembrane domain tilting by $\sim 14^\circ$ relative to the lipid membrane normal. (B) Proton-decoupled ^{31}P (B) and ^{15}N (C) NMR spectra of magnetically-aligned DLPC/DHPC bicelles containing uniformly ^{15}N -labeled cytochrome b₅ at the indicated temperatures. ^{31}P spectra indicate that bicelles are well aligned at the indicated temperatures but the alignment at 5°C (Figure S2) is not as good as that at 10°C. ^{15}N peaks appearing in the 60 to 90 ppm range are from backbone amide-NHs present in the relatively immobile transmembrane domain, whereas those appearing between 100 and 140 ppm are from the highly mobile soluble domain of cytochrome b₅. The contact time used for ramped cross-polarization experiments was 0.8 ms and protons were decoupled by a 25 kHz TPPM (Two-Pulse Phase-Modulated) pulse sequence during the signal acquisition.

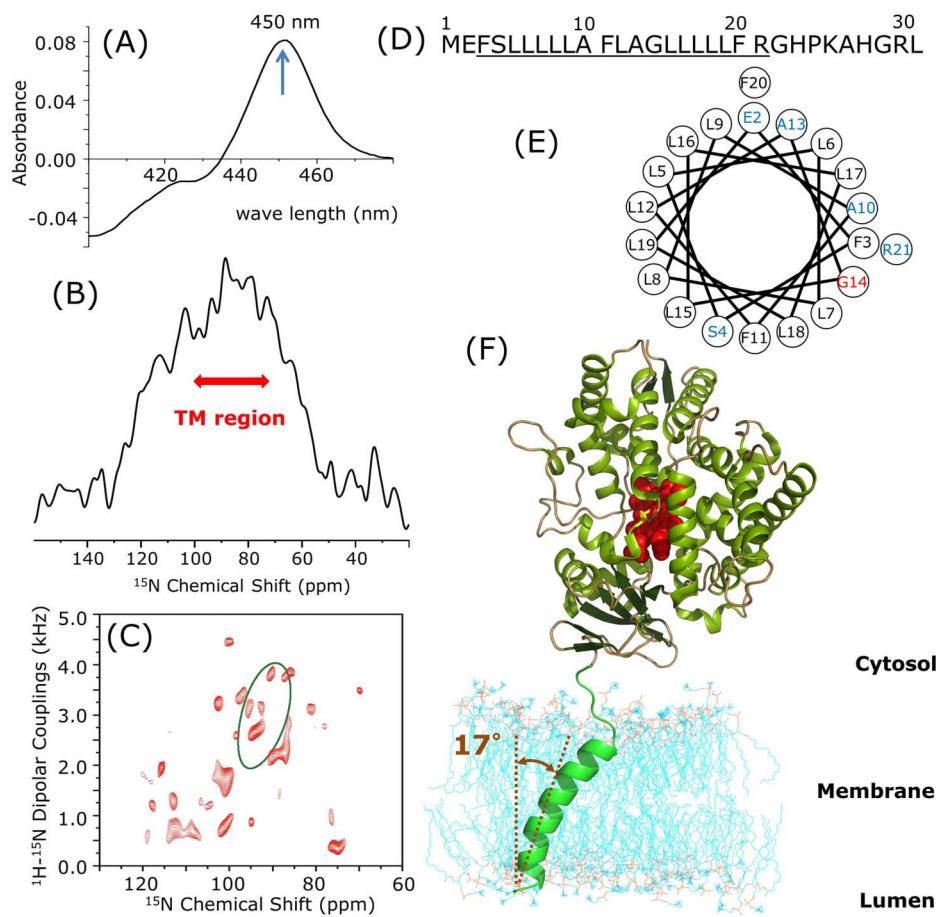


Figure 3 | The use of temperature resistant bicelles at lower temperatures enables the measurement of the transmembrane structure and topology of the functional form of microsomal cytochrome P450 2B4. (A) A UV-Vis spectrum shows that the overall folding of the catalytic site of a heat-sensitive membrane protein, cytochrome P450 2B4, can be stabilized in the functional form using temperature resistant bicelles, composed of DLPC and DHPC. (B) One-dimensional cross-polarization experiments of 100 μ l of magnetically-aligned DLPC/DHPC bicelles ($q = [\text{DLPC}]/[\text{DHPC}] = 4.0$) containing a 0.61 mM of a uniformly- ^{15}N -labeled cytochrome P450 2B4 shows anisotropic ^{15}N chemical shifts at 15°C. (C) Two-dimensional HIMSSELF experiment reveal that cytochrome P450 2B4 has a helical structure in the N-terminal transmembrane region. The amino acid sequence (D) and a helical wheel representation (E) of the N-terminal transmembrane region of cytochrome P450 2B4⁵⁴. The full length amino acid sequence is given in the Supporting Information. Hydrophobic and hydrophilic amino acids are in black and blue, respectively. (F) A model depicting the structure and topology of cytochrome P450 in lipid bilayers; the soluble domain structure is adapted from the crystal structure for amino acid residues 28–491^{44–46}. The transmembrane structure of residues 1–27, obtained using the structure assembly simulation, I-TASSER⁵⁵, is shown in the lipid bilayer region; the structure obtained with the highest C-score⁵⁵ was chosen. The transmembrane domain may not be a straight α -helix due to the presence of Gly residues. This result is consistent with the imperfect wheel-like pattern of resonances in the 2D HIMSSELF spectrum shown in Figure 3(C).

We also observed that cytochrome P450 2B4, when reconstituted into isotropic DLPC/DHPC bicelles ($q = 0.25$), can be stabilized for a period of approximately two weeks without any sample aggregation at 5°C. However, cytochrome P450 2B4 incorporated in DMPC/DHPC isotropic bicelles (q ratio = 0.25) at 35°C started precipitating within few hours, despite the use of glycerol to stabilize samples; all the conditions used were the same for both these bicelles containing P450. On the other hand, at 25°C (just above the phase transition temperature of DMPC), cytochrome P450 embedded DLPC/DHPC and DMPC/DHPC bicelles were found to be stable for 10 to 14 days. These observations suggest that P450s present in both these bicelles are stable above the bicelle phase transition temperature. However, preservation of the stability and function at lower temperature conditions are always favorable for structural studies on cytochromes P450. Therefore, temperature resistant bicelles would be a useful medium to stabilize heat-sensitive membrane proteins at lower temperatures.

Discussion

Although bicelles are increasingly utilized in the structural studies of proteins in general, and membrane proteins in particular, its

magnetic-alignment property is vital in the applications of solid-state NMR spectroscopy to study the structure and dynamics of membrane proteins and solution NMR spectroscopy to measure RDCs. To investigate heat-sensitive proteins, it is essential to develop bicelles that can align at a low temperature. In this study, for the first time, it is demonstrated that DLPC:DHPC bicelles can magnetically-align at temperatures ranging from 0 to 50°C. In addition, our results demonstrate the feasibility of applying solid-state NMR experiments on these bicelles to study the structure and dynamics of membrane proteins including the heat-sensitive cytochrome-P450. Further, we have optimized the experimental conditions to obtain a high-resolution 2D HIMSSELF spectrum from DLPC:DHPC bicelles containing a small amount of cyt-P450 to selectively detect signal from the rigid N-terminal transmembrane region of the protein. The observed dipolar coupling and anisotropic chemical shift values indicate the helical transmembrane segment is tilted by about 17° from the bilayer normal. While previous crystallographic studies focused on the soluble catalytic domain – devoid of the N-terminal transmembrane domain – of the protein^{44–47}, other studies have predicted that the hydrophobic residues in the N-terminal region could have a



transmembrane orientation and the hydrophobic F-G loop could interact with the membrane with a dependence on the membrane composition^{48,49,54}. Therefore, there is considerable current interest in the determination of the membrane orientation of cytochrome P450 in order to fully understand its mechanism of enzymatic action. We believe that the results reported in this study will be useful in further understanding the native folding of cytochrome P450 in the lipid membrane and its functional interactions with its redox partners.

The observed stability of cytochrome P450 and cytochrome b5 in isotropic DLPC:DHPC bicelles at a low temperature (as low as 5°C) suggest that the interaction between these two proteins can be measured in a membrane environment to determine the role of the transmembrane domains of these two proteins on the electron transfer process that enables the enzymatic activity of cytochrome P450. As demonstrated in our previous study, bicelles can also be utilized to obtain high-resolution spectra from embedded membrane proteins by magic angle spinning techniques⁵⁰. Therefore, we are certain that DLPC/DHPC bicelles and further development of bicelles with various other lipid compositions would broaden the horizon of NMR spectroscopy for biological applications. Aqueous solutions of DLPC:DHPC bicelles are transparent and would also be excellent membrane mimetics for studies by various other spectroscopies, such as UV-Vis, CD, ESR, 2D IR, Raman, SFG, and other vibrational spectroscopy.

Methods

Expression and purification of uniformly ¹⁵N labeled proteins. The wild type full length uniformly ¹⁵N labeled P450 2B4 (U-¹⁵N cyt P450 2B4) was expressed in *E. Coli* C41 cells. Plasmids pLW01-P450 2B4⁵¹ were transformed into the C41 cells, and incubated on a LB/carbenicillin plate overnight at 37°C. Three colonies from the plate were transferred into 200 mL of LB medium in a 500 mL Erlenmeyer flask containing carbenicillin at a final concentration of 0.24 mM. The culture was incubated for 16 hours under shaking at 200 rpm at 30°C. 10 mL of the culture was pelleted and resuspended gently with a 10 mL of ¹⁵N labeled Celtone culture (Celtone-N), which was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). The resuspended culture was then transferred into a 1 L of modified Celtone-N culture containing 900 mL Celtone-N, 100 mL water, ¹⁵N-NH₄SO₄ (2 g) glycerol (4 mL), FeCl₃·6H₂O (10 μM), ZnSO₄·7H₂O (25 μM), MnCl₂·4H₂O (20 μM), MgSO₄·7H₂O (1 mM), Na₂HPO₄ (0.68 g), KH₂PO₄ (0.3 g), NaCl (0.1 g), and a vitamin mixture (biotin, choline chloride, folic acid, niacinamide, D-pantothenate, pyridoxal, and riboflavin, each at 2.5 μg). Afterwards, the 100 mL of the culture was transferred into a 500 mL sterilized Erlenmeyer flask. The ten flasks were then incubated for 20 hours at 23°C under shaking at 120 rpm. When the absorbance at 600 nm was between 1.5–2.0, filter sterilized stocks of isopropylthio-β-galactoside (IPTG) and d-aminolevulinic acid (ALA) were added into the flask for a final concentration of 500 μM. After 30 minutes incubation, 3% (v/v) ethanol was added into the flasks. The culture was incubated for 76 hours at 22°C with shaking at 110 rpm. The incubation was continued for an additional 24–36 hours until inactive form of the protein (that is P420) appears. The U-¹⁵N labeled cyt P450 2B4 from the cells was purified as previously described⁵².

Preparation of bicelles. 1,2-dilauroyl-*sn*-glycero-3-phosphatidylcholine (DLPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphatidylcholine (DHPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). 18.7 mg DLPC and 3.4 mg DHPC corresponding to a molar ratio of $q = [\text{DLPC}]/[\text{DHPC}] = 4$ were cosolubilized in chloroform. Solvent was removed under a stream of N₂ gas to produce a lipid film on the walls of a glass vessel, which was kept in vacuum overnight to remove residual solvent. 16.3 μl of 50 mM phosphate buffer, pH 7.5, with 20% glycerol content was added to lipids. The resulting mixture of extreme viscosity was homogenized by vortexing, and 4 freeze/heat cycles between liquid nitrogen and 40°C. The resulting turbid gel is still extremely viscous, but its viscosity was slightly reduced at 0°C. Protein was added in the final step of the preparation. Adding 60 μl of 1.0 mM cytochrome P450 solution corresponding to 3.35 mg, or 60 nmol protein resulted in a protein:DLPC molar ratio of 1:500. Homogeneously mixed sample was packed in a 4 mm MAS (Magic Angle Spinning) rotor and used for subsequent NMR experiments. Prior to NMR measurements, the sample inside the probe was treated with a minimum of 5 cooling/heating cycles between 0 and 25°C to ensure homogenous magnetic alignment of bicelles.

NMR experiments. All spectra presented in Figure 1 were obtained from a 400 MHz Varian/Agilent NMR spectrometer using a 4 mm electric-field-free BioMAS probe. The 2D HIMSSELF spectrum was obtained on a 900 MHz Bruker NMR spectrometer at 15°C under static conditions using a HIMSSELF sequence with 0.5 ms cross-polarization contact time, 100 t_1 experiments, 25 ms acquisition time, 512 scans, 25 kHz TPPM (Two-Pulse Phase-Modulation) decoupling of protons, and a 3 s

recycle delay resulting in a total experimental time of 42.7 hours. A 4 mm E-Free triple-resonance VT MAS probe (Bruker) was used to prevent sample heating.

The carbon monoxide assays on cytochrome P450 2B4. The carbon monoxide difference spectra of cytochrome P450 2B4 were obtained to determine the percentage of the functional form of cytochrome P450. We monitored the absorption increase at 450 nm as a result of the formation of the ferrous-cytochrome P450 bound to carbon monoxide. A few grains of dithionite were added to the sample containing 1 μM cytochrome P450 in 100 mM potassium phosphate, pH 7.4, and 5.0% (w/v) glycerol. The sample was mixed and incubated at room temperature for 5 min. The baseline spectrum of the reaction mixture was recorded from 600 nm to 300 nm. Subsequently, carbon monoxide gas was bubbled gently through the dithionite-reduced solution for a few seconds and the reduced-carbon monoxide difference spectra were recorded at 25°C. The concentrations of the active form, cytochrome P450, and the inactive form, cytochrome P420, were calculated as described elsewhere^{45,53}.

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Author contributions

K.Y. and A.R. planned the experiments, K.Y., M.G., S.A., P.P., S.I., L.W. and A.R. performed the experiments and analyzed the results, K.Y. and A.R. wrote the paper, and A.R. designed and directed the research. All authors reviewed the manuscript.

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