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Cellular solid-state NMR investigation of a membrane protein using dynamic nuclear polarization ☆

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ABSTRACT

While an increasing number of structural biology studies successfully demonstrate the power of high-resolution structures and dynamics of membrane proteins in fully understanding their function, there is considerable interest in developing NMR approaches to obtain such information in a cellular setting. As long as the proteins inside the living cell tumble rapidly in the NMR timescale, recently developed in-cell solution NMR approaches can provide 3D structural information. However, there are numerous challenges to study membrane proteins inside a cell. Research in our laboratory is focused on developing a combination of solid-state NMR and biological approaches to overcome these challenges in order to obtain high-resolution structural insights into electron transfer processes mediated by membrane-bound proteins like mammalian cytochrome-b5, cytochrome-P450 and cytochrome-P450-reductase. In this study, we demonstrate the feasibility of using dynamic nuclear polarization (DNP) magic angle spinning (MAS) NMR spectroscopy for in-cell studies on a membrane-anchored protein. Our experimental results obtained from ^{13}C-labeled membrane-anchored cytochrome-b5 in native *Escherichia coli* cells show a ~16-fold DNP signal enhancement. Further, results obtained from a 2D ^{13}C/^{13}C chemical shift correlation MAS experiment demonstrate the feasibility of suppressing the background signals from other cellular contents for high-resolution structural studies on membrane proteins. We believe that this study would pave new avenues for high-resolution structural studies on a variety of membrane-associated proteins and their complexes in the cellular context to fully understand their functional roles in physiological processes. This article is part of a Special Issue entitled: NMR Spectroscopy for Atomistic Views of Biomembranes and Cell Surfaces. Guest Editors: Lynette Cegelski and David P. Weliky.

1. Introduction

High-resolution structures and dynamics of membrane proteins obtained in a membrane environment are very important to fully understand their biological function. Although the membrane environment poses numerous challenges to biophysical studies, recent NMR and crystallography studies have made tremendous progress in this area [1–3]. Particularly the solid-state NMR techniques are capable of providing structural information at atomic-resolution in a lipid bilayer environment, and could also be used to study the dynamic protein–protein and protein–ligand interactions [4–11].

Structure, dynamics and functional properties of a molecule highly depend on its interaction with neighboring molecules [12,13]. This is very much the case for membrane proteins [14–17], as the stability and folding of a membrane protein can be controlled by intermolecular interactions. For example, most membrane proteins are unstable in solution but they fold natively in a lipid bilayer environment to form stable structures. This is why studies on a membrane protein should be carried out in a near-native membrane environment to obtain physiologically meaningful information about the protein [18–20]. This is now well accepted by the structural biology community, even though some of the challenges still remain to be overcome for high-resolution studies in a membrane environment. As a result, many studies have focused on the development of membrane mimetics to overcome some of the challenges in finding a suitable model membrane for studies using a chosen biophysical technique [21]. However, it is not easy to mimic the exact native environment for in-vitro structural studies on a

Abbreviations: NMR, nuclear magnetic resonance; DNP, dynamic nuclear polarization; MAS, magic angle spinning; *E. coli*, *Escherichia coli*; MLVs, multilamelar vesicles; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; DARR, dipolar assisted rotational resonance; RAD, radio-frequency assisted diffusion; CTUC, constant-time uniform-sign cross-peak; fp-RFDR, finite-pulse radio frequency-driven recoupling; PDSD, proton driven spin diffusion; TOTAPOL, 1-(TEMPO-4-oxy)-3-(TEMPO-4-amino) propan-2-ol

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membrane protein [22–24]. A cell membrane is quite complex as it is composed of many different types of lipids, polysaccharides, cholesterol, proteins etc. In addition, the membrane composition can vary between different cells. For example, the compositions of Gram-positive and Gram-negative bacteria are different, and are quite different from that of a mammalian cell [25,26]. In fact, this is why the biological function of most antimicrobial peptides differs significantly in these cells. In addition to the cell membrane, the molecular composition and crowding inside the cell can influence the structure, dynamics and folding of the soluble domains of a membrane protein [1,27–30]. This is particularly important for single-pass and double-pass membrane proteins that contain very large soluble domains, including the membrane-anchored proteins like the mammalian cytochrome b5, cytochrome P450 and cytochrome P450 reductase [31]. These proteins pose tremendous challenges for structural studies using X-ray crystallography and traditional NMR spectroscopy. Therefore, it is very important to develop approaches to study the high-resolution structure and dynamics of membrane proteins and their interactions with other molecules in a cellular environment.

In this study, we demonstrate the feasibility of using the sensitivity-enhancement rendered by dynamic nuclear polarization (DNP) NMR spectroscopy under magic angle spinning (MAS) conditions for in-cell studies on membrane-anchored proteins in native *Escherichia coli* cells. Solid-state NMR experimental results obtained from a recombinant rabbit cytochrome b5 in different membrane environments are also presented in this study. Details on many different physiological roles of cytochrome b5 can be found elsewhere [32,35]. The amino acid sequence of rabbit cytochrome b5 and its structure are given in Fig. 1. Our previous NMR studies solved the first high-resolution structure of the full-length cytochrome b5 in a membrane environment [19,32,36]. Solid-state NMR experiments provided high-resolution insights into the dynamics of the protein in lipid bilayers [32,37]. The structure of the complex between full-length cytochrome b5 and cytochrome P450 bound to membrane has also been reported [18,19]. These studies have provided the structure, dynamics and topology of the protein alone and in complex with cytochrome P450 [18,19,21]. Moreover, these studies provided one of the first structural models to understand the electron transfer process between the membrane-bound metalloproteins.

Since cytochrome b5 is a well-behaved protein and an excellent model system for membrane-anchored proteins containing a large soluble domain, we used this protein to develop solid-state NMR approaches that can be applied to other membrane proteins. It should be noted that single-pass and double-pass membrane proteins pose additional challenges to biophysical studies, as compared to integral transmembrane proteins. For example, it is a monumental challenge to mimic the native cellular environment of these proteins that play vital roles in natively folding both soluble and transmembrane domains of single-pass and double-pass membrane proteins. While the soluble domains of these proteins requires bulk water – and possibly also other cellular contents – for stable structural foldings with native-like dynamics, the hydrophobic transmembrane domain needs a hydrophobic core of the lipid bilayer membrane. Due to these difficulties, high-resolution structures of the full-length single-pass and double-pass membrane proteins are very rare in comparison to integral transmembrane proteins. Successful crystallographic studies usually removed the membrane binding domain(s) of such proteins [31].

Our NMR-based structural studies and biological functional assays demonstrated that bicelles, which contain bulk water and hydrophobic core lipid bilayer, are excellent model membrane systems to study such proteins [18,19,21]. Further research on the development of bicelles to study temperature sensitive cytochrome P450 and its complexes are in progress in our laboratory. In addition, we are investigating the feasibility of solid-state NMR experiments in a cellular environment. The major challenges in this area are the sensitivity and spectral resolution. In this study, we present our initial in-cell solid-state NMR studies on cytochrome b5 that utilizes the sensitivity-enhancement offered by DNP along with sample spectra obtained from MAS experiments on bicelles and multilamelar vesicles (MLVs).

2. Methods and materials

2.1. Materials

Uniformly-deuterated [D_8]-glycerol, deuterium oxide, [1-^{13}C] valine, [2-^{13}C] leucine, [3-^{13}C] alanine, and tryptophan (indole ring-2-^{13}C) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). DNP polarizing agent, AMUPol, was kindly provided by Bruker Biospin (Billerica, MA). All phospholipids and detergent used in this study were purchased from Avanti Polar Lipids (Alabaster, AL). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Sample preparation of selectively ^{13}C-labeled cytochrome b5 with AMUPol for in-cell DNP experiments

A stock biradical solution of [D_8]glycerol/D_2O/H_2O (60:30:10 volume ratio) containing 40 mM AMUPol and a DNP solution of [D_8]glycerol/D_2O/H_2O (10:75:25 volume ratio) were prepared and were kept in a −80 °C deep freezer. Details on the overexpression and purification of cytochrome b_5 can be found elsewhere in the literature [38]. After the overexpression of cytochrome b5, a buffer of *E. coli* cells was exchanged with the DNP solution, [D_8]glycerol/D_2O/H_2O (10:75:25 volume ratio), by centrifugation. Resulting cell pellets were mixed with a stock biradical solution and [D_8]glycerol. The cell pellets with 10 mM AMUPol in the

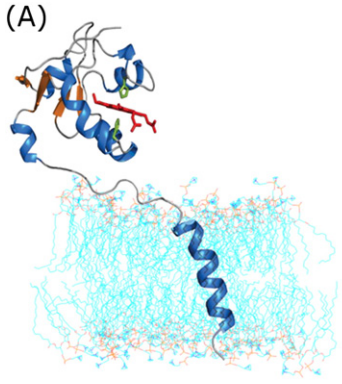

Fig. 1. Structure of full-length cytochrome b5. (A) High-resolution NMR structure of rabbit cytochrome b5 in lipid bilayers is composed of three distinct domains: the transmembrane α-helix at the C-terminus and the heme-containing catalytic soluble domain are connected by a flexible linker region. (B) The amino acid sequence of rabbit cytochrome b5. The underlined region is the hydrophobic transmembrane domain of the protein.

DNP solution and [D_8]glycerol/D_2O/H_2O (10:75:25 volume ratio) were packed into a 3.2 mm sapphire MAS rotor. The NMR probe was pre-cooled to 100 K before samples were inserted into the probe. Bicelles and MLVs were prepared as explained elsewhere in the literature [20,32]. NMR sample preparations used in this study are summarized in Fig. 2.

2.3. NMR measurements

DNP-enhanced solid-state NMR experiments were performed using an Avance III 600 MHz Bruker NMR spectrometer equipped with a 395.18 GHz second-harmonic gyrotron and a 3.2 mm ^{1}H,^{13}C,^{15}N triple-resonance low-temperature MAS probe at 99.5 K. MAS speed was set at 12.5 ± 0.003 kHz for the one- and two-dimensional experiments reported in this study. The samples were irradiated with 9 W of CW microwave power for DNP experiments. The sample temperature was calibrated by the spin-lattice relaxation (T_1) measurements of KBr [33,34] under the microwave irradiation and MAS. The DNP signal enhancement factors (ε) were obtained through the comparison of peak intensities from spectra acquired using identical experimental conditions except for with/without the microwave irradiation. The ^{13}C chemical shifts were referenced to tetramethylsilane using adamantine as an external reference. Solid-state NMR experiments on bicelles [20] and MLVs [46] were performed using a Varian/Agilent 600 MHz NMR spectrometer as explained in our previous studies.

3. Results and discussion

Solution NMR and crystallography studies have reported the high-resolution structure of the soluble domain of cytochrome b5, which is devoid of the transmembrane segment, in solution [35]. However, the soluble domain of the protein alone does not interact with cytochrome P450 and is incapable of donating electrons to enable the enzymatic function of cytochrome P450 [19,31,32]. Therefore, the high-resolution structural interactions between cytochromes b5 and P450, and the mechanism of electron transfer, have been unclear until our report on the first high-resolution structure of the full-length membrane-bound cytochrome b5 (shown in Fig. 1A) and the membrane-bound cytochromes b5-P450 complex using a combination of solid-state and solution NMR experiments [19,32]. In this study, MAS experiments on cytochrome b5 in different membrane environments including bicelles, MLVs and in-cell, are presented. All procedures of sample preparation are summarized in Fig. 2 and other details can be found either in the methods section or in our previous publications [20,32,38].

3.1. MAS experiments on fluid lamellar phase bicelles containing cytochrome b5

Bicelles [39], composed of lipids and mild detergents, are commonly used in the structural studies of biomolecules by NMR and crystallographic techniques. The presence of bulk water, planar lipid bilayer environment, and the curved toroidal pores of lamellar-phase bicelles is suitable for a variety of biophysical and biochemical studies. More details on bicelles can be found in the recent review articles [10,23].

2D MAS experiments that correlate the chemical shifts of ^{13}C nuclei were carried out on bicelles and MLVs (multilamellar vesicles) containing the uniformly-^{13}C-labeled cytochrome b5. Bicelles composed of DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) and DHPC (1,2-dihexanoyl-*sn*-glycero-3-phosphocholine) with a q = [DMPC]/[DHPC] ratio of 3.5, and uniformly-^{13}C-labeled cytochrome b5 with a DMPC: protein molar ratio of 220:1, were used (Fig. 3). A sample 2D $^{13}C/^{13}C$ DARR (dipolar assisted rotational resonance) [40,41] (or RAD (radio-frequency assisted diffusion) mixing [42]) spectrum of bicelles obtained at 310 K under MAS conditions is given in Fig. 3. This high-resolution 2D spectrum displays crosspeaks between chemically inequivalent ^{13}C nuclei that are close in proximity to have non-zero ^{13}C–^{13}C dipolar couplings under the recoupling sequence. We have previously reported the high-resolution 2D $^{13}C/^{13}C$ chemical shift correlation spectrum [20] obtained using CTUC (the constant-time uniform-sign cross-peak) technique [43]. Such high-resolution homonuclear correlation spectra are essential for resonance assignment in the structural studies using MAS solid-state NMR experiments [44]. These results demonstrate that structural studies can be accomplished on fluid lamellar phase samples without freezing the sample. This is a major advantage for cytochrome b5 – like most single-pass or double-pass membrane proteins – that consists of structural domains undergoing dynamics in very different time scales; our solid-state NMR studies on magnetically-aligned bicelles have demonstrated that the transmembrane (milliseconds)

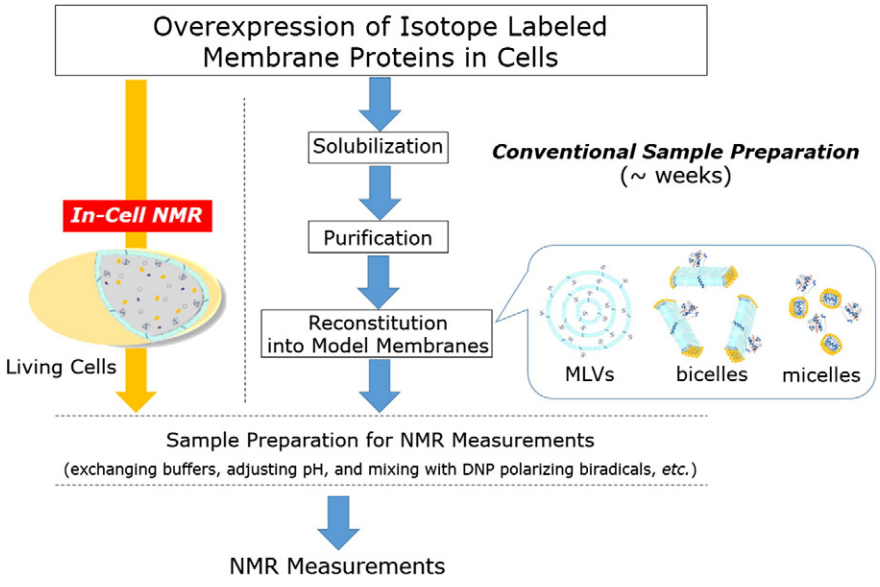

Fig. 2. Preparation of membrane protein samples for solid-state NMR studies. Sample types for NMR high-resolution structural studies on membrane-associated proteins: living cells, MLVs, bicelles and lipid/detergent micelles. The flow chart of the membrane-associated protein production for NMR experiments illustrates the differences between the in-cell NMR (left) and the conventional methods (right). There are several unique advantages with in-cell NMR approach to study membrane proteins in the cellular environment. It utilizes whole living cells and therefore the difficulties related to purification and reconstitution of a membrane protein are avoided, which can significantly speed up the sample production.

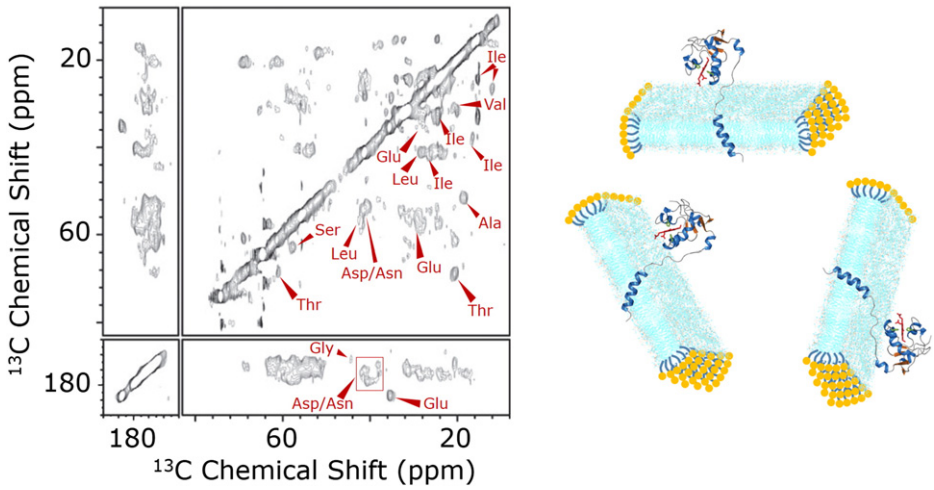

Fig. 3. 2D ^{13}C/^{13}C chemical shift correlation spectrum of bicelles. A two-dimensional ^{13}C–^{13}C dipolar assisted rotational resonance (DARR) (or radio-frequency assisted diffusion (RAD) mixing) chemical shift correlation spectrum of uniformly ^{13}C,^{15}N-labeled cytochrome b5 incorporated in 4:1 DMPC:DHPC bicelles at 5 kHz MAS and 310 K sample temperature. A 400 ms DARR mixing time, 128 t_1 increments, 64 scans, 4 dummy scans, and 2 s recycle delay were used in the experiment. Total experimental time was 5.5 hours. The ramped cross-polarization [84] contact time was 2 ms, and a 50 kHz SPINAL64 pulse sequence [85] was used for decoupling protons during ^{13}C signal acquisition of 25 ms.

and soluble (microseconds) domains undergo very different time scale of motions [34,37]. Therefore, solid-state NMR structural studies carried out on fluid lamellar phase samples could provide insights into the dynamics of the protein that plays key roles in its function.

3.2. MAS experiments on MLVs containing cytochrome b5

As shown in our previous study, MAS experiments at 310 K on MLVs containing cytochrome b5 resulted in spectra with poor signal-to-noise ratio [20]. The interference of dynamics with cross-polarization limits the efficiency of CP-based solid-state MAS experiments. Therefore, we optimized the experimental conditions to acquire spectra at cryogenic temperatures where the motions in the protein are frozen. A sample 2D ^{13}C/^{13}C chemical shift correlation spectrum obtained using finite-pulse-RFDR (radio frequency-driven recoupling) [45,46] of MLVs obtained at 103 K is given in Fig. 4. As demonstrated in our earlier study, our experiments demonstrate that the sensitivity of the experiment was enhanced due to the suppression of molecular motions by freezing the sample [47]. As shown in the spectrum in Fig. 4, some of the resonances can be assigned in spite of broadening of spectral lines in comparison with that in Fig. 3 for bicelles. Nevertheless, the higher sensitivity rendered by the low temperature is encouraging as selectively-labeled proteins can be utilized to enhance the resolution and potentially used for interatomic distance measurements. In addition, the sensitivity of NMR experiments can be further enhanced using DNP experiments [48–62].

3.3. In-cell DNP MAS experiments

As mentioned above, lowering the temperature to freeze molecular motions significantly increased the signal-to-noise ratio of the spectrum but at the expense of spectral resolution. In order to demonstrate further sensitivity enhancement using DNP, and the feasibility of using DNP experiments in the cellular context, we expressed cytochrome b5 with selectively ^{13}C labeled amino acid residues. The labeling scheme was judiciously designed so that the isotropic chemical shifts of the

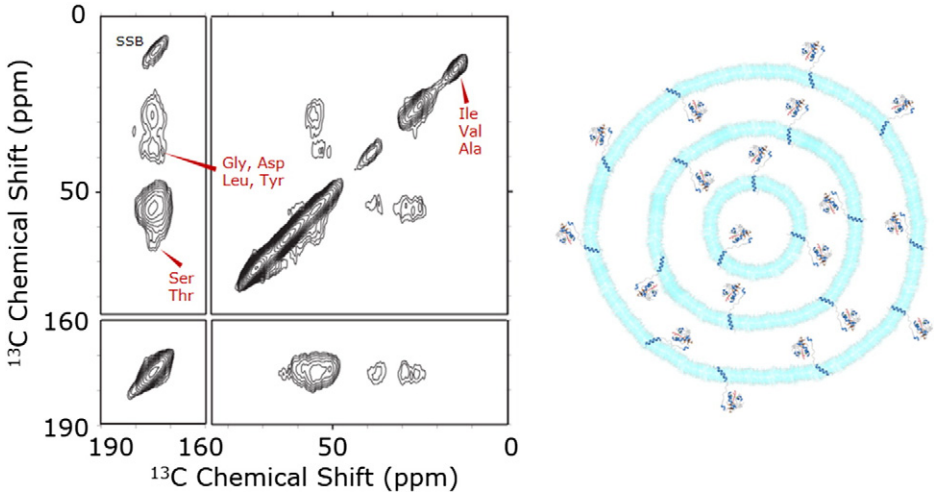

Fig. 4. 2D ^{13}C/^{13}C chemical shift correlation spectrum of MLVs. A two-dimensional ^{13}C/^{13}C chemical shift correlation spectrum of uniformly ^{13}C,^{15}N-labeled cytochrome b5 in DLPC multilamelar vesicles (right) at 12.5 kHz MAS and 103 K sample temperature. The finite-pulse radio frequency-driven recoupling (fp-RFDR) with XY16 phase cycling was used in the mixing time to recouple ^{13}C–^{13}C dipolar couplings under MAS. A 12 ms fp-RFDR mixing time, 128 t_1 increments, 80 scans, 4 dummy scans, and 2.5 s recycle delay were used to acquire the 2D spectrum. Total experimental time was 7 hours. The ramped cross-polarization contact time was 1.3 ms, and a 100 kHz SPINAL64 pulse sequence was used for decoupling protons during the signal acquisition of 13 ms. The spinning side band in the spectrum is indicated as SSB.

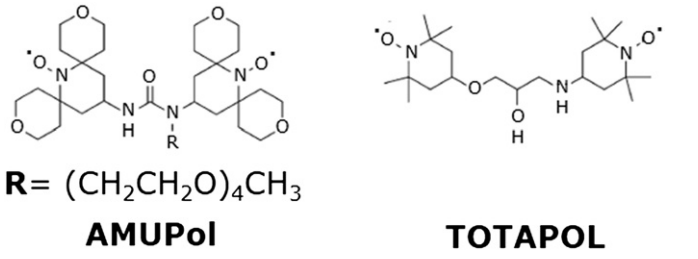

Fig. 5. Chemical structures of biradical polarizing agents. A hydrophilic polarizing agent, AMUPol, has higher dynamic nuclear polarization efficiency than a conventional polarizing agent, 1-(TEMPO-4-oxy)-3-(TEMPO-4-amino)propan-2-ol (TOTAPOL).

labeled residues can be used to estimate the secondary structural information of the protein inside the cell. Samples were prepared with AMUPol (Fig. 5) [63] for DNP measurements as explained above (Fig. 2). 1D $^{13}C\{^{1}H\}$ CPMAS spectra acquired with and without the DNP effect are given in Fig. 6. The recycle delays between successive scans of NMR experiments were set to $1.3 \times T_1$, which provided the optimum sensitivity. The ^{1}H spin-lattice relaxation time (T_1) of the in-cell cytochrome b5 with 10 mM AMUPol in this study was measured to be 6.0 s at 99.5 K. The ^{1}H spin-lattice relaxation times (T_1) measured via CP to the carbonyl groups of lipids resonating at ~173 ppm were found to be 30.2 s for DMPC MLVs without the DNP polarization agent, 2.0 s for DMPC MLVs with 10 mM AMUPol, and 4.7 s for the whole in-cell sample with 10 mM AMUPol at 99.5 K. Previous studies have shown that the spin-lattice relaxation times (T_1) of lipids in *E. coli* are comparable with synthetic, purified, and total extracted lipids at room temperature. The variations in the T_1 relaxation times are attributed to the difference in the viscosity of cytoplasm in in-cell conditions [64]. At cryogenic temperatures used for DNP experiments, the differences in the viscosity of the sample do not play a role in the experimentally measured T_1 values; further, the T_1 relaxation times are reduced by the paramagnetic relaxation enhancements from the DNP biradical agents. Therefore, this set of experimentally measured relaxation data infers that the DNP agent, AMUPol, could be dispersed relatively homogeneously in the NMR sample. If needed, it is possible to use paramagnetic agents that are bound to the membrane as demonstrated elsewhere [48,65–69].

It is remarkable that the spectral lines originating from the labeled sites of the protein are visible in the DNP spectrum in spite of the dominant peaks from glycerol, as shown in Fig. 6. Our experiments demonstrate a DNP enhancement factor (ε) of ~16 in the cellular sample, which is larger than the enhancement obtained using another DNP agent, TOTAPOL (1-(TEMPO-4-oxy)-3-(TEMPO-4-amino)propan-2-ol) [70]. In fact, TOTAPOL is the most commonly used exogenous biradical polarizing agent for DNP solid-state NMR experiments on biomolecular samples up to date [70]. However, there are some disadvantages in using TOTAPOL: a) the flexible chemical structure of TOTAPOL prevents the efficient cross-effect magnetization polarization transfer, and b) it gives poor efficiency at higher magnetic fields, or fast sample spinning speeds (for >3 kHz) in MAS experiments [70]. Various cross-effect DNP polarizing agents have been developed to overcome these disadvantages [71–76]. Among these agents, AMUPol is greatly beneficial for biological applications since it has a high water solubility and offers a better efficiency for DNP enhancement even at high magnetic fields or fast MAS speeds (up to 14 kHz) [63]. Previous studies have shown that the DNP enhancement factors obtained using AMUPol are about 3.5–4 times higher than that with TOTAPOL, due to its high solubility, the electron relaxation time, larger electron-electron dipole coupling, and its rigid chemical structure [63]. Moreover, the DNP signal enhancement using AMUPol can be potentially further increased by the optimization of sample preparation. A recent report showed that multiple washing steps of bacterial cell walls using polarizing biradical solutions can significantly improve the DNP efficiency [77]. Therefore, optimization of cellular sample preparation could be important for successful implementation of multidimensional in-cell DNP solid-state NMR experiments. Current research in our laboratory focused on developing procedures to effectively incorporate a DNP agent in cellular samples for solid state NMR measurements from membrane proteins.

2D $^{13}C/^{13}C$ chemical shift correlation experiments under MAS conditions were also carried out on the above-mentioned in-cell sample containing cytochrome b5 using the radio-frequency pulse sequence given in Fig. 7. The resultant 2D spectrum is shown in Fig. 7. In order to detect long-range distances in a selectively ^{13}C-labeled cytochrome b5, the PDSD (proton driven spin diffusion) pulse sequence [78,79] was chosen for 2D $^{13}C/^{13}C$ correlation experiments. For example, the average distance of observed correlation in a 2D $^{13}C/^{13}C$ PDSD experiment could be about 5.5 Å for the transmembrane α-helical region. This experiment allowed us to observe the reasonably well-resolved cross peaks for [3-^{13}C] Ala/[2-^{13}C] Leu, [2-^{13}C] Leu/Trp (indole ring-2-^{13}C), [3-^{13}C] Ala/[1-^{13}C] Val, and [2-^{13}C] Leu/[1-^{13}C] Val sites of cytochrome b5 even at 99.5 K. It should be noted that previous studies have shown that solid-state NMR experiments at low-temperatures result in line-broadening mainly due to the restriction of the molecular motions [47,80–82]. Thus, our preliminary DNP results at cryogenic temperature demonstrate that it is feasible to acquire reasonably well-resolved MAS spectra if the isotopically labeled sites in the embedded protein of interest are carefully chosen. Well-resolved MAS spectra along with determination of structural constraints have been

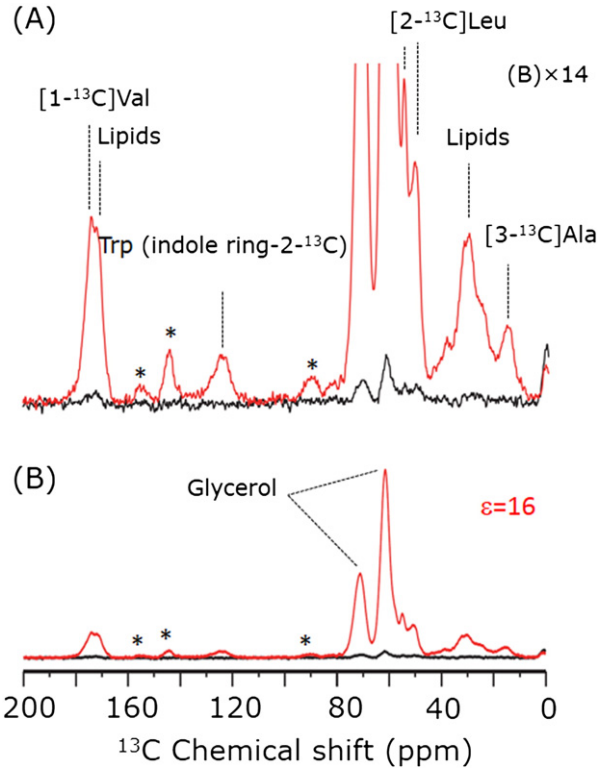

Fig. 6. DNP-enhanced solid-state NMR spectroscopy of *E. coli* cells. One-dimensional in-cell ^{13}C CPMAS NMR spectra of cytochrome b5 in *E. coli* cells using CPMAS with microwave irradiation on (red), and off (black). As mentioned in the main text, the protein was selectively labeled with [1-^{13}C] Val, [2-^{13}C] Leu, [3-^{13}C] Ala, and Trp (indole ring-2-^{13}C). Ten millimolar AMUPol in [D_8]glycerol/D_2O/H_2O (60/30/10 volume ratio) was used as DNP polarization agents. The signal enhancements (ε) were 16 for cytochrome b5 and 30 for glycerol. The sample was spun at 12.5 kHz and the sample temperatures were maintained at 99.5 K. Other experimental parameters used to acquire the spectra are: 0.8 ms cross-polarization contact time, 100 kHz SPINAL64 to decouple protons during the signal acquisition of 25.9 ms, and a recycle delay of 7.8 s. The total measurement time was 3 min. Spinning side bands in the spectra are indicated by asterisks. A background signal around 0 ppm is from the silicon rubber seal used in the MAS rotor. Fig. 3(A) is the 14× magnified spectra of Fig. 3(B).

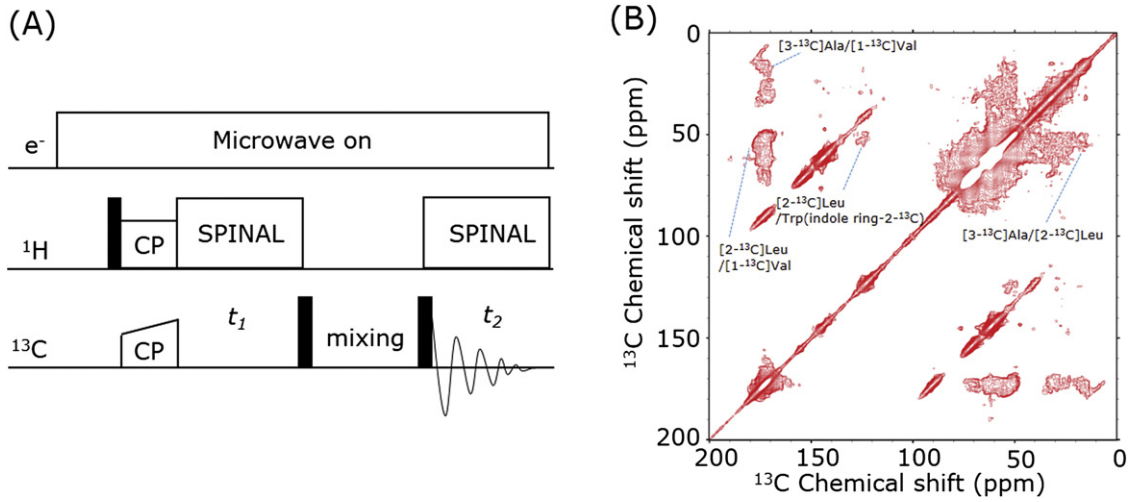

Fig. 7. 2D $^{13}C/^{13}C$ chemical shift correlation spectrum *E. coli* cells containing selectively-^{13}C-labeled cytochrome b5. (A) Two-dimensional $^{13}C/^{13}C$ chemical shift correlation pulse sequence that utilizes the proton driven spin diffusion (PDSD) to mix the longitudinal magnetization of ^{13}C nuclei under MAS. (B) 2D $^{13}C/^{13}C$ chemical shift correlation spectrum of cytochrome b5 in *E. coli* cells selectively labeled with [1-^{13}C] Val, [2-^{13}C] Leu, [3-^{13}C] Ala, and Trp (indole ring-2-^{13}C). The 2D spectrum was obtained using the pulse sequence given in (A) using a highly efficient polarizing agent, AMUPol at 99.5 K; 10 mM AMUPol in [D_8]glycerol/D_2O/H_2O (60/30/10 volume ratio) was used. Other experimental conditions include: 12.5 kHz spinning speed of the sample, 1.5 ms cross-polarization contact time, 3 s PDSD mixing time, 100 kHz SPINAL64 sequence to decouple protons during signal acquisition of 13 ms, 192 t_1 increments, 16 scans, 4 dummy scans, and 7.8 s recycle delay. The total experimental time was 9.2 hours. Covariance NMR was used for processing the 2D spectrum.

demonstrated by the recent DNP solid-state NMR studies on samples including globular proteins [57,59,80], membrane proteins [15,48,49,51,53,55,58–62,82], cell extracts [27,77,83] and cellular samples [28,29]. Although the loss of spectral resolution limits the application of DNP MAS experiments at cryogenic temperatures, the sensitivity gain can be well utilized to measure high-resolution structural information if the system under study is selectively labeled with isotopes. Further development of isotope labeling strategies for structural studies of membrane-associated cytochrome proteins in cellular conditions, using DNP MAS experiments at cryogenic temperatures, is currently underway in our laboratory. An investigation of interactions between membrane-bound proteins using DNP MAS experiments will be reported elsewhere in the near future.

4. Concluding remarks

In this study, we have demonstrated that MAS solid-state NMR experiments are suitable to study the structure and dynamics of membrane-bound proteins containing a large soluble domain like the single-pass and double-pass membrane proteins. As we have demonstrated, MAS experiments can provide high-resolution structural information from fluid lamellar-phase lipid bicelles that retain native molecular motions, frozen lipid bilayers, and also from in-cell samples. One of the main concerns of freezing the sample to enhance the sensitivity of NMR experiments can be overcome by carefully labeling the proteins at selective sites. Our results from in-cell sample containing selectively ^{13}C-labeled cytochrome b5 demonstrate the feasibility of studying membrane proteins using DNP experiments in a cellular environment, which considerably simplifies the procedure of sample preparation for NMR measurements. It is gratifying to note that the use of AMUPol enhances the DNP sensitivity by a factor of ~16 in the cellular sample.

Though the reported DNP-based in-cell experimental results are preliminary, they show the feasibility to implement such approaches to study an exciting class of single-pass membrane proteins like cytochromes P450. Up to date, there is no high-resolution structure of these membrane-bound full-length proteins as the full-length cytochrome P450 is highly unstable and very sensitive to experimental conditions like ionic concentration, pH, membrane composition and temperature. Therefore, the in-cell based solid-state NMR approach could be the desired magic wand for structural studies of cytochrome P450 (and other similar membrane proteins). In addition, the interactions between cytochrome P450 and its redox partners, such as cytochrome b5 and cytochrome P450 reductase, measured in the cellular environment could provide piercing insights into their physiological functions. Such studies could aid in the design of drugs that are oxidized by the cytochrome P450 enzymes.

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