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Shortening Spin-lattice Relaxation Using a Copper-Chelated lipid at Low-Temperatures – A Magic Angle Spinning Solid-State NMR Study on a Membrane-Bound Protein

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Abstract

Inherent low sensitivity of NMR spectroscopy has been a major disadvantage, especially to study biomolecules like membrane proteins. Recent studies have successfully demonstrated the advantages of performing solid-state NMR experiments at very low and ultralow temperatures to enhance the sensitivity. However, the long spin-lattice relaxation time, T_1 , at very low temperatures is a major limitation. To overcome this difficulty, we demonstrate the use of a copper-chelated lipid for magic angle spinning solid-state NMR measurements on cytochrome-b5 reconstituted in multilamellar vesicles. Our results on multilamellar vesicles containing as small as 0.5 mole % of a copper-chelated lipid can significantly shorten T_1 of protons, which can be used to considerably reduce the data collection time or to enhance the signal-to-noise ratio. We also monitored the effect of slow cooling on the resolution and sensitivity of ^{13}C and ^{15}N signals from the protein and ^{13}C signals from lipids.

1. Introduction

The ability to study most samples without any restriction due to molecular size or motion clearly distinguishes solid-state NMR spectroscopy from other techniques. As a result, solid-state NMR spectroscopy is increasingly utilized in the high-resolution investigation of a variety of biological, chemical and materials related problems. Some of the recent studies have utilized cutting-edge solid-state NMR techniques to study the high-resolution structure and dynamics of crystalline and membrane proteins [1–8] and mechanism of membrane disruption by antimicrobial peptides in a variety of membrane environments [9,10]. Magic angle spinning solid-state NMR techniques have been used to determine the structures of amyloid fibers [11,12]. Importantly, solid-state NMR spectroscopy is the only technique that can provide piercing atomic-level insights into complex biological systems like bone materials [13–15]. These studies have successfully provided significant insights into the functional aspects of biomacromolecules at atomic resolution. The continued success in the development of instrumentation [16] – particularly the higher field magnets and ultrafast

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magic angle spinning (MAS) probes [17] – will continue to expand the applications of solid-state NMR spectroscopy. [18]

While these are amazing developments in the field, the major limitation of this powerful technique has been its low sensitivity.[19] One of the ways to increase the sensitivity of this technique is to perform experiments at low temperatures.[20–24] In addition to other contributing factors, the suppression of molecular motions also enhances the sensitivity. [21,22] As a result weak intermolecular interactions and molecular components present at a very low concentration can be detected.[25] Though low-temperature experiments are technically demanding, sample stability for long data acquisition, the ability to detect unstable intermediates, and the effective implementation of dynamic nuclear polarization (DNP) experiments are additional advantages of low-temperature NMR experiments. [20,22,24] Such low-temperature studies are becoming common as the development of instrumentation [20,22] and techniques including DNP [20,22,24] are progressing very well. There is considerable interest in developing DNP-based solid-state NMR approaches and expanding their applications to study biological samples.[27–30] However, freezing the sample can broaden the spectral lines due to the frozen conformational heterogeneity and also increases the spin-lattice relaxation (T_1). These are two major disadvantages that severely limit the applications of low-temperature solid-state NMR spectroscopy to study biological molecules in general, membrane proteins in particular. For example, single-pass (or even double-pass) membrane proteins that contain a large soluble domain exhibit a broad scale of dynamics and conformational heterogeneity – and therefore pose challenges to low-temperature solid-state NMR studies.[3] It should be noted that these proteins are extremely difficult to crystallize unlike intrinsic transmembrane proteins such as G-protein coupled receptors. Therefore, there is considerable interest in the development of solid-state NMR approaches to study the high-resolution structure and dynamics of membrane proteins containing large soluble domain(s).

In this study, we report an investigation of the effect of low temperature on a membrane-bound protein and the use of a copper-chelated lipid to shorten the T_1 relaxation. Magic angle spinning solid-state NMR results obtained from a single-pass transmembrane protein, a 15.7-kDa cytochrome-b5 (Figure 1), embedded in bicelles [35] and detergent micelles [31] are reported. Cytochrome-b5 has been shown to enhance (or is even essential) for some catalytic reactions by cytochrome-P450 (also known as ‘mother nature’s blow torch’) that metabolize nearly 70% of current-day drugs.[3] Its suggested functions include direct donation of an electron to cytochrome-P450, fine-tuning of kinetic parameters by relaying electrons, or an allosteric effector function on cytochrome-P450. Our recent NMR studies reported the first high-resolution structure of the membrane-bound ~72-kDa complex formed between full-length cytochrome-b5 and cytochrome-P450 2B4 using solution and solid-state NMR techniques.[31–34] Results reported in these studies revealed the amino acid residues defining the interaction interface in the b5-P450 complex and also the mechanism of electron transfer from cytochrome-b5 to cytochrome-P450.[31] Besides modifying drug metabolism, cytochrome-b5 is also essential for the biosynthesis of testosterone and numerous unsaturated lipids that are necessary for maintaining the integrity of cellular membranes.[3] We recently reported the first high-resolution structure of the full-length membrane-bound rabbit cytochrome-b5 by using a combination of solution and solid-state NMR techniques.[31–34] It consists of a heme-containing soluble domain that is connected to its helical transmembrane domain at the C-terminal by a flexible linker region. Because of the difficulties in preparing suitable samples of the full-length cytochrome-b5, X-ray and solution NMR studies have reported the structure of the water-soluble domain of cytochrome-b5 that lacks the membrane domain.[37–39] Previous solution NMR studies have reported the dynamics of the truncated cytochrome-b5 in solution [40,41]. Our

experimental results demonstrating the increasing sensitivity, decreasing resolution and shortening of T_1 due to the lowering of the sample temperature are presented in this study.

2. Results and Discussion

Decreasing temperature increased the S/N and decreased the spectral resolution

Carbon-13 and ^{15}N CPMAS (Cross Polarization Magic Angle Spinning) spectra of uniformly- ^{13}C & ^{15}N -labeled full-length rabbit cytochrome-b5 embedded in DLPC (1, 2-dilauroyl-*sn*-glycero-3-phosphocholine) multilamellar vesicles (MLVs) were obtained at various temperatures starting at room temperature down to 100 K. The sample was slowly cooled to 100 K and warmed to room temperature several times. The room temperature spectrum obtained each time was identical suggesting that the sample was well behaved. Sample spectra obtained at 268 and 100 K using are compared in Figure 2; the same number of scans and other experimental conditions as mentioned in the Figure 2 caption were used. Carbon-13 and ^{15}N spectra, obtained in the presence and absence of a copper-chelated lipid, are compared in Figure 2 and are discussed below. As seen from Figure 2, the low-temperature spectra of both ^{13}C and ^{15}N nuclei exhibit higher S/N. Since the main purpose of this study was to demonstrate the NMR sensitivity enhancement for membrane proteins, and signals from lipids and proteins can be easily differentiated due to their distinct chemical shift values, we did not perform cryogenic experiments on vesicles in the absence of the protein. However, the spectra shown in Figure 2 reveal sensitivity enhancements for both lipids and proteins.

Since the sample was made up of a very small amount of protein (400:1 lipid:protein molar ratio), the spectra at 268 K and above (not shown) are dominated by signals from lipid molecules in the sample while the low-temperature spectrum exhibits many peaks from the protein as well. In addition, cross-polarization near the room (or at a higher) temperature is inefficient for the soluble and linker regions of the protein that have been shown to undergo fast motion (microsecond) in the NMR time scale.[31,32,34] As the temperature of the sample was decreased, more peaks from the protein start to appear and their intensities increase with the decreasing temperature as shown in Figure 3. Particularly, resonances from the carbonyls in the 170–185 ppm range and aromatic carbons in the 110–140 ppm range were not seen from spectra obtained above 250 K but can be seen clearly below 250 K. Peaks from the amino acid side chains, Lys and Arg, start to appear in the ^{15}N spectra below 156 K.[35,36] Below the lamellar to gel phase transition temperature of the DLPC lipid ($\sim -5^\circ\text{C}$), lipid signals start to broaden and as a result their intensities decreased with the decreasing temperature. The signal-to-noise ratios were measured for peaks in different chemical shift regions of the spectra and the values are given in Figure 4 for selected peaks in the spectra; the overall, S/N is also given for each spectrum in Figure 3. A 14-fold increase in the S/N for ^{13}C and a 6-fold increase for ^{15}N experiments were observed when the temperature of the sample was reduced from 280 to 100 K.

It is difficult to quantify the contributions to the observed sensitivity enhancement arising from the reduction in the mobility of protein and lipids and from the freezing of water. However, this would be possible, if deuterated-water and deuterated lipids are used in these experiments. In this study on cytochrome-b5, reduction in protein dynamics significantly contribute to the observed increase in S/N. This protein consists of three domains: a flexible soluble domain ($\sim 77\%$ of amino acids), a flexible linker domain (~ 15 amino acids), and a rigid transmembrane region (~ 25 amino acids). At room temperature, the soluble and linker regions are highly mobile as reported in our previous NMR study.[32] These regions of the protein would become immobile at 250 K and below, and therefore the CP efficiency is expected to increase significantly at these low temperatures. However, the freezing of water and lipid molecules at these temperatures would also contribute to the increase in S/N.

Overall, the increase in the signal intensity and line broadening observed for cytochrome-b5 are similar to that reported for a crystalline SH3 protein.[23] We refer the readers to a recently reported solid-state MAS NMR study on a crystalline SH3 protein for details on the changes in protein dynamics and the consequent changes on the spectral resolution due to lowering the sample temperature.[23]

Shortening T_1 values using a copper-chelated lipid

Since lowering the temperature freezes molecular motions, the T_1 values have been found to be larger at low sample temperatures.[44] Previous studies have utilized paramagnetic salts to reduce the T_1 values from a variety of solids.[45–50] However, the presence of a large amount of paramagnetic salt could increase the sample temperature if experiments on non-frozen samples are desired.[51,52] Sample heating could lead to denaturation of the protein under investigation. On the other hand, it was recently demonstrated that the use of a paramagnetic metal-chelated lipid significantly reduces the amount of paramagnetic salt required to reduce the T_1 values for membrane-bound peptides and proteins.[51–53] Since the lipid containing a paramagnetic metal (like Cu^{2+} or Gd^{2+}) is immobilized in a model membrane, spin diffusion via the dipolar couplings enables the paramagnetic relaxation effect to be highly efficient as explained in our previous studies.[51,52] As a result, only a very low concentration (about 3 mM) of paramagnetic metal-chelated lipid is sufficient to significantly shorten the T_1 values of protons in a lipid bilayer or isotropic bicelles containing a membrane protein without altering the T_2 values or spectral resolution. In addition, no changes in the biophysical and biological functional properties of the protein in the sample were observed suggesting that the use of a very low amount of paramagnetic salt can avoid any metal-induced alteration of protein properties.

In this study, we demonstrate the effect of a copper-chelated lipid incorporated in MLVs to speed up the T_1 relaxation of protons in the embedded protein at 100 K; it was not possible to measure the T_1 values of protons due to low spectral resolution, particularly at low temperatures. One-dimensional ^{13}C and ^{15}N CPMAS spectra indicate the increase in the S/N even with as small as 0.5 mole % of the Cu-chelated lipid (Figure 2). To further demonstrate the effect of copper-chelated lipid, global proton T_1 values were measured for the samples in the presence and absence of the copper and are given in Table 1. The values reported in Table 1 demonstrate the significant reduction of T_1 values in the presence of a copper-chelated lipid. In addition, the T_1 values measured at various temperatures also confirm the paramagnetic effect from the copper-chelated lipid (Figure 5). As can be noted in Table 1, T_1 values obtained from a significant number of ^{13}C resonances are smaller at 100 K, even in the absence of copper, as compared to the values reported by a previous solid-state NMR study on a crystalline SH3 protein [23]. This could be attributed to the paramagnetic effect, from the iron in the heme unit of the soluble domain of cytochrome-b5, that may become stronger at 100 K as the molecular motions are frozen.

Fast acquisition of 2D $^{13}\text{C}/^{13}\text{C}$ correlation spectra in the presence of a copper-chelated lipid at 100 K

2D $^{13}\text{C}/^{13}\text{C}$ RFDR (Radio Frequency-driven Dipolar Recoupling) [54] spectra correlating the isotropic chemical shifts of ^{13}C nuclei for a mixing time of 12 ms were obtained from the MLV samples in the presence and absence of the copper (Figure 6). As shown in Figure 6, the total duration of 2D $^{13}\text{C}/^{13}\text{C}$ RFDR data collection can be ~3.5 faster by shortening the T_1 values of protons using only a very small amount (0.5 mole %) of the copper-chelated lipid. A comparison of 2D spectra in Figures 6B and 6C suggests that lowering the temperature of the sample from 130 to 100 K further enhanced the S/N . Due to inhomogeneous broadening of resonances at low temperatures, the CO-C α signals are mostly unresolved in all 2D spectra in Figure 6. Resonances corresponding to certain

residues like Thr, Ser, Tyr, Phe, Trp, Ala, Val, Ile and Gly are indicated. It is important to note the similarity in spectral resolution between the 2D spectra obtained from samples with and without the copper-chelated lipid suggest that the presence of Cu^{2+} ions did not significantly alter the spin-spin relaxation (T_2) of ^{13}C nuclei from the protein in the sample. This further confirms the dominant presence of inhomogeneous line broadening of signals upon cooling the sample to 100 K. As reported in a recent study [23], the presence of multiple conformations, particularly that of the sidechains, contributes significantly to the observed broadening of the spectral lines.

3. Conclusion

In this study, we have demonstrated the effect of lowering the sample temperature to 100 K on ^{13}C and ^{15}N MAS spectra of a membrane-bound cytochrome-b5. Our results revealed an increase in S/N and spectral line widths as the sample temperature was decreased to 100 K. The loss of spectral resolution observed for the membrane-bound protein is similar to that observed for a crystalline protein [23] and it is mainly due to the conformational heterogeneity. We have also demonstrated the use of a copper-chelated lipid to significantly shorten the spin-lattice relaxation and enabled ~ 3.5 times faster collection of 2D $^{13}\text{C}/^{13}\text{C}$ RFDR spectrum under MAS at 100 K. We believe that the results reported in this study will be useful to design and conduct cryogenic solid-state MAS studies on membrane-bound proteins, which is likely to have a high impact in the structural studies of large-size proteins, protein-protein complexes, and protein-ligand complexes in a membrane environment. It would also be fruitful to investigate the effectiveness of sample preparation to overcome the conformation heterogeneity [55] as demonstrated in a recent study on amyloid fibers [21] in order to fully utilize the cutting-edge dynamic nuclear polarization (DNP) based solid-state NMR experiments.[21,26,56,57]

4. Experimental section

Uniformly ^{13}C & ^{15}N labeled rabbit cytochrome-b5 was expressed in *E.coli* in minimal media and purified as reported in the literature.[31] The protein was reconstituted in multilamellar vesicles with a final concentration of 400:1 lipid:protein molar ratio as outlined in our previous publication.[35] Our previous studies have shown that cytochrome-b5 can be reconstituted in a model membrane containing a variety of different types of lipids such as DMPC, DMPG, POPC, and POPG.[31,32,34,36] Magnetically-aligned DMPC:DHPC bicelles were used to determine the structure and membrane orientation of cytochrome-b5 using 2D HIMSELF (Heteronuclear Isotropic Mixing leading to Spin Exchange via the Local Field) or HERSELF (HEteronuclear Rotating-frame Spin Exchange via the Local Field) experiments.[58] In this study, we chose to use DLPC multilamellar vesicles as this lipid is used in the functional measurement of cytochrome-b5 and P450.[33] In addition, our recent study showed that DLPC containing bicelles are well suited for solid-state NMR experiments on a wide range of temperatures.[33] More details about the samples used in the NMR measurements are given in the figure captions.

All NMR spectra were obtained on a Bruker AVANCE III 600 MHz solid-state NMR spectrometer (operating with Topspin version 2.1) using a triple-resonance 3.2 mm HCN low temperature MAS probe. MLV samples were packed in 3.2 mm diameter MAS rotors made up of zirconium with vespel spinning cap (Bruker) and a Bruker MAS unit controlled the sample spinning frequency within ± 5 Hz. A silicon rubber seal (Bruker) immediately below the rotor cap was used to avoid any leakage of water from the sample during spinning.[23] Temperature was controlled using a Cryo-NMR cooling cabinet (Bruker) by independently controlling variable temperature, bearing and drive nitrogen gas streams at approximately 100 K. The sample and the instrument were allowed to equilibrate for ~ 30

minutes at each temperature. After that, the probe was tuned and all experimental conditions such as RF field strength, decoupling efficiency, and Hartman-Hahn match for efficient cross-polarization were optimized. All other experimental conditions are mentioned in Figure captions. TPPM (Two-Pulse Phase Modulation) [59] decoupling field strength of 100 kHz was applied to protons during data acquisition and in the t_1 period of $^{13}\text{C}/^{13}\text{C}$ 2D RFDR experiment. All spectra were processed using Bruker Topspin.

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Figure 1.

A representation of multilamellar vesicles containing the full-length rabbit cytochrome-b5 used in the present study (left) and an illustration of the paramagnetic effect to reduce the spin-lattice relaxation of protons (right). The NMR structure of membrane-bound cytochrome-b5 was recently solved by a combination of solution and solid-state NMR techniques.[31–35]

Figure 2.

Carbon-13 (A) and ^{15}N (B) CPMAS spectra of DLPC MLVs containing a uniformly ^{15}N & ^{13}C -labeled cytochrome-b5 at 100 K (black and red) and 268 K (green). The overall S/N values measured from protein spectral lines are: 4 (without Cu^{2+} -DMPE-DTPA at 268 K, green), 44 (without Cu^{2+} -DMPE-DTPA at 100 K, black), and 65 (with 1 mole% Cu^{2+} -DMPE-DTPA at 100 K, red) for ^{13}C , and 6 (without Cu^{2+} -DMPE-DTPA at 268 K, green), 49 (without Cu^{2+} -DMPE-DTPA at 100 K, black), and 57 (with 1 mole% Cu^{2+} -DMPE-DTPA at 100 K, red) for ^{15}N . The spectra were obtained at 12.5 kHz spinning speed using a ramped-cross-polarization pulse sequence [42] with a 1.3 ms contact time, 100 kHz SPINAL-64 proton decoupling [43], and a 2.5 s recycle delay. A total of 68 (^{13}C) and 128 (^{15}N) FIDs were coadded.

Figure 3. ^{13}C (A) and ^{15}N (C) CPMAS spectra of DLPC MLVs containing a uniformly ^{15}N & ^{13}C -labeled cytochrome-b5 and a 0.5 mole % (of total lipids) Cu^{2+} -DMPE-DTPA (1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid) lipid at various indicated temperatures. All other experimental conditions were as mentioned in Figure 2 caption. The overall *S/N* for protein in the sample is indicated in each spectrum. (B) The molecular structure of DLPC lipid.

Figure 4.

Increase in the ^{13}C (A) and ^{15}N (B) signal intensity with the decreasing temperature for DLPC MLVs containing a uniformly ^{15}N & ^{13}C -labeled cytochrome-b5 and a 0.5 mole % (of total lipids) copper-chelated DMPE lipid obtained from spectra given in Figure 3.

Figure 5.

Spin-lattice relaxation vs temperature: T_1 values were measured for few selected ^{13}C resonances of a uniformly ^{15}N & ^{13}C -labeled cytochrome-b5 reconstituted in DLPC MLVs containing 0.5 mole % (of total lipids) copper-chelated DMPE lipid. T_1 values for more ^{13}C resonances are given in Table 1. Protons were saturated by an RF irradiation and the magnetization recovery was measured by observing ^{13}C signal via cross-polarization from protons. All other experimental conditions were as mentioned in Figure 2 caption.

Figure 6.

2D $^{13}\text{C}/^{13}\text{C}$ RFDR MAS spectra correlating isotropic ^{13}C chemical shifts of DLPC MLVs containing a uniformly ^{15}N & ^{13}C -labeled cytochrome-b5 in the absence (blue, A) and presence (red, B and C) of a 1 mole % (of total lipids) copper-chelated DMPE lipid. A 12 ms RFDR mixing time, 128 t_1 increments, 80 (blue) and 96 (red) scans and a recycle delay of 2.5 (blue) and 0.6 (red) s were used. Total data collection times were 7 and 2 hours for samples 1 and 3, respectively.

Table 1

Spin-lattice relaxation (T_1) times obtained from DLPC multilamellar vesicles (with and without copper) containing a uniformly- ^{13}C & ^{15}N -labeled rabbit cytochrome-b5 using CPMAS experiments at 100 K. Proton T_1 values measured for selected ^{13}C resonances demonstrate the paramagnetic effect from a copper-chelated DMPE lipid.

^{13}C resonance (ppm)	DLPC MLVs (s)	DLPC MLVs + 1 mol% Cu^{2+} DMPE-DTPA (s)
175	1.32	0.92
156	3.14	0.82
136	3.46	2.49
134	10.95	1.23
131	4.29	0.63
128	2.43	1.04
127.4	10.29	0.52
126.9	2.82	1.86
116	1.17	0.88
71	2.24	1.32
63	1.84	1.22
55	1.32	0.87
41	1.49	0.87
33	1.74	1.26
26	1.45	1.09
15	5.06	1.69

Errors estimated for the reported T_1 values range from 0.01 to 0.25.