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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.

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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

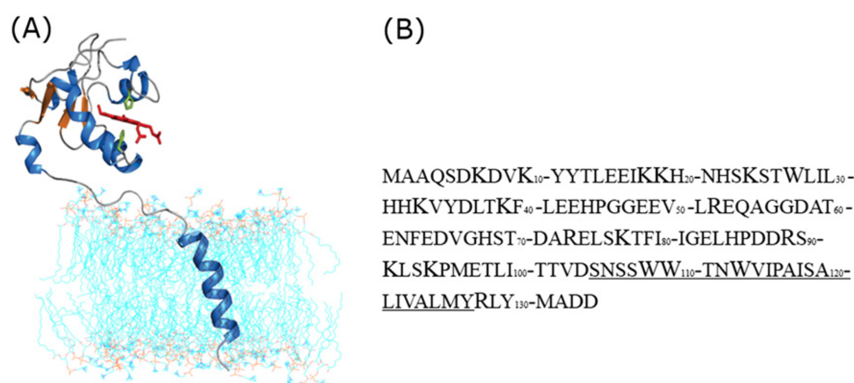

Fig. 1. Structure of full-length cytochrome b5. (A) High-resolution NMR structure of rabbit cytochrome b5 in lipid bilayers is composed of three distinct domains: the transmembrane α-helix at the C-terminus and the heme-containing catalytic soluble domain are connected by a flexible linker region. (B)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

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