Journal of Magnetic Resonance 242 (2014) 169–179

Contents lists available at ScienceDirect

Journal of Magnetic Resonance

journal homepage: www.elsevier.com/locate/jmr

Proton-detected 2D radio frequency driven recoupling solid-state NMR studies on micelle-associated cytochrome-b_5

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ARTICLE INFO

Article history:
Received 25 November 2013
Revised 6 February 2014
Available online 1 March 2014

Keywords:
Membrane protein
Cytochrome-b_5
RFDR
NOESY

ABSTRACT

Solid-state NMR spectroscopy is increasingly used in the high-resolution structural studies of membrane-associated proteins and peptides. Most such studies necessitate isotopically labeled (^{13}C, ^{15}N and ^{2}H) proteins/peptides, which is a limiting factor for some of the exciting membrane-bound proteins and aggregating peptides. In this study, we report the use of a proton-based slow magic angle spinning (MAS) solid-state NMR experiment that exploits the unaveraged ^{1}H–^{1}H dipolar couplings from a membrane-bound protein. We have shown that the difference in the buildup rates of cross-peak intensities against the mixing time – obtained from 2D ^{1}H–^{1}H radio frequency-driven recoupling (RFDR) and nuclear Overhauser effect spectroscopy (NOESY) experiments on a 16.7-kDa micelle-associated full-length rabbit cytochrome-b_5 (cytb_5) – can provide insights into protein dynamics and could be useful to measure ^{1}H–^{1}H dipolar couplings. The experimental buildup curves compare well with theoretical simulations and are used to extract relaxation parameters. Our results show that due to fast exchange of amide protons with water in the soluble heme-containing domain of cyb$_5$, coherent ^{1}H–^{1}H dipolar interactions are averaged out for these protons while alpha and side chain protons show residual dipolar couplings that can be obtained from ^{1}H–^{1}H RFDR experiments. The appearance of resonances with distinct chemical shift values in ^{1}H–^{1}H RFDR spectra enabled the identification of residues (mostly from the transmembrane region) of cytb_5 that interact with micelles.

1. Introduction

Atomic-level characterization of structure and dynamics using solid-state nuclear magnetic resonance (NMR) spectroscopy can provide piercing insights into macromolecular systems, even though it is a challenging task due to low spectral resolution and sensitivity. Significant advances in the development of pulse sequences, ultrahigh field magnets, and a very fast (in the range of 30–40 kHz) to ultra-fast ($\geqslant$60 kHz) magic angle spinning (MAS) techniques with a low radio-frequency decoupling power requirements have opened up new avenues to investigate a variety of biomolecules such as membrane proteins [1–8]. Particularly, the recoupling techniques [9,10] have facilitated an efficient measurement of internuclear distances from both small (uniformly labeled) and large (selectively labeled) biomolecules. Most of these recoupling-based structural studies utilize the homonuclear dipolar couplings among low-abundant nuclei (^{13}C, ^{15}N) due to the larger spread of their chemical shift frequency. On the other hand, homogeneously broadened ^{1}H resonances resulting from strong ^{1}H–^{1}H dipolar interactions have not been suitable to fully utilize the high sensitivity and abundance of protons. However, the availability of ultrafast MAS probes and the use of heavily deuterated samples have made the complete suppression of homonuclear ^{1}H–^{1}H dipolar couplings feasible; this has paved the way for the development of proton-detection based multi-dimensional experiments in the solid state [5,11–22]. Recent studies have also demonstrated the applicability of MAS experiments on soft solids like micelles and fluid lamellar phase bilayers which, unlike rigid solids, exhibit weak ^{1}H–^{1}H dipolar couplings due to their high molecular mobility [23]. In this regard, one particular approach is to employ Radio Frequency-Driven Recoupling (RFDR) [24,25] and Nuclear Overhauser Effect SpectroscopY (NOESY) [26] experiments under MAS to obtain 2D ^{1}H–^{1}H isotropic chemical shift correlation spectra via coherent ^{1}H–^{1}H dipolar coupling and NOE, respectively. This approach has been previously applied to investigate the motional characteristics of resin and membrane-bound peptides [27,28]. A recent study on a membrane-bound antimicrobial peptide

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http://dx.doi.org/10.1016/j.jmr.2014.02.016
1090-7807/

(MSI-78, also commercially known as pexiganan) also highlights the importance of this approach for the measurement of ^{1}H–^{1}H residual dipolar couplings [29]. In this study, we extend this objective on a relatively large micelle-associated unlabeled cytochrome-b_5 ($cytb_5$) to gain better insights into its structure and dynamics.

$Cytb_5$ is a 16.7-kDa electron transfer protein consisting of 134 amino acid residues. It is composed of three distinct regions – namely a structured water-soluble paramagnetic ferric low-spin heme-containing domain, a transmembrane helical domain, and an unstructured ~14-residue long linker region [30]. The NMR structure along with the amino acid sequence of the full-length membrane-bound rabbit $cytb_5$ (PDB #2M33) is shown in Fig. 1. The structure contains five α-helices (α1, L14-H20; α2, K39-E43; α3, E49-Q54; α4, T60-V66 and α5, T70-F79), five β-strands (β1, K10-Y12; β2, W27-L30; β3, K33-D36 and β4, G56-D58) and one 3_{10} helix (P86-R89). The flexible linker domain (S90-D104) of $cytb_5$ lacks a defined secondary structure and connects the helical transmembrane and heme domains. $Cytb_5$, in combination with its redox partners such as cytochrome-P450 (cytP450) and cytP450 reductase (CPR), is essential for enzyme kinetics to metabolize 70% of the drugs presently available in the market [31]. In addition, $cytb_5$ is vital to carryout important biochemical reactions such as the synthesis of testosterone and lipids which are important to preserve the cellular integrity [31]. Previous solid-state NMR studies determined the topology of the transmembrane domains of $cytb_5$ [32] and cytP450 [33], reported the drastic difference between the timescales of motions for the soluble and transmembrane domains [34,35], and also characterized the dynamics of side chains [32]. Further, solid-state NMR studies revealed the interaction between the transmembrane domains of $cytb_5$ and cytP450 by using magnetically-aligned bicelles [36]. Recently, we reported the first high resolution structure of a highly dynamic membrane-bound full-length $cytb_5$–cytP4502B4 electron transfer complex (~72 kDa) employing experimentally derived NMR constraints from solution and solid-state NMR techniques, and mutagenesis data [30]. Additionally, amino acid residues in the interacting interface of the $cytb_5$–cytP4502B4 complex and an electron transfer pathway were also identified [30]. These findings were well corroborated with our results based on backbone amide-^{15}N chemical shift anisotropy (CSA) tensors derived from free and P450-bound $cytb_5$ using solution NMR and quantum chemical studies [37–39].

In the present study, we restrict our focus on 2D ^{1}H–^{1}H correlation spectra obtained from the RFDR and NOESY experiments and analyze the intensity difference for the cross-peaks observed from these two MAS experiments. Experimental details and discussion of the NMR structure of micelle-associated $cytb_5$ (PDB #2M33) are described elsewhere [30,40] and will not be discussed further. We believe that the RFDR experiment could be more sensitive compared to the routinely employed solution NMR NOESY experiment as the cross-peak intensities are higher due to additional dipolar recoupling from nearby spins. More importantly, this

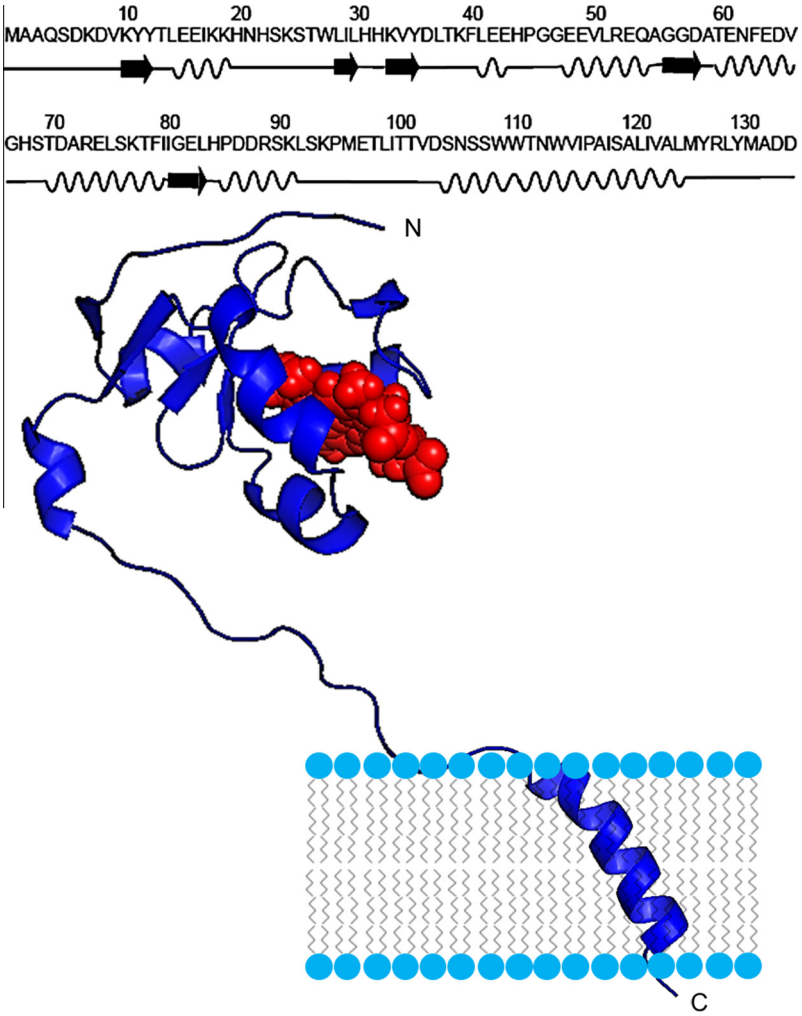

Fig. 1. Amino acid sequence and NMR structure (PDB #2M33) of the full-length membrane-bound rabbit cytochrome-b_5 [30,40].