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^{1}H, ^{13}C and ^{15}N resonance assignments for the full-length mammalian cytochrome b_5 in a membrane environment

Subramanian Vivekanandan[†], **Shivani Ahuja**[†], **Sang-Choul Im**[‡], **Lucy Waskell**[‡], and **Ayyalusamy Ramamoorthy**[†,*]

[†]Department of Chemistry and Biophysics, University of Michigan, Ann Arbor, MI 48109-1055, USA

[‡]Department of Anesthesiology, University of Michigan, and VA Medical Center, Ann Arbor, MI 48105, USA

Abstract

Microsomal cytochrome b_5 plays a key role in the oxidation of a variety of exogenous and endogenous compounds, including drugs, fatty acids, cholesterol and steroid hormones. To better understand its functional properties in a membrane mimic environment, we carried out high-resolution solution NMR studies. Here we report resonance assignments for full-length rabbit cytochrome b_5 embedded in DPC (dodecylphosphocholine) micelles.

Keywords

cytochrome b_5; membrane protein; Heteronuclear NMR

Biological context

Cytochromes b_5 ($cytsb_5$) are ubiquitous electron transport proteins found in plants, animals, fungi and prokaryotic organisms. In eukaryotes, $cytsb_5$ exist as membrane-anchored proteins found either in the endoplasmic reticulum (ER) or in the outer mitochondrial membrane (Schenkman et al 2003). In animal erythrocytes and prokaryotes, $cytsb_5$ are mostly found in a water-soluble form that lacks the C-terminal transmembrane domain (Dürr et al 2007; Vergeres et al, 1995). The isoform of the full-length $cytb_5$, which resides on the cytoplasmic side of the ER membrane (referred to as microsomal $cytb_5$), is a ~16-kDa (134 amino acids), predominantly an acidic membrane protein consisting of two separate domains: a large, N-terminal, cytosolic heme-containing soluble domain (~94 amino acids) that includes the binding site for its redox partners and a C-terminal hydrophobic transmembrane domain (~23 amino acids). These two domains are connected by a proline containing hinge region of ~14 residues referred to as the linker (Clarke et al. 2004). $Cytb_5$ contains a type B heme, which is located in the hydrophobic core of the soluble cytosolic domain, with the highly conserved His68 and His44 coordinating the heme iron as the 5^{th} and 6^{th} ligands (Lederer

*To whom the correspondence should be addressed: Ayyalusamy Ramamoorthy, Department of Chemistry and Biophysics, University of Michigan, Ann Arbor, Michigan 48109-1055, USA, Tel.: (734) 647-6572; Fax: (734) 764-3323; ramamoor@umich.edu.
Subramanian Vivekanandan and Shivani Ahuja equally contributed to this work.

1994). Microsomal cytsb$_5$ participate in a number of key reactions, including fatty acid desaturation (Dürr et al. 2007), the biosynthesis of cholesterol (Schenkman et al. 2003) and sex hormones (Kominami et al. 1992), and the hydroxylation of N-acetyl-neuraminic acid (Takematsu et al. 1994).

High-resolution structures have been determined for the cytosolic, heme-binding domain of truncated, microsomal cytb$_5$ from solution NMR and X-ray crystallography (Banci et al, 2000; Nunez et al. 2010; Durley et al. 1996). Solution NMR studies have reported the dynamics of the truncated cytb$_5$ in solution (Arnesano et al, 2000; Banci et al, 2001). Solid-state NMR studies on magnetically-aligned bicelles containing the full-length rabbit cytb$_5$ reported the fast dynamics (microsecond dynamics) of the soluble domain, slow mobility (millisecond time scale) of the transmembrane domain, and the topology of the transmembrane domain (Dürr et al. 2007b; Xu et al. 2008; Soong et al. 2010; Xu et al. 2010). The tilt angle of the transmembrane helix of cytb$_5$ and its membrane insertion process has been investigated using solid-state NMR and sum frequency generation experiments in phospholipid bilayers (Dürr et al. 2007b; Nguyen et al. 2010). However, the lack of any high-resolution structural data for the full-length cytb$_5$ makes it particularly difficult to establish the molecular mechanism of electron transfer upon its interaction with the monooxygenase, cytochrome P450 (cytP450) (Schenkman et al. 2003; Dürr et al. 2007).

Thus, to elucidate the structure of full-length microsomal cytb$_5$ in a membrane mimetic two- and three-dimensional heteronuclear (^{13}C, ^{15}N) NMR spectroscopy were performed (Ahuja et al. 2013). Here we present the assignment of ^{1}H, ^{13}C, and ^{15}N resonances for cytb$_5$ protein embedded in DPC micelles.

Methods and experiments

C41 cells were purchased from Lucigen (Middleton, MI). U-^{13}C, ^{15}N and ^{2}H CELTONE rich medium, ^{15}N-CELTONE rich media, ^{13}C, ^{15}N-CELTONE rich media, ^{2}H-dodecylphosphocholine (DPC-D$_{38}$), ^{13}C-glucose, ^{15}N-ammonium sulfate and D$_2$O were purchased from Cambridge Isotope Laboratories (Andover, MA). Resins and buffer components were purchased from Sigma-Aldrich. Glycerol for NMR experiments was purchased from Sigma-Aldrich and Roche Applied Science. The NMR samples were placed into 5 mm symmetrical D$_2$O-matched Shigemi NMR microtubes (Shigemi, Inc, Alison Park, PA).

Wild-type rabbit, full-length cytochrome b$_5$ was overexpressed and purified using the protocols described previously (Xu et al. 2008). U-^{15}N cytb$_5$, U-^{15}N, ^{13}C cytb$_5$ and U-^{15}N, ^{13}C, ^{2}H cytb$_5$ were expressed using Celtone-N, Celtone-CN and Celtone-DCN complete media, respectively, with additional supplements as described in reference (Nunez et al. 2010 and Ahuja et al. 2013). Purification of cytb$_5$ was performed as described elsewhere (Mulrooney et al. 2000). Each purified protein exhibited a single band on an SDS PAGE gel.

NMR Spectroscopy

All NMR experiments were performed on a Bruker Avance 900 MHz four-channel NMR spectrometer equipped with an *x*,*y*,*z* axis PFG 5 mm TCI cryoprobe. NMR sample was prepared by reconstituting 0.1 – 0.5 mM $cytb_5$ in 100 mM potassium phosphate buffer containing 5 % deuterated glycerol in the presence of 45 mM perdeuterated DPC (DPC-D_{38}) at pH 7.4.

Two-dimensional TROSY-based ^{1}H-^{15}N (Pervushin et al. 1997) and ^{1}H-^{13}C heteronuclear single-quantum coherence (HSQC) spectra and three-dimensional TROSY based (3D) HNCA, HNCO, HNCACB, HN(CA)CO, HN(CO)CA, ^{15}N- edited TOCSY-HSQC (Sattler et al. 1999) were collected for the backbone chemical shifts assignments. ^{15}N, ^{13}C and ^{2}H labeled protein was used for all the triple resonance backbone NMR experiments. For ^{15}N-HSQC-NOESY and ^{13}C-HSQC-NOESY experiments, uniformly ^{15}N and ^{13}C labeled $cytb_5$ embedded in DPC-D_{38} was used. The 3D-NOESY (with mixing times 80 and 100 ms) experiments were used to confirm the chemical shift assignment in addition to obtaining intra and inter-residue NOEs. All aromatic side chain protons and carbon atoms were assigned using 2D-NOESY and 3D-NOESY experiments. Time to time, several 2D TROSY ^{1}H-^{15}N HSQC spectra were recorded to monitor sample stability. The proton chemical shifts were referenced to the methyl signal of 2,2-dimethyl-2-silapentane-sulfonic acid (DSS, Cambridge Isotope Laboratories) as an internal chemical shift reference at 0.0 ppm. The ^{13}C and ^{15}N chemical shifts were referenced indirectly to DSS (Harris et al. 2001). All the above NMR experiments were performed at 25 C.

All NMR spectra were processed by either NMRPipe (Delaglio et al. 1995) or Topspin 2.0 (Bruker) and analyzed using Sparky (Kneller et al. 1993).

Assignment and Data Deposition

The assigned ^{1}H-^{15}N TROSY-HSQC spectrum for full-length $cytb_5$ is shown in Fig. 1. Using standard three-dimensional solution NMR experiments, NMR resonance assignment was achieved for 88.5% of the backbone and side chain atoms of residues from the soluble heme-binding domain of full-length $cytb_5$ (2D spectral strips illustrating resonance assignments are shown in Figures 2 and 3). Besides three prolines, the unassigned residues in the heme-binding domain of $cytb_5$ include M1-D6, S23, K33, K91, K94, I100, S105, due to their flexibility and rapid solvent exchange with their amide protons. Ambiguous assignments were made for the residues N106, A124, M126, Y127, R128, D133 and D134 due to broad and overlapped peaks in all 3D triple resonance and $^{15}N/^{13}C$-edited 3D-HSQC-NOESY spectra. No backbone assignments were made for the transmembrane domain residues S107-V123, L125, L129, Y130, M131 and A132 as no resonance peaks were identified for these residues in the ^{1}H-^{15}N TROSY-HSQC spectrum of $cytb_5$. The restricted slow (millisecond or slower) motion of the transmembrane domain of $cytb_5$ incorporated in DPC micelles causes significant broadening of the transmembrane domain resonances due to fast spin-spin relaxation. As described in our previous work, a ^{1}H-^{15}N-HMQC spectrum recorded under magic angle spinning (2.5 kHz) on a selectively ^{15}N-alanine labeled sample of $cytb_5$ incorporated in DPC micelles displayed broad resonances for the backbone amide-NHs of the four alanines present in the transmembrane domain of

cytb$_5$, along with narrow resonances for the alanines in the soluble domain (Dürr et al. 2007b; Ahuja et al. 2013, Fig. 4). Hence, static solid-state NMR experiments were performed on uniformly ^{15}N-labeled full-length cytb$_5$ incorporated in magnetically-aligned bicelles - composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) lipids in a 3.5:1 molar ratio - to obtain the structure of the transmembrane domain of cytb$_5$ (Ahuja et al. 2013).

An inspection of the ^{1}H-^{15}N-TROSY-HSQC spectrum (Fig 1) of cytb$_5$ revealed two or more NMR resonances (marked as *) for many of the residues. These two sets of NMR resonances originate from the two isomers (major and minor) of cytb$_5$ that differ by a 180° rotation of the heme plane about the axis that cuts through the *meso*-carbon atoms α and γ (Banci et al. 2000; Zhang et al. 2004). The ratio of the populations of the two isomers can be calculated by determining the peak intensity ratio (here in the ^{1}H-^{15}N-TROSY-HSQC spectrum of cytb$_5$) for identical residues in the two isomeric forms. The major/minor isomer ratio in our study for the full-length rabbit cytb$_5$ was determined to be about 6.6:1 which is similar to the previously obtained 5:1 ratio for truncated rabbit cytb$_5$ (Banci et al. 2000) and nearly identical to the isomer ratio of 6.5:1 for truncated bovine cytb$_5$ (Zhang et al. 2004).

The analysis of ^{15}N-HSQC-NOESY and ^{13}C-HSQC-NOESY reveals that the soluble, heme-binding domain (M1-D89) of cytb$_5$ consists of six α-helices, five β-strands. The linker region (S90-D104) was found to be completely unstructured.

A list of the ^{1}H, ^{13}C and ^{15}N chemical shift values has been deposited into the BioMagResBank (http://www.bmrb.wisc.edu) under accession number BMRB - 18919. Although backbone assignments were done for the resonance peaks of both the major and minor isomers, all the reported assignments in BMRB are only for the major isomer of the ferric full-length microsomal cytb$_5$.

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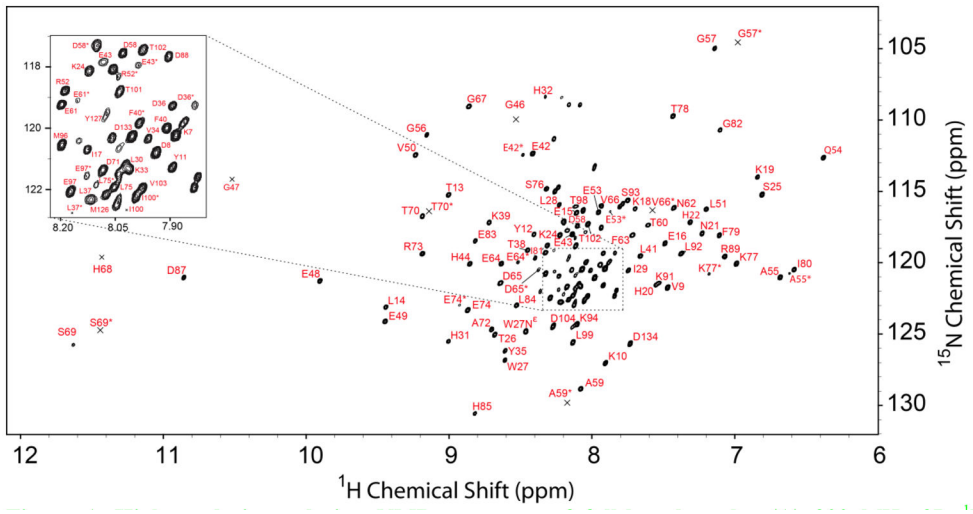

Figure 1. High-resolution solution NMR spectrum of full-length cytb$_5$

(A) 900 MHz 2D ^{1}H-^{15}N-TROSY-HSQC spectrum of a uniformly ^{15}N, ^{13}C and ^{2}H- labeled full-length mammalian cytb$_5$ in NMR buffer at pH 7.4 and 45 mM DPC micelles. The assignments for resolved backbone residues are labeled with one letter amino acid code and residue number. The peaks marked by an asterisk (*) indicate the amino acid residue assignment from the minor population of cytb$_5$ isomer. An expansion of the crowded region of the 2D ^{1}H-^{15}N-TROSY-HSQC spectrum is inserted in the figure for clarity. The low intensity peaks are marked as ‘x’ with residue assignment.

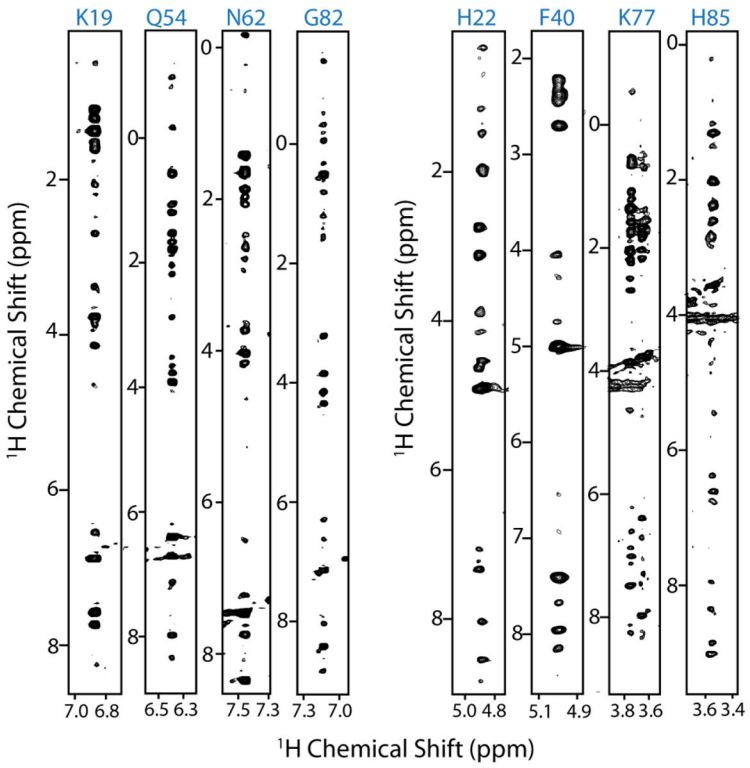

Figure 2.

^{1}H-^{1}H planes of eight different residues extracted from a 3D ^{15}N-edited HSQC-NOESY (mixing time 100 ms)recorded on a fully protonated uniformly ^{13}C and ^{15}N labeled $cytb_5$ in DPC micelles.

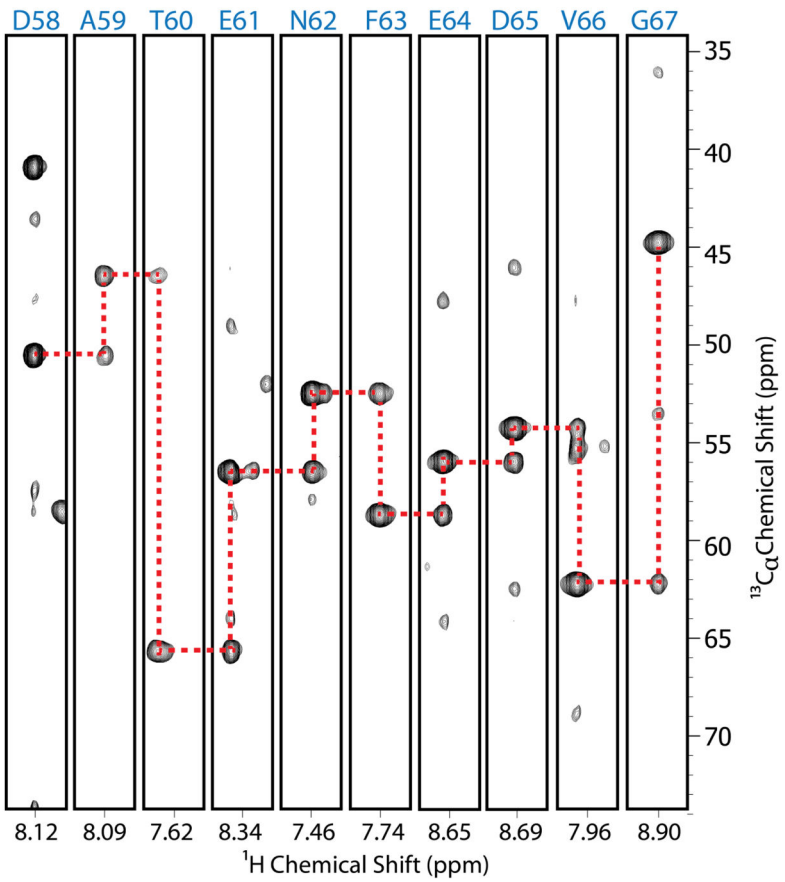

Figure 3.

Strips from HNCA spectrum used to make backbone assignments. Each strip is labeled on top by the amino acid whose NH was detected and at the bottom with the ^{1}HN chemical shift.