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Letter to the Editor: Complete ^{1}H, ^{15}N and ^{13}C assignment of the soluble domain of the *ba*$_3$ oxidase subunit II of *Thermus thermophilus* in the reduced state

Marco D. Mukrasch[a], Christian Lücke[b], Frank Löhr[a], Oliver Maneg[c], Bernd Ludwig[c] & Heinz Rüterjans[a,*]

[a]*Institute of Biophysical Chemistry, Center for Biomolecular Magnetic Resonance, J.W. Goethe-University, D-60439 Frankfurt, Germany;* [b]*Max Planck Research Unit for Enzymology of Protein Folding, D-06120 Halle, Germany;* [c]*Institute of Biochemistry, Molecular Genetics, J.W. Goethe-University, D-60439 Frankfurt, Germany*

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Biological context

Cytochrome *c* oxidase (complex IV) is the final proton-pumping protein complex in the respiratory chain of most aerobic organisms. Under low oxygen partial pressure, the extremely thermophilic, gram-negative eubacterium *Thermus thermophilus* HB8 expresses a heme *ba*$_3$-type oxidase. It consists of only two subunits (I and II), carrying several cofactors, and an additional small subunit IIa of unknown function (Soulimane et al., 2000). Remarkably, this oxidase complex lacks most of the canonical amino acid signatures in its proton translocating pathways, thought to be essential for its function as a proton pump.

The primary electron acceptor of the *ba*$_3$ oxidase is the copper A (Cu_A) center, which is located in the periplasmic domain of subunit II. The Cu_A center of *T. thermophilus* consists of two copper ions (Williams et al., 1999), which are bridged by two sulfur atoms from cysteine residues Cys117 and Cys121 (the numbering refers to the soluble Cu_A domain). Each of the copper ions is further liganded by an imidazole ring (His82 and His125); one copper ion is coordinated to a methionine side-chain (Met128), the other one to the backbone oxygen of a glutamine residue (Gln119). In the oxidized state, the Cu_A center is paramagnetic with a formal oxidation number of +1.5 for each copper atom due to charge delocalization. Upon reduction, the purple protein solution becomes colorless and the charge of each copper ion is +1.

In order to facilitate the functional und structural investigation of the *ba*$_3$ oxidase from *T. thermophilus*, the soluble Cu_A domain (14.8 kD) of subunit II has been expressed heterologously without the N-terminal membrane-anchor region (Slutter et al., 1996). Here we report the resonance assignment of this 136 amino acid protein fragment in the reduced state.

*To whom correspondence should be addressed. E-mail: hruet@bpc.uni-frankfurt.de

Methods

The soluble Cu_A domain was expressed in *E. coli* cells BL21(DE3)pLysS. The expression vector pMA10, encoding the Cu_A fragment, is based on the pET-3a vector. The labeling strategy was performed according to Markley and Bracken (2001). Cells were grown at 37 °C in 1 L LB medium to an OD_{600} = 1.0, centrifuged, then washed in M9 minimal medium lacking any nitrogen and carbon sources and transferred into 250 ml M9 medium supplemented with 250 mg $^{15}NH_4Cl$ and 250 mg ^{13}C-glucose. After incubation for 30 min at 37 °C, another 750 mg ^{13}C-glucose were added, expression induced with 0.4 mM IPTG, and cells harvested 7 h later. Purification was performed essentially as previously published (Slutter et al., 1996), yielding a total of 6.8 mg purified $^{13}C/^{15}N$-labeled Cu_A fragment.

For the NMR measurements, the protein was transferred into 20 mM potassium phosphate buffer (pH 6.0) containing 100 μM protease inhibitor (Pefabloc, Biomol, Hamburg), kept under an argon atmosphere after degassing, and reduced by addition of excess ascorbate. The final protein concentrations of the ^{15}N- and $^{13}C/^{15}N$-labeled Cu_A samples were 1.2 mM and 0.9 mM, respectively.

All NMR experiments were performed at a temperature of 25 °C using a Bruker DMX 600 spectrometer equipped with a three-axis gradient $^{1}H\{^{15}N$,

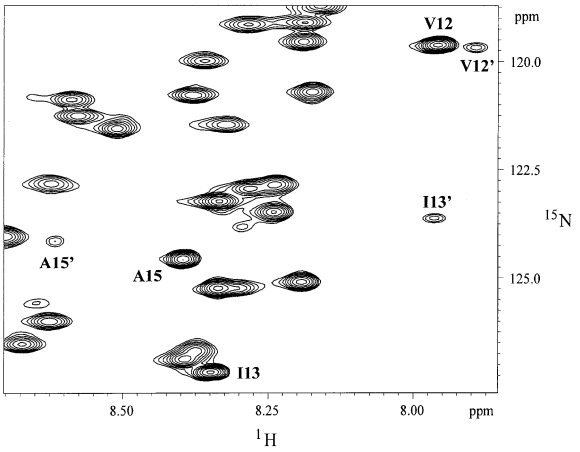

Figure 1. Part of the [^{15}N, ^{1}H]-TROSY spectrum (^{1}H resonance frequency 600.13 MHz, 25 °C, pH 6.0) of the soluble Cu_A domain from *Thermus thermophilus*. Residues Val12, Ile13 and Ala15 show two separate spin systems due to the occurrence of both the *trans* and *cis* isomer form of Pro14. The smaller peaks (labeled with a prime) are associated with Pro14′ in the *cis* conformation, representing approximately 8–9% of the total Cu_A concentration.

^{13}C} triple-resonance probe. The following multidimensional spectra were collected: 2D [^{15}N, ^{1}H]-TROSY, 3D [^{15}N, ^{1}H]-TROSY-HNCO, 3D [^{15}N, ^{1}H]-TROSY-HNCACB (Salzmann et al., 1999), 3D (H)CC(CO)NH-TOCSY, 3D H(CC)(CO)NH-TOCSY, 3D (HACA)CO(CA)NH, 3D (HM)CMC(CM)HM (Gardner et al., 1998), 2D CT-[^{13}C, ^{1}H]-HSQC, 2D [^{15}N, ^{1}H]-TROSY-H(N)C^{ar}, 3D [^{13}C, ^{1}H]-TROSY-HCD(CG)CB (Löhr et al., 2002) and 2D [^{15}N, ^{1}H]-HMBC. All spectra were referenced to internal DSS (Wishart et al., 1995).

NMR data were acquired and processed with the program XWINNMR 2.6 (Bruker) and analyzed with AURELIA 2.7.5 (Bruker). For secondary structure prediction, the chemical shift values of the H^{α}, C^{α}, C^{β} and C′ nuclei were entered into the CSI program (Wishart and Sykes, 1994).

Results

Nearly the entire resonance assignment of the Cu_A fragment was obtained, despite the large number of proline residues (11 out of 136 amino acids) complicating the sequential connectivities. Secondary structure prediction with the CSI program indicated mostly β-strand conformation between residues 19–134, which is in good agreement with the crystal structure of subunit II (PDB entry 1EHK). Several short helical segments at the N-terminus, however, are not indicated in the CSI results, possibly as a consequence of the transmembrane anchor cleavage.

Some amino acids showed multiple spin systems. In particular, for residues Val12, Ile13, Pro14 and Ala15 a second set of spin systems with lower intensities (referred to as Val12′, Ile13′, Pro14′ and Ala15′) was identified (Figure 1), which could be connected sequentially as well. Apparently, this proline 14 residue, which is located in a structurally flexible region at the N-terminal end, can reach a thermodynamical equilibrium between its *trans* and *cis* isomer forms, thereby influencing also the neighboring residues. Based on chemical shift statistics (Schubert et al., 2002), the C^{β} and C^{γ} resonances indicated that Pro14 and Pro14′ represent the *trans* and *cis* conformation, respectively. The signal intensities in the 2D [^{15}N, ^{1}H]-TROSY spectrum (Figure 1) suggest that 8–9% of the Cu_A molecules were present in *cis* conformation under the applied experimental conditions.

Extent of assignments and data deposition

The ^{1}H, ^{15}N and ^{13}C resonances of the Cu_A fragment in the reduced state have been assigned almost completely. The first two N-terminal residues (Met1, Ala2) appear to be cleaved off enzymatically in the cell after translation, leading to a free N-terminus at Tyr3. However, some side-chain amides as well as the Phe and Tyr ring resonances remain unassigned; and no backbone amide signal could be detected for Asn45. The sequence-specific resonance assignment has been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) database under accession number BMRB-5819.

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