# The $Cu_A$ Center of a Soluble Domain from *Thermus* Cytochrome $ba_3$. An NMR Investigation of the Paramagnetic Protein

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The $Cu_A$ center[1] in subunit II of cytochrome *c* oxidase, the terminal enzyme of aerobic respiration, transfers electrons from cytochrome *c* to the proton-pumping machinery in subunit I.[2] The unique electronic absorption and EPR spectra of $Cu_A$ exclude it from classification with the well-studied biological copper centers.[3] High-resolution X-ray structures of $Cu_A$-containing proteins[4] reveal two copper atoms approximately 2.5 Å apart, bridged by two cysteine sulfurs. Each Cu has a terminal histidine ligand and a weak ligand, methionine for one and a main chain carbonyl for the other. These structures are consistent with earlier EPR measurements[5] and theoretical calculations,[6] which predicted a highly delocalized mixed-valence [Cu(II),Cu(I)] $Cu_A$ site. Here we report $^1$H NMR measurements at 600 MHz on a soluble $Cu_A$ domain from *Thermus thermophilus* cytochrome $ba_3$.[7]

The NMR spectra (Figure 1)[8,9] display several remarkably narrow lines due to the very short relaxation time of the $Cu_A$ site.[10] Twelve sizably hyperfine-shifted signals are observed at pH 8, and 13 are at pH 4.6, all of them of intensity 1. There are three very broad signals (a−c) clustered between 250 and 300 ppm and a fourth broad signal (d) at 105 ppm, with $T_1$ values of 1 ms or less, six (seven) relatively sharp signals in the 35−15 ppm region with $T_1$ values between 2 and 17 ms, and two upfield-shifted signals with $T_1$ values of 5 and 1 ms (Table 1). Signal i is absent at pH 8, and its intensity at pH 4.5 decreases with increasing temperature. Signals h, i, and z disappear in $D_2O$, with the disappearance of signal z occurring over a period of several days at room temperature. All signals except b, d, and g show normal Curie behavior (Figure 1C). Signal d definitely shows anti-Curie behavior, while signals b and g have almost no temperature dependence.

The 4 ms mixing 2D NOESY spectrum (Supporting Information) shows dipolar connectivities between signals e−h, f−i, and i−k. The last one is barely observable, probably due to the short $T_1$ of signal k. Furthermore, additional connectivities between signal j and signals h and e are detected through 1D NOE. In fact, properly performed 1D NOEs (i.e., saturating the faster-relaxing signal of the pair so as to observe NOE on the slower-relaxing signal) provide larger sensitivity than 2D NOESY.

The above connectivities, together with the relative values of the $T_1$ values and the exchangeable nature of the protons giving signals h and i, led to the assignment of signals e, h, and j as belonging to one coordinated histidine and of signals f, i, and k as belonging to the other coordinated histidine. Signals h and i arise from the two NH protons, signals e and f from the H$\epsilon$2, and signals j and k from the H$\delta$1. The NOE pattern definitely shows coordination of both histidines through their N$\delta$1 nitrogens, as observed in the available structures of homologous systems.[4] By referring to the structure of $Cu_A$ from *Paracoccus,* it appears that one histidine (His181, corresponding to His145 in our fragment[11]) has an NH H-bonded to the peptide carbonyl of Trp121 or Asp178, while the other histidine (His224, corresponding to His158 in our fragment[11]) has its NH exposed to solvent. This indicates that the proton associated with signal i, which exchanges relatively rapidly, belongs to His224. Therefore, the sequence-specific assignment of the histidine signals follows, as reported in Table 1.

Attempts to observe NOEs from signals a−c, which are about $10^4$ Hz broad, were unsuccessful. Signal d shows a clear NOE to signal g, which does not show any other dipolar connectivity with hyperfine-shifted signals. The large hyperfine shifts, line widths, and $T_1$ values of signals a−d indicate that they belong to $\beta$-$CH_2$ protons of the coordinated cysteines. Indeed, specific deuteration of the $\beta$-$CH_2$ protons of the cysteines gives $Cu_A$ domain samples missing signals a−d. Signals b, d, and g share an anomalous temperature dependence (Figure 1C). Their relative line widths and the NOE connectivity between d and g allow us to assign signals b and d as the $\beta$-$CH_2$ protons of one of the two cysteines and signal g as the corresponding $\alpha$ proton.

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(8) Samples of the $Cu_A$ domain were prepared in San Diego according to Slutter et al. [see ref 7], and its purity checked from the 280/530 nm absorption ratio. NMR samples were 2−3 mM protein in 0.1 M ammonium chloride at pH 4.5 or in 0.1 M potassium phosphate buffer at pH 8.0. Samples in $D_2O$ were obtained by exchange in Centricon 10 from Amicon. One sample had the $\beta$-$CH_2$ protons of cysteine deuterated.

(9) The NMR spectra were run on a Bruker AMX 600 instrument. $T_1$ measurements were performed using the nonselective inversion−recovery method. Different carrier frequencies were used to cover different regions of the spectrum with the 180° pulse. 1D NOE difference spectra were obtained as previously described [Banci, L; Bertini, I.; Luchinat, C.; Piccioli, M.; Scozzafava, A.; Turano, P. *Inorg. Chem.* **1989**, *28*, 4650−4656. Bertini, I.; Luchinat, C. *NMR of Paramagnetic Substances*; Elsevier: Amsterdam, 1996].

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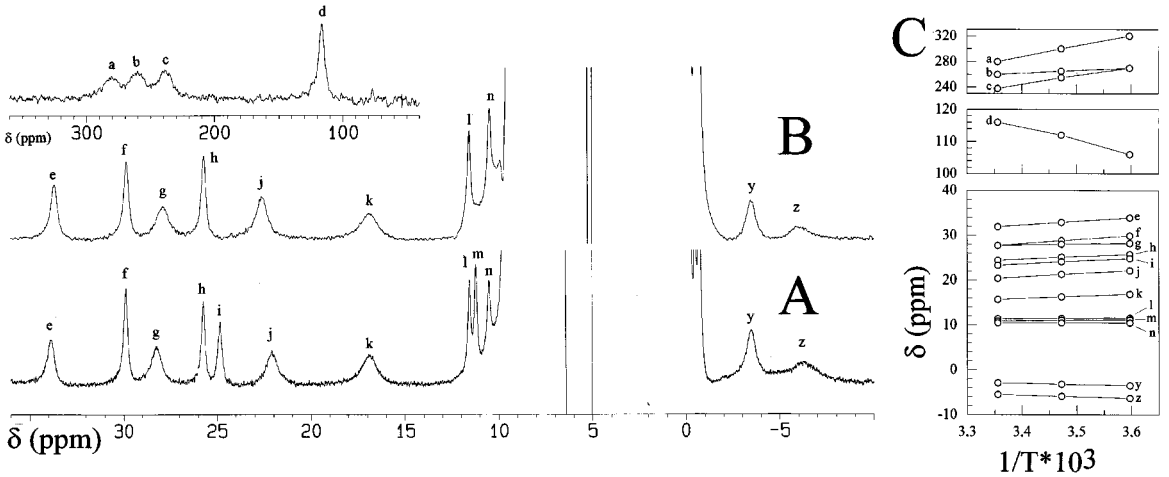


**Figure 1.** 600 MHz $^1$H NMR spectra of water solutions of the *Thermus* $Cu_A$ domain at pH 4.5 (A) and pH 8 (B) at 278 K. The inset shows the far-downfield region of the pH 8 spectrum recorded in $D_2O$ at 298 K. Part (C) shows the $1/T$ dependence of the chemical shifts of the hyperfine-shifted signals at pH 8 (a−d) and pH 4.5 (e−z).

**Table 1.** Chemical Shift at 298 K, Temperature Dependence, and Assignment of the Hyperfine-Shifted Signals of the *Thermus* $Cu_A$ Domain at pH 8.0[a]

| signal | δ (ppm) | *T* dependence | assignment |
|---|---|---|---|
| a | 280 | Curie | Cys H$\beta$ |
| b | 260 | *T* independent | Cys′ H$\beta$ |
| c | 238 | Curie | Cys H$\beta$ |
| d | 116 | anti-Curie | Cys′ H$\beta$ |
| e | 31.7 | Curie | His181 H$\delta$2 |
| f | 27.7 | Curie | His224 H$\delta$2 |
| g | 27.4 | *T* independent | Cys′ H$\alpha$ |
| h | 24.4 | Curie | His181 H$\epsilon$2 NH |
| i | 23.3[b] | Curie | His224 H$\epsilon$2 NH |
| j | 21.1 | Curie | His181 H$\epsilon$1 |
| k | 15.7 | Curie | His224 H$\epsilon$1 |
| l | 11.4 | Curie | NH |
| m | 10.9[b] | Curie | NH |
| n | 10.5 | Curie | NH |
| y | −2.8 | Curie | His H$\beta$(?) |
| z | −5.1 | Curie | NH |

[a] Residue numbering as in the *Paracoccus* $Cu_A$ domain. [b] Chemical shifts were measured at pH 4.5.

It follows that signals a and c must be due to the $\beta$-$CH_2$ protons of the other cysteine.

Exchangeable signals l, m, and z are assigned to NH protons close to the $Cu_A$ domain, and signal y tentatively assigned to a $\beta$-$CH_2$ proton of a coordinated histidine.[12]

The NMR relaxation data obtained on the $Cu_A$ domain are consistent with room temperature electron relaxation rates of about $10^{11}$ $s^{-1}$, compared to $10^8$−$10^9$ $s^{-1}$ in mononuclear copper(II).[13] Such high relaxation rates have been observed for spin-coupled (triplet) copper(II)[14] as well as spin-coupled copper(II)−cobalt(II) complexes,[12] but it is a first for a spin-doublet copper system. We suggest that two low-lying doublets of binuclear $Cu_A$ are responsible for the rapid electron relaxation; these doublets would be spaced by $\leq 6kT$ for an Orbach process or somewhat more for a Raman process.[13] The two doublets would involve the orbitals of the two sulfur atoms to different extents to account for the different temperature dependence of the proton signals of the two cysteines, in a way similar to that proposed for low-spin cytochromes.[15] By lowering the temperature, one of the two doublets would be depopulated.

The similar hyperfine shifts displayed by the ring proton resonances of the two histidines demonstrate that the unpaired electron is delocalized over two nearly equivalent copper centers. The large hyperfine shifts of the $\beta$-$CH_2$ cysteine proton resonances confirm that there is extensive electron delocalization onto the cysteine S atoms in $Cu_A$, as originally suggested by Peisach and Blumberg[16] and supported by subsequent EPR[5] and ENDOR[17] studies.

The highly delocalized, mixed-valence structure of the $Cu_A$ site has clear functional advantages. It means that an electron entering this site from cytochrome *c* is directly coupled across the [$Cu(Cys)_2Cu$] unit of the binuclear center to the Cu(His) origin of the tunneling pathway to cytochrome *a*.[18] In addition, the nuclear reorganization associated with electron transfer is considerably reduced in a delocalized binuclear site,[6a] as required to account for the observed *c* to $Cu_A$ and $Cu_A$ to *a* rates.[18] Our NMR work, therefore, shows that the electronic structure of $Cu_A$ is ideal for a mediator of electron flow from cytochrome *c* to the proton-pumping unit of cytochrome oxidase.

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**Supporting Information Available:** A 4 ms 2D NOESY spectrum at pH 4.5 and 278 K (2 pages). See any current masthead page for ordering and Internet access instructions.

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(12) Signal *z* is possibly shifted upfield by pseudocontact coupling. Signals (l and m) experience a weak Curie temperature dependence and are thus also in the vicinity of the copper center. Signal m disappears in $H_2O$ at pH 8 and exchanges immediately in $D_2O$, while signal l belongs to a proton that is only exchangeable in $D_2O$ over a period of hours. Therefore, both are probably backbone NH protons that are near the copper site, the one giving rise to signal l being in a solvent-shielded region. Signal y is shifted upfield and gives a strong NOESY connectivity with a broad signal at 6.8 ppm. The $T_1$ value of signal y, and the line width of this signal and of that at 6.8 ppm, allow us to tentatively assign these two signals to the $\beta$-$CH_2$ of one of the histidines. A $\beta$-$CH_2$ proton of a copper(II)-coordinated histidine has already been observed to be shifted upfield in CuCo−superoxide dismutase [Bertini, I.; Luchinat, C.; Piccioli, M. *Prog. Nucl. Magn. Reson. Spectrosc.* **1994**, *26*, 91−141].

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