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View Article Online
DOI: 10.1039/C9CC08883K

COMMUNICATION

# Unexpected electron spin density on the axial methionine ligand in $Cu_A$ suggests its involvement in electron pathways

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

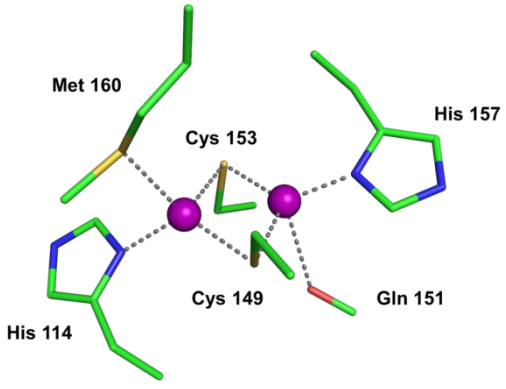
Marcos N. Morgada,†[ab] María-Eugenia Llases,†[a] Estefanía Giannini,[a] María-Ana Castro,[c] Pedro M. Alzari,[d] Daniel H. Murgida,[c] María-Natalia Lisa[ae] and Alejandro J. Vila [abe]



**The $Cu_A$ center is a paradigm for the study of long-range biological electron transfer. This metal center is an essential cofactor for terminal oxidases like Cytochrome *c* oxidase, the enzymatic complex responsible for cellular respiration in eukaryotes and in most bacteria. $Cu_A$ acts as an electron hub by transferring electrons from reduced cytochrome *c* to the catalytic site of the enzyme where dioxygen reduction takes place. Different electron transfer pathways have been proposed involving a weak axial methionine ligand residue, conserved in all $Cu_A$ sites. This hypothesis has been challenged by theoretical calculations indicating the lack of electron spin density in this ligand. Here we report an NMR study with selectively labeled methionine in a native $Cu_A$. NMR spectroscopy discloses the presence of net electron spin density in the methionine axial ligand in the two alternative ground states of this metal center. Similar spin delocalization observed on two second sphere mutants further supports this evidence. These data provide a novel view of the electronic structure of $Cu_A$ centers and support previously neglected electron transfer pathways.**

Copper ions are essential for electron transfer (ET) in all living organisms.[1] This biological function is performed by copper centers in proteins with unique electronic features: the mononuclear Type 1 (blue) sites and the binuclear $Cu_A$ (purple) centers.[2, 3] These sites share some common features: a rigid structure that minimizes the reorganization energy, highly covalent copper-thiolate bonds with cysteine ligands (one or two, respectively in these sites) and two equatorial histidine ligands that maintain the geometry of the metal sites (Figure 1).[4-6] Type 1 and $Cu_A$ centers also share the presence of weak axial ligands, whose role in fine tuning the electronic structure and functionality of these sites has been intensely discussed, particularly in the blue copper sites.[7-13] The most common axial ligand in blue copper sites is a methionine residue, with variable Cu-S(thioether) distances ranging from 2.6 to 3.0 Å, that have also been shown to tune the reduction potential and the electronic structure of the metal site.[14] The nature of the axial ligand in Type 1 copper sites is variable, and a glutamine residue or no axial ligand can be found in these centers.[15-17] In the case of $Cu_A$ centers, a Met residue and a peptide bond are the conserved weak axial ligands in all known sites, except for the recently characterized PmoD-$Cu_A$, with two Met axial ligands.[18, 19] The role of the Met axial ligand in the $Cu_A$ site has also been matter of debate.[13] There is a general consensus supporting that the main role of this ligand is to tune the reduction potential[11] while preserving the reorganization energy, as shown by Solomon and coworkers.[12, 20] The involvement of this residue in ET pathways, however, is more controversial.[12, 13]

**Figure 1**. Structure of the $Cu_A$ site. Taken from the crystal structure of the soluble domain from the $ba_3$ oxidase from *Thermus thermophilus* (PDB 2CUA).




[a] Instituto de Biología Molecular y Celular de Rosario (IBR, CONICET-UNR), Ocampo y Esmeralda, Rosario 2000, Argentina.
[b] Área Biofísica, Departamento de Química Biológica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, Rosario 2000, Argentina
[c] Instituto de Química Física de los Materiales, Medio Ambiente y Energía (INQUIMAE), Departamento de Química Inorgánica, Analítica y Química Física, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires and CONICET, Buenos Aires 1428, Argentina
[d] Institut Pasteur, Unité de Microbiologie Structurale, CNRS UMR 3528 - Université Paris Diderot, Paris 75015, France
[e] Plataforma de Biología Estructural y Metabolómica (PLABEM), Ocampo y Esmeralda, Rosario 2000, Argentina
† MNM and MEL contributed equally to this work.
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

The electronic structure of $Cu_A$ sites can be described by a double-well potential with two partially populated electronic states in thermal equilibrium: $\sigma_u$* and $\pi_u$ (separated by a small energy gap of 600 $cm^{-1}$).[12, 20, 21] A stronger interaction with the axial ligand decreases this gap, making the $\pi_u$ ground state more accessible. It has been proposed that this phenomenon could increase the superexchange coupling and favor an ET pathway through this position.[12, 22] Two independent ET pathways simulations based on known cytochrome *c* oxidase (CcO) structures support the involvement of the Met ligand as the electron entry port to the $Cu_A$ site.[23, 24] In the case of the *T. thermophilus caa*$_3$ CcO, ET to the $Cu_A$ site is intramolecular, since there is a cytochrome *c* domain fused to the $Cu_A$-containing subunit COX II, with an ET pathway of 18.8 Å from the heme *c* to $Cu_A$ involving the axial Met ligand.[23] A more recent structure of the bovine cytochrome *c*-CcO bovine complex also suggested a preferred pathway to the oxidized $Cu_A$ site through the Met ligand.[24] The feasibility of these pathways depends on the strength of the metal-ligand interaction, that has a direct impact in the superexchange coupling between the donor and acceptor sites ($H_{DA}$).[25-28] However, at the moment there are no evidences of electron spin density in the axial Met ligand, ruling out possible ET pathways through this residue.[13]

NMR spectroscopy has disclosed the presence of electron density into the Met ligand in several blue copper proteins as witnessed by the finding of non-null contact shifts in the HΥ or in the ε-$CH_3$ of this residue.[29-32] In contrast, no hyperfine-shifted signals from the Met ligand in $Cu_A$ centers have been detected.[21, 33-35] **Here we report the presence of electron spin density in $^1$H and $^{13}$C nuclei of the Met ligand in both the $\sigma_u$* and $\pi_u$ states by NMR studies of specifically labeled $Cu_A$ samples. These results support the feasibility of ET pathways involving this axial ligand in both alternative ground states.**

To map the electron spin density on the Met axial ligand, we expressed the soluble $Cu_A$-containing domain of the *ba*$_3$ oxidase from *Thermus thermophilus* (*Tt* $Cu_A$ hereafter) with the Met residue selectively labeled in $^{13}$C.[36] Slutter et al. have shown that EPR and near-IR features of this $Cu_A$ domain are similar to those of the full oxidase.[37] Indeed, the crystal structures of the soluble domain (PDB ID: 2CUA) and the full oxidase (PDB ID: 1EHK) show an identical geometry of the metal center and its environment. This allows us to extrapolate our results to the full complex.

A $^1$H,$^{13}$C HMQC spectrum of oxidized *Tt* $Cu_A$ disclosed a $^{13}$C signal at 31.3 ppm coupled with two protons at 20.7 and 7.8 ppm (Figure 2A). The former $^1$H resonance has been previously assigned to a Hε1 of His114.[21, 33] The current data show that these resonances correspond to a $CH_2$ moiety from the axial Met ligand. To further corroborate this assignment, we performed 1D and 2D NOE experiments, that revealed a strong dipolar coupling between these two proton resonances, confirming that they correspond to geminal protons (Figure 2B). We assign these resonances to the Υ-$CH_2$ moiety from Met160, since these protons are three bonds away from one of the copper ions. The signal located at 20.7 ppm had less intense NOEs with other resonances. One of them shows a

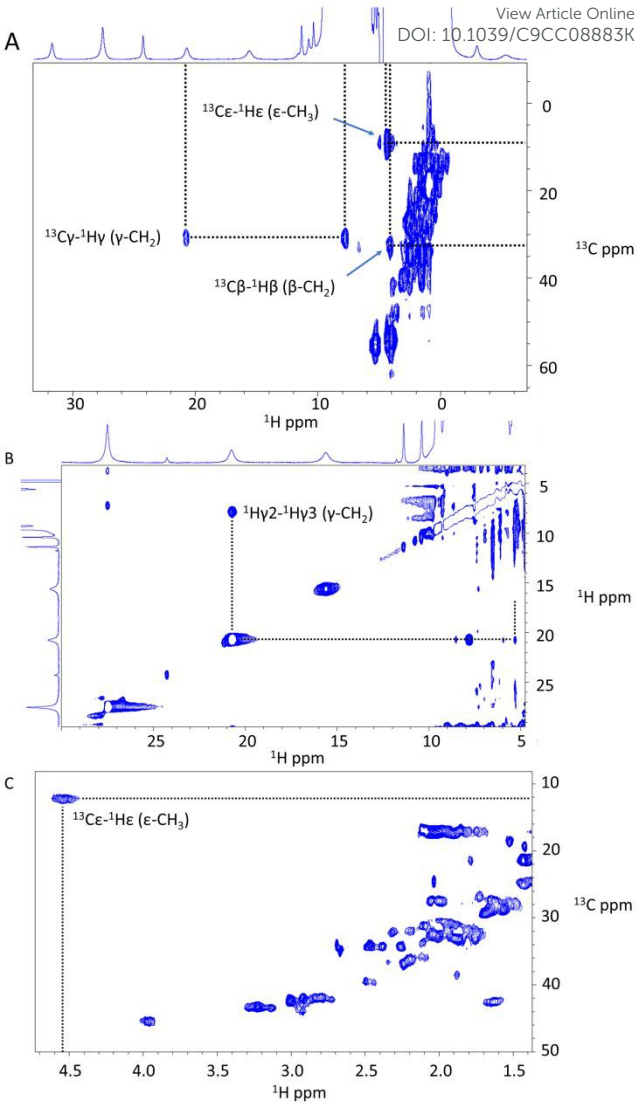



**Figure 2.** (A) $^1$H$^{13}$C HMQC spectrum of *Tt* $Cu_A$ with $^{13}$C-labeled Met. The cross peaks in the diamagnetic region are attributable to natural $^{13}$C abundance and to some level of labeling scrambling. (B) NOESY spectrum of unlabeled *Tt* $Cu_A$ recorded with a mixing time of 4 ms. (C) $^1$H$^{13}$C HMQC spectrum of *Tt* $Cu_A$ with ε-$^{13}CH_3$ labeled Met.

correlation in the HMQC spectrum with a $^{13}$C located at 32.9 ppm, that we assign to a β-$CH_2$ moiety from the Met ligand. The geminal proton of this signal could not be clearly identified in the diamagnetic region. The chemical shifts of these protons are less distant from the diamagnetic region. To complete the assignment, we then prepared a sample with the ε-$^{13}CH_3$ labeled Met.

The HMQC spectrum of this sample revealed a correlation between signals at 12.5 ppm ($^{13}$C) and 4.54 ppm ($^1$H) (Figure 2C), *i.e.*, both considerably shifted from the assigned diamagnetic resonances for the $CH_3$ group of the Met ligand in the reduced form (25.8 and 1.91 ppm, respectively).[38] Overall, these findings strongly suggest the presence of electron spin density in the Met160 sidechain. Since most of these signals fall within the diamagnetic region in uniformly labeled samples, none of these assignments could have been identified without resorting to this selective labeling strategy.

The observed hyperfine shifts ($\delta_{obs}$) in paramagnetic compounds have different contributions: the diamagnetic contribution to the chemical shift of the nucleus without the effect of the unpaired electron (in this case, $\delta_{dia}$ corresponds to reduced $Cu_A$), the contribution to the chemical shift due to the dipolar coupling between the nuclei and the magnetic moment of the unpaired electron ($\delta_{pc}$) and the contribution from the Fermi contact shift due to non-null electron spin density in the nucleus ($\delta_{con}$).[39] $\delta_{con}$ can be calculated from equation 1 (See details in supplementary material):

$$\delta_{con} = \delta_{obs} - \delta_{dia} - \delta_{pc} \qquad (1)$$

In general, the low magnetic anisotropy of $Cu_A$ sites leads to the assumption that the $\delta_{pc}$ term is negligible.[40] However, since we are dealing with nuclei with small hyperfine shifts and close to the copper site, the pseudocontact component that depends on the distance of the nuclei to the paramagnet may not be disregarded. Based on the structure available for this protein,[4] we calculated the pseudocontact contribution to the isotropic shifts and, therefore the contact contributions for all nuclei. As shown in Table 1, all $^{13}C$ and $^{1}H$ nuclei from Met160 (except for the Cβ) have non-negligible contact shifts in *Tt* $Cu_A$. The largest contact contributions can be located to the ε-$CH_3$ and Υ-$CH_2$ moieties, that are next to the copper binding $S_\delta$(Met) atom.

The $\delta_{con}$ of three out of the five resonances from the Met residue showed a non-Curie temperature dependence, suggesting that this shift reflects an averaged contribution of both the $\sigma_u$* and $\pi_u$ states (Figure S1). Thus, in principle both states can present net spin density on the Met axial ligand. Aiming to complete the interpretation of the NMR data, we ran QM/MM and QM (DFT) calculations based on the available crystal structure (Figure 3). These calculations indeed confirm the presence of electron density in the Met ligand (Figure 3 and Table S1) and allowed a stereospecific assignment of the geminal $H_Y$'s. Comparison of the $\delta_{con}$ with the calculated spin densities allows us to confirm that the proton at 20.7 ppm corresponds to the HY2 and the resonance at 7.8 ppm to the geminal $H_Y$3 (Figure 2 and table S3).

**Table 1.** Observed and contact shifts of $^{1}H$ and $^{13}C$ nuclei from the Methionine ligand in the three $Cu_A$ variants at 298K compared to shifts in Type 1 proteins.

| | $Cu_A$ sites | | | Type 1 Proteins | | | |
|---|---|---|---|---|---|---|---|
| **Protein** | *Tt*$Cu_A$ | Tt3Lh | Tt3L*At* | PsAz[a] | Rc[b] | Pc[c] | Ami[d] |
| **S(Met)-Cu distance [Å][e]** | 2.47[f] | 2.51[g] | 2.52[g] | 3.10 | 2.92 | 2.88 | 2.76 |
| | 2.46 | 2.48 | 2.59 | | | | 2.88 |
| **$\delta_{obs}$ HY2 [ppm]** | 20.7 | 19.8 | 19.5 | --[h] | --[h] | 23.5 | 12.0 |
| **$\delta_{con}$ HY2 [ppm]** | 16.7 | 15.9 | 15.6 | --[h] | --[h] | 19.9 | 7.3 |
| **$\delta_{obs}$ HY3 [ppm]** | 7.8 | 8.8 | 8.7 | --[h] | --[h] | 13.0 | 11.1 |
| **$\delta_{con}$ HY3 [ppm]** | 5.3 | 6.3 | 6.1 | --[h] | --[h] | 8.6 | 6.7 |
| **$\delta_{obs}$ Hε [ppm]** | 4.5 | 3.3 | 3.2 | 12.1 | 8.10 | --[h] | --[h] |
| **$\delta_{con}$ Hε [ppm]** | 3.5 | 2.2 | 2.0 | 10.5 | 6.0 | --[h] | --[h] |
| **$\delta_{obs}$ CΥ [ppm]** | 31.3 | 35.5 | 34.0 | --[h] | --[h] | --[h] | --[h] |
| **$\delta_{con}$ CΥ [ppm]** | -7.00 | -2.8 | -4.4 | --[h] | --[h] | --[h] | --[h] |
| **$\delta_{obs}$ Cε [ppm]** | 12.5 | 14.5 | 12.2 | --[h] | --[h] | --[h] | --[h] |
| **$\delta_{con}$ Cε [ppm]** | 12.4 | -10.4 | -12.9 | --[h] | --[h] | --[h] | --[h] |
| **$\delta_{obs}$ Cβ [ppm]** | 32.9 | 32.9 | 33.0 | --[h] | --[h] | --[h] | --[h] |
| **$\delta_{con}$ Cβ [ppm]** | 0.0 | 0.0 | 0.2 | --[h] | --[h] | --[h] | --[h] |

[a]Pseudoazurin, from reference 31, [b]Rusticyanin from reference 33, [c]Plastocyanin from ref 30, [d]Amicyanin from reference 29, [e]The distances correspond to the two molecules in the asymmetric unit, [f]Soluble subunit from $ba_3$ oxidase from *Thermus thermophilus*, from ref. 4, [g]This work, [h]Not available.

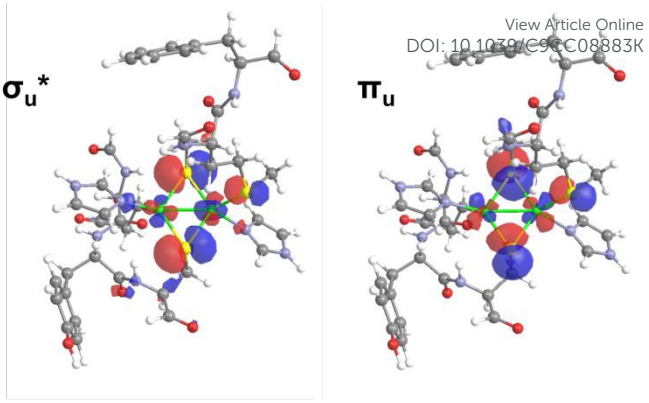



**Figure 3.** Electron spin density distribution on the $\sigma_u$* and $\pi_u$ electronic states in *Tt* $Cu_A$.

To confirm these findings, we studied two second-shell mutants of *Tt* $Cu_A$ in which three loops from the bacterial protein were replaced by the homologous ones from the human oxidase (*Tt*3Lh) [41, 42] and a plant oxidase (*Tt*3L*At*) from *Arabidopsis thaliana* [42, 43] (Supplementary Information). These mutants are good mimics of the $Cu_A$-containing eukaryotic oxidases [41-44] since they include changes only in second sphere residues, conserving all metal ligands. The NMR spectra of *Tt*3L*At* were assigned and resembled those of the previously reported *Tt*3Lh (Figure S2-3 and Table S3). Both variants preserve the identity of all $Cu_A$ ligands but display a smaller energy gap (*ca.* 240 $cm^{-1}$) between the two alternative ground states.[40] HMQC experiments in $^{13}C$ labeled Met and ε-$^{13}CH_3$ Met samples of this chimeric proteins revealed hyperfine shifted resonances with a pattern resembling that observed for *Tt* $Cu_A$. 1D and 2D NOE of these signals also showed similar correlations, enabling the assignment of all $^{1}H$ and $^{13}C$ signals corresponding to the Met ligand in these $Cu_A$ variants (Table S3 and Figures S4-6). To calculate the contact shifts on these two variants, we solved the crystal structures of the two $Cu_A$ mutants. Both proteins display $Cu_A$ sites structurally similar to *Tt* $Cu_A$ (Figure S7 and Table S4). The Cu-S(Met) distances range between 2.5 and 2.6 Å, *i.e.*, within a much shorter range than in blue copper sites, reflecting minor structural distortions induced by the mutations.[4, 14] As in the wild-type protein, the nuclei from the Met axial ligand have net contact shifts in both chimeric proteins (Table 1). These data indicate the presence of unpaired electron spin density in the axial Met residue in all three $Cu_A$ variants.

Here we report the presence of electron spin density both in the methionine ε-$CH_3$ and Υ-$CH_2$ nuclei in the three $Cu_A$ sites here studied (*Tt* $Cu_A$, *Tt*3Lh and *Tt*3L*At*). The contact shifts on the Υ-$CH_2$ are larger than those observed in the homologous nuclei of T1 sites, despite there is only one electron delocalized between two copper ions in the $Cu_A$ centers. Blue copper proteins display net contact shifts in the ε-$CH_3$ (Met) and in the Υ-$CH_2$ protons. In general, normal T1 sites show

shifts in the ε-$CH_3$ while distorted T1 sites are characterized by non-null shifts in the Y-$CH_2$ nuclei (Table 1). In azurin, displaying a long Cu-S(Met) distance (3.0 Å), there is no contact contribution to any Met nucleus. We propose that the shorter Cu-S(Met) distance in native $Cu_A$ sites compared with T1 centers could account for this electron spin density.

The current results differ with calculations performed for the biosynthetic $Cu_A$-azurin.[13] This apparent discrepancy can be attributed to the longer Cu-S(Met) bond (2.9 Å) in this model protein, compared to the short Cu-S distance in native *Tt* $Cu_A$ and the two loop mutants here analyzed.[45] This differential interaction with the axial ligand is also reflected in the redox properties of this site: while the reduction potential of $Cu_A$-azurin is rather insensitive to axial ligand mutations, changes in the axial Met in *Tt* $Cu_A$ give rise to changes in the reduction potentials resembling those reported for Type 1 copper centers.[9, 11, 43-45]

The current experimental data support the notion that the axial Met ligand of native $Cu_A$ sites has net electron spin density in both $\sigma_u$* and $\pi_u$ states. This scenario validates the feasibility of ET pathways involving the axial Met ligand in heme-copper oxidases and claims for the reinterpretation of the current picture of ET in terminal oxidases.

## Conflicts of interest

There are no conflicts to declare.

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