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Short communication

Redox-state sensing by hydrogen bonds in the CuA center of cytochrome c oxidase

Luciano A. Abriata [1], Alejandro J. Vila *

Instituto de Biología Molecular y Celular de Rosario (IBR, CONICET-UNR) and Área Biofísica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina

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ABSTRACT

Cytochrome *c* oxidases (CcO) couple electron transfer to active proton translocation through a gated mechanism that minimizes energy losses by preventing protons from flowing backwards or leaking. Such a complex mechanism requires that information about the redox and protonation states of the different centers be transmitted between different parts of the oxidase. Here we report a network of residues located around the electron entry point of CcO, the CuA site in subunit II, that experience collective pH equilibria around neutral pH. This network starts at the occluded side of the CuA site and extends to the interface between subunits I and II of the CcO, where the proton exit is located and through which electrons flow into subunit I. One of the residues in this network is directly involved in a hydrogen bond to one of the CuA ligands, whose strength is highly sensitive to the redox state of the metal center. We propose that this interaction mediates the transmission of redox changes from ET centers to other functional regions of the oxidase, and possibly also in other similar machineries, as part of their gating and regulatory mechanisms.

Cytochrome *c* oxidase (CcO) is an integral membrane protein that serves as the terminal enzyme of aerobic respiratory chains, coupling downhill electron transfer to proton translocation across the membrane [1,2]. Incoming electrons are shuttled one at a time from soluble cytochrome *c* to a dinuclear copper center termed CuA located in subunit II, and from there to the prosthetic groups embedded inside subunit I and the CuB-heme oxygen-reducing center [2]. At different stages of the four-electron cycle, protons are actively pumped across the complex by means of a stepwise mechanism coordinated by electron–proton gating steps to prevent protons from flowing backwards or leaking [3–11]. The coupling between electron transfer and proton pumping processes requires a mechanism able to transmit information about protonation and oxidation states between different parts of the oxidase, to orchestrate the whole catalytic cycle. A large amount of efforts have been devoted to identify the different possible electron and proton gating points of the cycle [3–13] reaching partial consensus (recently reviewed by Blomberg and Siegbahn [11]). However, little is known on how such redox linkage is established at the atomic level.

The CuA site is the electron entry port in CcOs [14,15]. This center is defined by two cysteine residues whose sulfur atoms bridge two copper ions rendering a Type III mixed-valence system in its oxidized state (Cys149 and Cys153 in Fig. 1A), which give rise to a unique electronic structure [16–20]. The coordination spheres are completed by His114 and Met160 for the most occluded copper ion, and by His157 and a backbone O atom for the most exposed one. His114 is located in an internal β-strand, whereas the other 5 ligands are located in an exposed loop (Fig. 1C) [16,21]. Pioneering studies on an artificial CuA site engineered into the blue copper protein azurin showed that protonation of the most exposed histidine increases the reduction potential of the center, suggesting that this could prevent electron flow from CuA to heme a in the CcO regulating its activity and possibly acting at one or more points of the proton-coupled electron transfer mechanism [22–24]. More recently, however, our work on the native CuA-containing domain of *Thermus thermophilus* CcO ba_3 (*Tt*CuA) showed that protonation equilibria within a physiological range do not induce significant changes on the electronic structure of the metal site and have only a very small impact on its redox potential and electron transfer capabilities [25]. This observation, however, does not rule out the existence of more subtle mechanisms linking the redox state of the CuA center with the rest of the oxidase.

Our previous NMR work focused on the paramagnetically shifted resonances arising from nuclei of the copper ligands in oxidized *Tt*CuA. It revealed only minor perturbations of the electronic structure of the center between pH 4 and 8 that did not alter its mixed-valence nature [17,25]. These subtle perturbations were localized to the imidazole resonances of His114 and the backbone H atom of Gly115, a conserved second-shell ligand H-bonded to the S atom of Cys149 [26,27]. We have now extended our study to the whole protein by inspecting the pH-dependent changes in the HSQC spectra of *Tt*CuA in its reduced form, where the absence of paramagnetic effects allows for an NMR study of all residues at high sensitivity and resolution.

* Corresponding author. Tel.: +54 341 4237070.
E-mail address: vila@ibr-conicet.gov.ar (A.J. Vila).
[1] Current address: Laboratory of Biomolecular Modeling, Institute of Bioengineering, School of Life Sciences — École Polytechnique Fédérale de Lausanne, Switzerland.

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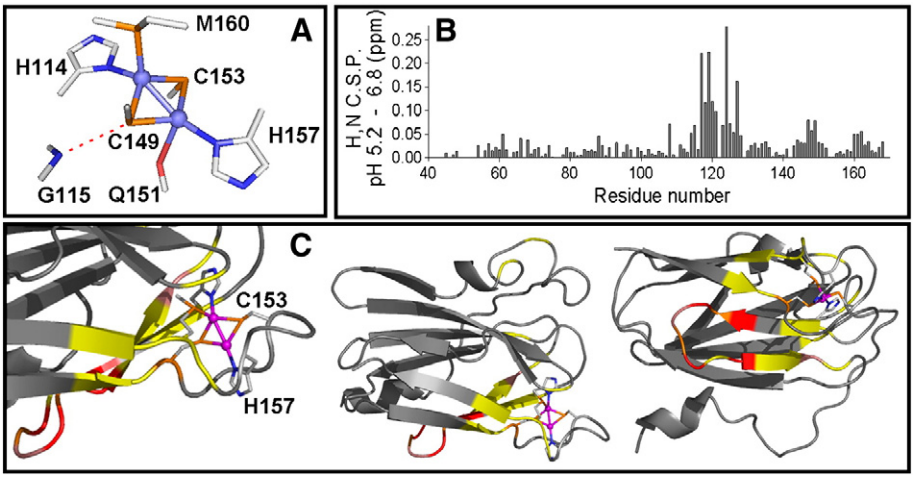

Fig. 1. (A) The copper ligands of the CuA center, including the conserved hydrogen bond to the S atom of Cys149. (B) Combined ^{1}H,^{15}N chemical shift perturbations (CSP) computed from assignments of HSQC spectra recorded at pH 5.2 and 6.8 (given in Table S1). (C) Different views color-coding residues with significant CSP between pH 5.2 and 6.8 on a cartoon representations of the soluble CuA-containing domain of *Thermus thermophilus* ba_3 oxidase. Gray stands for CSP ≥ 0.04 or missing data; CSP above 0.04 grows from yellow to orange to red. Residue numbering in this figure and the whole paper corresponds to PDB entries 2CUA, 1EHK and 3S8F [16,35,36].

Samples of reduced *Tt*CuA uniformly labeled at ^{15}N were prepared as described elsewhere [26,28] and their ^{1}H,^{15}N HSQC spectra were acquired at pH 4.7, 5.2, 6.2, 6.8, 7.5 and 8.2 at 25 °C in a Bruker Avance II 600.13 MHz spectrometer. Spectra in the pH range from 5.2 to 6.8 were the best resolved and most intense, and were subject to further analysis. The resonance assignments available at pH 6 [29] were transferred to the spectrum collected at pH 6.2 and then to those at pH 5.2 and 6.8 (Table S1) by following the pH-dependent shifts of the cross peaks in the HSQC spectra. The combined ^{1}H,^{15}N chemical shift perturbation (CSP) was then computed by comparing the assignments at pH 5.2 and 6.8 (Figs. 1B and S1). Significant perturbations (CSP ≥ 0.04 ppm) are observed in four isolated residues and in sequence segments that include the β-strand holding His114 and Gly115, its subsequent β-strand and the short loop in-between, the first and last residues of the ligands loop including residues Cys149 and Met160, and the tips of two β-strands flanking this loop (Table 1). All these segments correspond approximately to a β-sheet structure on the back of the CuA site (Fig. 1C, left) suggesting an extensive network of pH-sensitive residues connected to it through His114, Cys149 and Met160, *i.e.* the same pH-sensitive residues detected in our study of the oxidized form. Notably, this network corresponds to the surface of the soluble CuA fragment (Fig. 1C, center and right) that maps to the interface between subunits I and II in the structure of the whole oxidase from *T. thermophilus* (Fig. 2). Moreover, residues with the largest perturbations (around residue 124) are located right next to the purported proton exit pathway, which includes the affected Glu126.

The resonance corresponding to the NH of Gly115 changes with pH. This backbone amide forms a hydrogen bond with the S atom of Cys149, as observed in the crystal structures and as evidenced by its resilience towards exchange with $^{2}H_2O$ [26,30]. We measured the exchange rate of the NH signals from Gly115 in $^{2}H_2O$ (Fig. S2). Fits of the resulting decay profiles yield half times of ~8 days for the oxidized protein against only ~8 h for the reduced protein, indicating a strong dependence of the hydrogen bond strength on the redox state of the center. This result contradicts recent QM calculations in the blue copper protein azurin, [31] which suggest a weaker sulfur–hydrogen bond in the oxidized state. The difference might be attributed to either an increased flexibility in the reduced protein (which is not the case, based on NMR studies [21]) or, more likely, to a lowered stability of the reduced protein, as reported in unfolding studies [32].

These data disclose a network of pH-sensitive residues in the interface between subunit II and subunit I that connects with the CuA center through a hydrogen bond that is strongly sensitive to its oxidation state. This network accesses the CuA site through His114, Gly115, Cys149 and Met160, the first two being also sensitive to pH in the oxidized form. We

Table 1
Residues that experience significant chemical shift perturbation between pH 5.2 and 6.8.

Protein region	Residues
Beta strand containing His114 and Gly115, continuing loop and beta strand	His_{114} Gly_{115} His_{117} Val_{118} Glu_{119} Gly_{120} Thr_{121} Ile_{123} Asn_{124} Val_{125} Glu_{126} Val_{127} Leu_{128}
Base of the copper-binding loop and tips of the beta strands connected to this loop	Ile_{147} Ile_{148} Cys_{149}, Met_{160} Phe_{161} Gly_{162}
Loop close to the CuA site but without copper ligands	Phe_{88}
Isolated residues, not part of the network described here	Glu_{61} Asp_{66} Thr_{108}

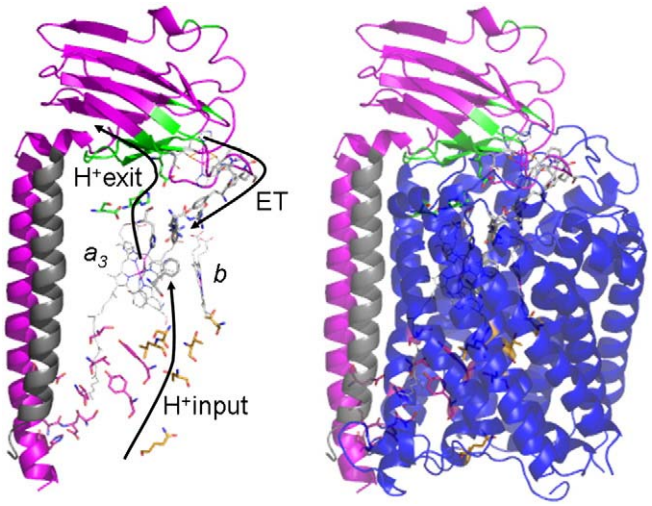

Fig. 2. Electron transfer and proton transport pathways (left) displayed on *Thermus thermophilus* ba_3 oxidase, whose global fold is shown on the right to help localize the different groups. Prosthetic groups (hemes *b* and a_3, and the CuA and CuB sites) are shown as lines. Residues making up the electron transfer and proton transport pathways are shown as sticks (reviewed in [4–6,13]). Residues shown as green sticks constitute the proposed proton exit pathway whereas green cartoons identify the pH-sensitive residues observed in subunit II (shown in detail in Fig. 1). Residues shown as gray sticks make up the proposed electron transfer pathway from CuA to the catalytic center; residues in pink and orange are part of pathways proposed for proton input.

propose that such a network could be exploited to sense the redox state of the CuA center from remote points of the oxidase (or to communicate it), possibly through the residues involved in the proton exit pathway. This proposal is complementary to that advanced in the studies of CuA–azurin, which involves changes in the electronic structure of the center, [22–24] being also in agreement with the proposed electrostatic nature of the gates; [3,33] and is similar to the explanation of how electron-transfer events and protein structure are coupled in cytochrome *a* [34]. Mechanisms like this, *i.e.* based on the response of hydrogen bond strengths to charges and *vice versa*, could be at the atomistic basis of redox linkage between different parts of the oxidase.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jinorgbio.2013.07.032.

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