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Native Cu_A redox sites are largely resilient to pH variations within a physiological range†

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Previous studies on engineered Cu_A centres have shown that one of the histidine ligands is protonated and dissociated from the metal site at physiological pH values, thus suggesting a role in regulating proton-coupled electron transfer of cytochrome *c* oxidases *in vivo*. Here we report that for native Cu_A such protonation does not take place at physiologically relevant pH values and, furthermore, no significant changes in the spectroscopic and redox properties of the metal site occur at low pH.

Cytochrome *c* oxidase (C*c*O) is an integral membrane protein that serves as the terminal enzyme of aerobic respiratory chains, coupling downhill electron transfer (ET) reactions with uphill proton translocation (PT) across the membrane.[1] Electrons shuttled by a soluble *c* type cytochrome are delivered to the primary acceptor of C*c*O, the dinuclear copper centre Cu_A, and from there to the catalytic active site where molecular oxygen is reduced to water.[2] The electrochemical gradient generated by the PT activity, which ultimately sustains ATP synthesis, produces variable local proton concentrations at the membrane interface. Pioneering studies on artificial Cu_A sites engineered into the blue copper protein Azurin (Cu_A-Azu) suggested that these local variations in pH may result in physiological consequences of utmost importance in regulating the redox activity of the Cu_A site.[3–6] Specifically, it has been shown that this metal centre undergoes a significant perturbation induced by protonation and concomitant detachment of one of the equatorial histidine ligands (H120 in Cu_A-Azu) with an apparent pK_a of around 5. The transition has been proposed to determine the conversion from a fully delocalized mixed valence (MV) state to a localized valence (LV) state,[4,5] although more recent spectroscopic and computational studies established that detachment of protonated H120 renders both Cu ions asymmetric in their contribution to the electronic wavefunction, but preserves the MV character.[6]

The symmetric MV state (sMV) is a hallmark of oxidized Cu_A centres, characterized by a seven-line hyperfine pattern in the EPR spectrum that is associated with the delocalization of the S = 1/2 over the two Cu ions.[7] This feature is essential for the redox function as it results in a low reorganization free energy (λ), thus accelerating inter- and intra-protein ET reactions.[8–10] Indeed, it was recently shown that the metal site structure is largely responsible for the rigidity lowering the λ value.[11] The transition to the asymmetric MV state (aMV) is characterized by a four-line hyperfine EPR pattern, as well as by distinct changes of the vibrational and electronic spectra,[4,6] a 50% increase of λ[12] and *ca.* 150 mV up-shift of the formal reduction potential (E°).[4] Based on the characterization of the H120A mutant of Cu_A-Azu, it has been rationalized that protonation of H120 accounts for 70 mV of the total E° shift. These findings led to the proposal that the acid–base equilibrium of this histidine ligand plays a role in regulating proton-coupled ET reactions of C*c*O.[4,6]

Mutation of the weak axial ligand methionine in Cu_A-Azu sites, on the other hand, has no effect on the value of E°.[13] In sharp contrast, replacement of the equivalent residue in native Cu_A sites modulates E° by up to 200 mV,[14] thus underlining the importance of the native protein environment in defining the electronic and redox properties of the metal site.

Here we report on the spectroscopic and electrochemical responses to pH variations in the native Cu_A domain from *T. thermophilus* ba_3 C*c*O (*Tt*-Cu_A), which has been reported to be fairly stable over a broad pH range.[15]

NMR allows monitoring directly protonation events and metal–ligand interactions in paramagnetic proteins. ^{1}H-NMR spectra of the oxidized native *Tt*-Cu_A site show three distinguishable, reversible transitions in a wide pH range (Fig. 1 and Fig. S3, ESI†). The first one is characterized by the disappearance of the imidazolic $N^\varepsilon H$ signal from H157 (equivalent to H120 in Cu_A-Azu) at 23.2 ppm (signal *i*),[16] with an apparent pK_a value of 3.5. The remaining ^{1}H resonances corresponding to the same imidazole ring (*f* and *k*) are largely unperturbed upon this transition, suggesting that (1) the protonation state of H157 is not modified and that (2) this residue remains

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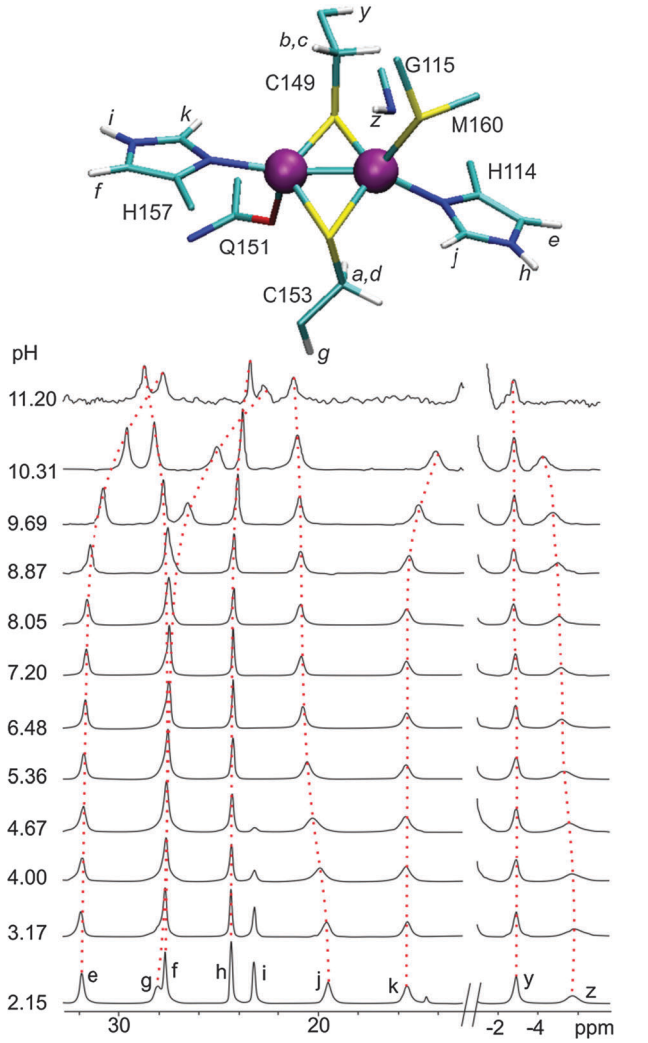

Fig. 1 (top) Representation of the *Tt*-Cu_A site, rendered from PDB ID 2CUA.[18] The hydrogen atoms are labelled according to the ^{1}H-NMR spectra. (bottom) Paramagnetic ^{1}H-NMR spectra monitoring the pH titration of the *Tt*-Cu_A site. Spectra were acquired at 25 °C in tris–acetate–phosphate–salt buffer.

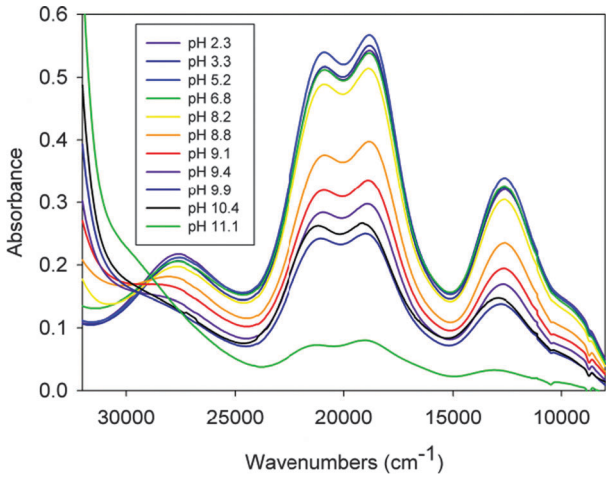

Fig. 2 UV-Vis spectra of *Tt*-Cu_A acquired at various pH values in tris–acetate–phosphate–salt buffer. All spectra were recorded at room temperature.

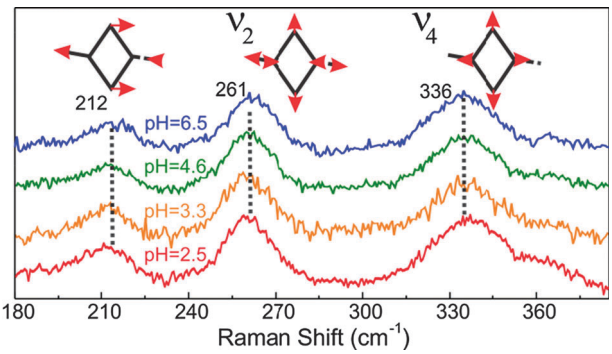

Fig. 3 Resonance Raman spectra of the *Tt*-Cu_A site recorded at room temperature under 514 nm excitation. Band assignation was adopted from Xie *et al.*[6]

bound to the copper ion at low pH (since resonances from all nuclei belonging to this residue are isotropically shifted). Instead, the absence of signal *i* at higher pH values can be attributed to a faster exchange rate of the exposed $N^{\varepsilon}H$ proton of H157 with the solvent, as opposed to the equivalent proton of H114 (signal *h*) that is involved in H-bonding interactions inside the protein.[15] UV-Vis spectrophotometric titrations support this interpretation. The electronic spectrum of the *Tt*-Cu_A site is dominated by $S_{Cys} \rightarrow Cu$ metal–ligand charge transfer bands at 21 500 and 18 550 cm^{-1} and a $\psi \rightarrow \psi^{\star}$ intervalence band at 12 750 cm^{-1},[17] all of which undergo distinct variations in the pH-induced transition in Cu_A-Azu.[6] As shown in Fig. 2, absorption spectra of *Tt*-Cu_A remain essentially unaltered within a pH range from 2 to 7. Resonance Raman (RR) spectra recorded under 514 nm excitation, *i.e.* in resonance with the $S_{Cys} \rightarrow Cu$ CT band, are also invariant within this pH range (Fig. 3 and Fig. S1, ESI†). Under these conditions, RR spectra are dominated by vibrational modes from the Cu_2S_2 core. The sMV → aMV transition is expected to have an impact on the contribution of the $Cu–N_{His}$ vibrations to the ν_2 RR band, diminishing its intensity, and on the position of the ν_4 mode.[6] In summary, ^{1}H-NMR, UV-Vis and RR data show that the protonation state of H157 in *Tt*-Cu_A does not change in the pH range from 2 to 7 (in contrast with the results for Cu_A-Azu), and that H157 is a metal ligand all along the monitored pH range.

A second acid–base transition with apparent pK_a = 4.7 is observed based on the shift of ^{1}H-NMR peaks assigned to the imidazole protons of the equatorial ligand H114 (signals *e* and *j* in Fig. 1) and the peptide proton of Gly115 (signal *z*), a second-shell ligand H-bonded to the S atom of the Cys149 ligand. Also in this transition the acid–base equilibrium has no significant impact on the electronic properties of the *Tt*-Cu_A site, as judged from the UV-Vis and RR spectra. Finally, the third transition detected using NMR displays an apparent pK_a close to 11 and parallels the changes in the absorption bands. This transition has already been reported for the native *Tt*-Cu_A site,[19] and was ascribed to the collapse of the Cu_2S_2 core. A related, albeit different transition has been observed in the native Cu_A site from *P. denitrificans* and rationalized in terms of a conversion to a type 2 copper site.[20]

The redox behaviour of *Tt*-Cu_A was evaluated by cyclic voltammetry (CV) in solution at pH values from 2.2 to 11.3, obtaining nearly ideal reversible responses in all cases (Fig. S2, ESI†). E° values determined from these experiments are displayed in

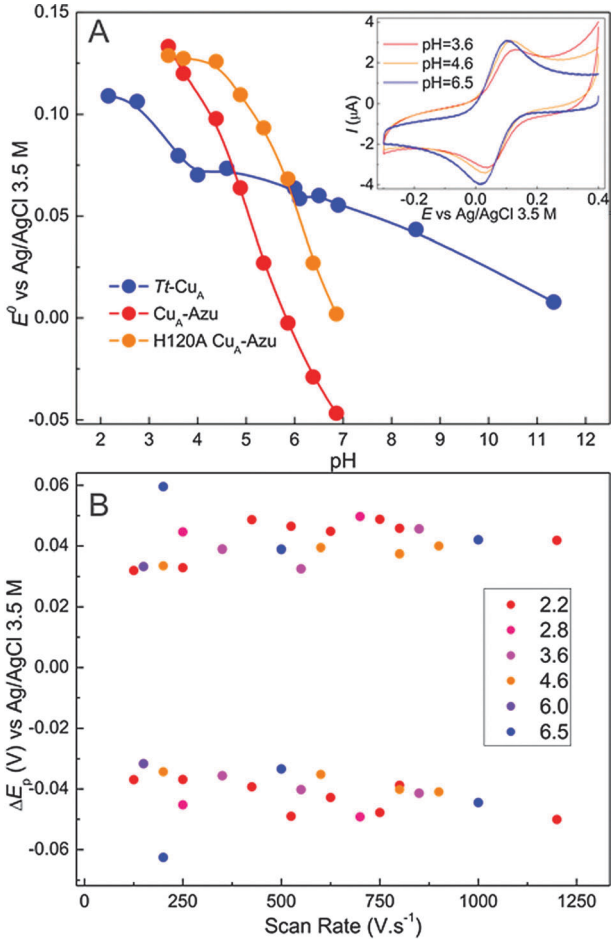

Fig. 4 (A) pH-dependence of the reduction potential of *Tt*-Cu_A, Cu_A-Azu[4] and H120A Cu_A-Azu.[4] Inset: selected CVs obtained at different pH values at room temperature. (B) Anodic and cathodic CV peak potential difference from E^0 (ΔE_p) obtained for *Tt*-Cu_A as a function of scan rate at various pH values.

Fig. 4A, along with data adopted from the literature for Cu_A-Azu and H120A Cu_A-Azu,[4] which are included for comparison. The engineered proteins exhibit sharp variations in E° with apparent pK_a values above 5. In contrast, the native *Tt*-Cu_A site shows a low-amplitude transition at pH close to 3 followed by a small and nearly continuous variation at higher pH that reflects the existence of several acid–base equilibria with a low impact on the electronic structure, thereby in excellent agreement with the spectroscopic data. Remarkably, in the physiologically relevant range of pH 4–7 the native centre is nearly pH-insensitive, presenting E° shifts of only 15 mV, whereas for Cu_A-Azu the variation is more than 150 mV. Moreover, the CVs obtained in the pH range 2–7 show that the separations between cathodic and anodic peaks as a function of the scan rate are pH-independent, thus indicating that λ remains largely constant (Fig. 4B).

Most likely the different pH dependencies of *Tt*-Cu_A and Cu_A-Azu can be partially ascribed to the protonable His35 residue in Cu_A-Azu, which is very close to the active site.[21,22]

Our results highlight the importance of the protein matrix acting as a second (and higher) coordination sphere in regulating both the electronic and redox properties of the Cu_A redox centre, which is consistent with the differential role of the weak axial ligand methionine in regulating thermodynamic and kinetic ET parameters, as reported recently.[8,14] Moreover, they challenge the notion of redox properties of the primary electron acceptor of C*c*O being fine-tuned by local variations of pH *in vivo*.[4,6,12] Instead, the availability of two alternative electronic ground states could play a role in pathway switching during the redox cycle.[8]

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