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Native Cu_A Redox Sites are Largely Resilient to pH Variations within Physiological Range

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Experimental procedures

*Protein Preparation***.** Samples of wild type Cu_A soluble fragments from subunit II of *T. thermophilus* cytochrome *c* oxidase ba_3 were produced as described elsewhere[1] based on the original protocol in described by Slutter *et al.*.[2] Samples were stored in 100 mM phosphate buffer pH 6.0 with 100 mM KCl.

Resonance Raman spectra. Typical protein samples were 1 mM in a 10 mM phosphate, 500 mM KNO_3 buffer. $HClO_4$ was added to 200 µL of each protein solution in order to set the pH at the desired value. Equilibration was achieved after ca. 2 minutes after acid addition. Resonance Raman spectra were acquired in backscattering geometry by using a confocal microscope coupled to a single-stage spectrograph (Jobin Yvon XY 800) equipped with a liquid-nitrogen-cooled back-illuminated CCD detector. Elastic scattering was rejected with an edge filter (Semrock). The 514 nm line of a cw argon laser (Coherent Innova 70c) was focused into 100 µL protein solution films in a rotating quartz cell using a 20X objective (20.5 mm wd, 0.35 N.A.) Acquisitions were performed with laser powers of about 13mW at sample (30 s acquisition time) and a binning factor of 2, resulting in a spectral resolution of *c.a.* 4 cm^{-1}. Spectra were acquired either at room temperature in rotating quartz cells or at 77 K using a Linkam THMS600 cryo-microscopy devise. After acquisition, spectra were processed with home-made software for baseline correction and peak determination.

Electrochemical determinations. Cyclic voltammetry was perfomed with a Gamry REF600 potentiostat in a home-made electrochemical cell equipped with a polycrystalline gold bead working electrode, a PT wire auxiliary electrode and a Ag/AgCl (3.5 M KCl) reference electrode to which all potentials in this work are referred. Au gold beads of 1 mm diameter were first oxidized in 10% $HClO_4$ applying a 3 V potential for 2 minutes, sonicated in 10% HCl for 15 minutes, rinsed with water and subsequently treated with a 3:1 v/v H_2O_2:H_2SO_4 mixture at 120 °C. The electrodes were then subjected to repetitive voltammetric cycles between -0.2 and 1.6 V in 10% $HClO_4$ and thoroughly washed with water and ethanol. Working electrodes were coated

with self-assembled monolayers (SAMs) by overnight incubation in an ethanolic 1mM HS-$(CH_2)_6$-OH solution to prevent protein adsorption. After thorough rinsing with ethanol and deionized water, the coated electrodes were placed in the electrochemical cell containing a 300 μM protein solution (in 10 mM phosphate buffer, KNO_3 500mM, pH 7). The pH was adjusted by addition of $HClO_4$ or KOH, followed by 2 minutes of equilibration.

Nuclear Magnetic Resonance Spectroscopy. Samples for NMR experiments were prepared in TAPS buffer (Tris:HCl, sodium acetate and sodium phosphate, 50 mM each, plus 100 mM NaCl) with 10% D_2O. NMR experiments were carried out on a Bruker Avance II Spectrometer operating at 600.13 MHz (^{1}H frequency) at 25ºC. Spectra were acquired with a triple-resonance (TXI) probehead using the PASE pulse sequence[3] on spectral windows of 48 – 360 kHz (total recycle times around 200 and 40 ms respectively).

Optical spectroscopy. Electronic absorption spectra were acquired with a Jasco V-670 spectrophotometer, which covers a wavelength range well beyond 1300 nm (but at longer wavelengths the absorption of water is too strong). Quartz cells were used with 1 cm or 0.1 cm path length as required.

Supplementary Figures

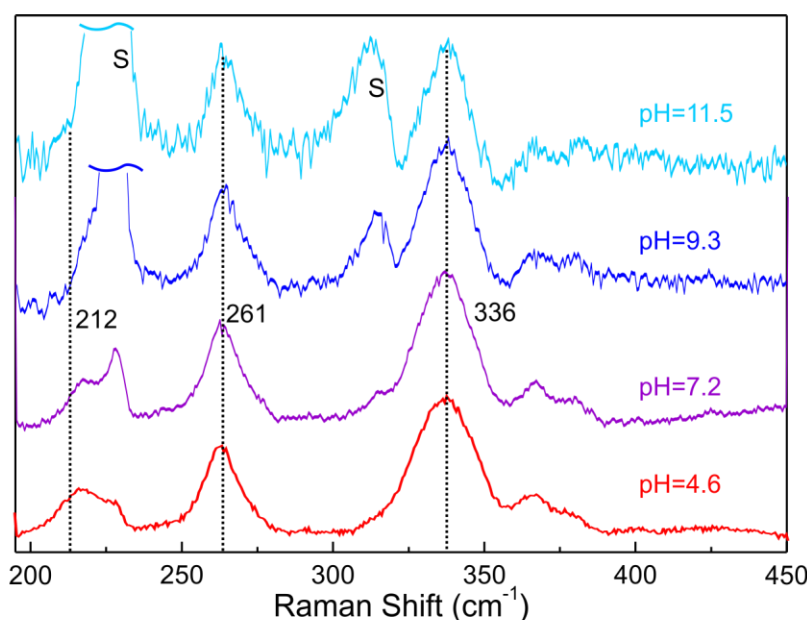

Figure S1. Resonance Raman spectra of the Tt-CuA site recorded at 77 K under 514 nm excitation. Peaks due to the solvent are labeled “S”.

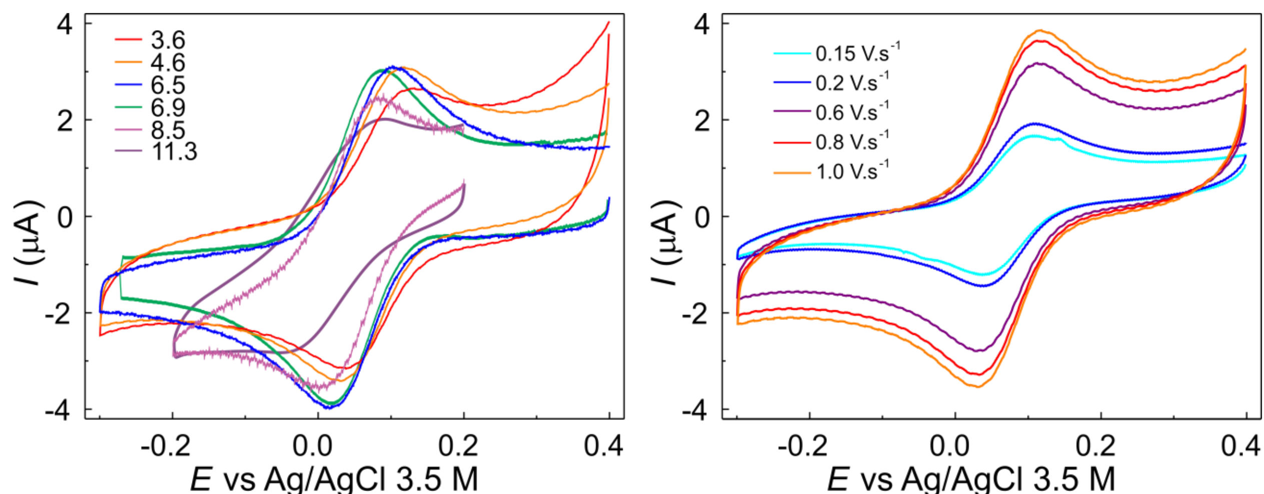

Figure S2. Left: CVs acquired at different pH values from 3.6 to 11.3 at a constant scan rate of 0.6 $V.s^{-1}$. Right: CVs acquired at pH 4.6 with variable scan rates from 0.15 $V.s^{-1}$ to 1.0 $V.s^{-1}$

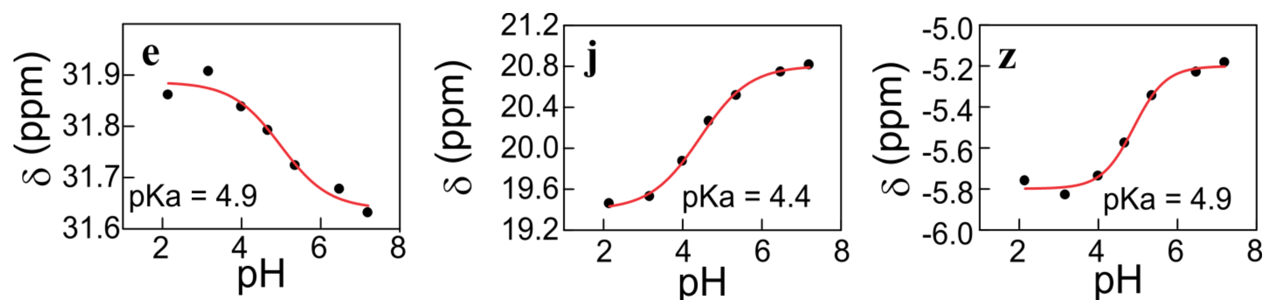

Figure S3. Positions of signals *e, j* and *z* of the ^{1}H spectra in figure 1 at different pH values between 2.15 and 7.2, *i. e.* during the second transition as described in the article, in which the mixed valence nature of the center is preserved. An apparent p*K*a of 4.7 ± 2 is obtained from a global fit.

References

1 Abriata, L. A.; Ledesma, G. N.; Pierattelli, R.; Vila, A. J., *J. Am. Chem. Soc.,* 2009, **131**, 1939.

2 Slutter, C. E.; Sanders, D.; Wittung, P.; Malmström, B. G.; Aasa, R.; Richards, J. H.; Gray, H. B.; James, A., *Biochemistry,* 1996, **35**, 3387.

3 Bondon, A.; Mouro, C. *J. Magn. Reson.,* 1998, **134**, 154.