

# Reversible Switching of Redox-Active Molecular Orbitals and Electron Transfer Pathways in Cu<sub>A</sub> Sites of Cytochrome c Oxidase\*\*

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**Abstract:** The Cu<sub>A</sub> site of cytochrome c oxidase is a redox hub that participates in rapid electron transfer at low driving forces with two redox cofactors in nearly perpendicular orientations. Spectroscopic and electrochemical characterizations performed on first and second-sphere mutants have allowed us to experimentally detect the reversible switching between two alternative electronic states that confer different directionalities to the redox reaction. Specifically, the M160H variant of a native Cu<sub>A</sub> shows a reversible pH transition that allows to functionally probe both states in the same protein species. Alternation between states exerts a dramatic impact on the kinetic redox parameters, thereby suggesting this effect as the mechanism underlying the efficiency and directionality of Cu<sub>A</sub> electron transfer in vivo. These findings may also prove useful for the development of molecular electronics.

**P**rotein electron-transfer (ET) reactions constitute the basis of energy transduction in living organisms and involve a plethora of fine-tuning mechanisms that have just begun to be uncovered. This variety arises from the structural complexity of redox proteins,<sup>[1]</sup> and includes modulation of the electronic coupling ( $H_{DA}$ ) by fluctuations of the amino acid bridge between donor and acceptor<sup>[2–5]</sup> and tuning of reorganization energies ( $\lambda$ ) by second-sphere ligands,<sup>[6]</sup> among other possibilities.

The Cu<sub>A</sub> site is the primary electron acceptor of the terminal respiratory enzyme cytochrome c oxidase (CcO), which acts as a proton pump while sequentially transferring electrons through several redox cofactors towards molecular oxygen.<sup>[7]</sup> The two Cu ions of the binuclear center are bridged by cysteine ligands, forming a nearly planar Cu<sub>2</sub>S<sub>2</sub> diamond core characterized by a short Cu–Cu distance. The coordina-

tion sphere is completed by two equatorial histidines and two weak axial ligands provided by a methionine (M160) sulfur and a backbone carbonyl group.<sup>[8]</sup>

The electronic structure of Cu<sub>A</sub> can be described by a double-well potential as a function of small geometric perturbations, such as Cu–Cu stretching<sup>[9]</sup> or dihedral distortions,<sup>[10]</sup> where the energy minima correspond to two alternative  $\sigma_u^*$  and  $\pi_u$  ground states (GSs). As determined for the Cu<sub>A</sub>-containing subunit II fragment of the *ba*<sub>3</sub> oxidase from *Thermus thermophilus* (*Tt*-Cu<sub>A</sub>), the  $\pi_u$  state is thermally accessible at room temperature from the lower lying  $\sigma_u^*$  state.<sup>[11]</sup> Moreover, NMR and DFT calculations show differential electron densities in the  $\sigma_u^*$  and  $\pi_u$  GSs, thus leading to the proposal that both states may be functional in vivo, conferring directionality by means of differential electronic coupling of Cu<sub>A</sub> in the different GSs with the electron donor (cytochrome c) and acceptor (a heme cofactor), respectively.<sup>[10]</sup> In recent work we reported that the  $\sigma_u^*/\pi_u$  energy gap ( $\Delta E_{\sigma_u^*/\pi_u}$ ) can be fine-tuned either by mutating the weak axial ligand M160 or by substituting the three loops that define the second coordination sphere of *Tt*-Cu<sub>A</sub> by those from a homologous eukaryotic domain (*Tt*-3L variant).<sup>[12]</sup>  $\Delta E_{\sigma_u^*/\pi_u}$  values for native *Tt*-Cu<sub>A</sub>, M160H, M160Q and the *Tt*-3L variants are 600, 200, 900, and 240 cm<sup>–1</sup>, respectively.<sup>[10,12]</sup> Thus, the populations of the  $\pi_u$  state at room temperature are 30–35 % for M160H and *Tt*-3L, about 5 % for WT *Tt*-Cu<sub>A</sub> and about 1 % for M160Q. One can anticipate that for sufficiently large  $\Delta E_{\sigma_u^*/\pi_u}$ , displacement along the ET reaction coordinate is likely to proceed through the lower lying  $\sigma_u^*$  GS. Smaller gaps, in contrast, may lead to ET exclusively from the  $\pi_u$  GS. The underlying idea, as shown by QM calculations,<sup>[10]</sup> is that a variety of small structural distortions result in an adiabatic  $\sigma_u^* \rightarrow \pi_u$  transition. If the reaction coordinates for this transition and for ET are coupled, the  $\pi_u$  GS may become redox active and ET through the  $\sigma_u^*$  GS is disabled, even if this GS remains energetically lower (see Figure S1 in the Supporting Information). In such scenario, the relative magnitudes of the  $\Delta E_{\sigma_u^*/\pi_u}$  versus the ET activation free energy ( $\Delta G^\ddagger$ ) determine the redox-active GS. Indeed, first and second sphere mutants of Cu<sub>A</sub> have been shown to present either  $\sigma_u^*$  or  $\pi_u$  redox-active GSs, depending on the magnitude of their  $\Delta E_{\sigma_u^*/\pi_u}$ .<sup>[10,12]</sup> Here we report that a reversible functional switching between a  $\sigma_u^*$  and a  $\pi_u$  redox-active GSs can be elicited by pH changes within the same protein variant. The combined use of electrochemical and spectroscopic techniques to assess this unique behavior unveils differences in electronic coupling for both states and further supports the feasibility of an in vivo regulatory mechanism by this switching.

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Protein samples were adsorbed on Au electrodes coated with self-assembled monolayers (SAMs) to produce relatively well oriented protein films,<sup>[13]</sup> and were functionally assessed by cyclic voltammetry (CV). This method was chosen instead of solution electrochemistry to bypass protein diffusion that hampers comparative kinetic studies between the different protein variants, particularly regarding electronic couplings. The reduction potentials obtained in solution and for the protein films are very similar, thereby indicating that the structure of the different Cu<sub>A</sub> sites is preserved upon adsorption (Table S1).

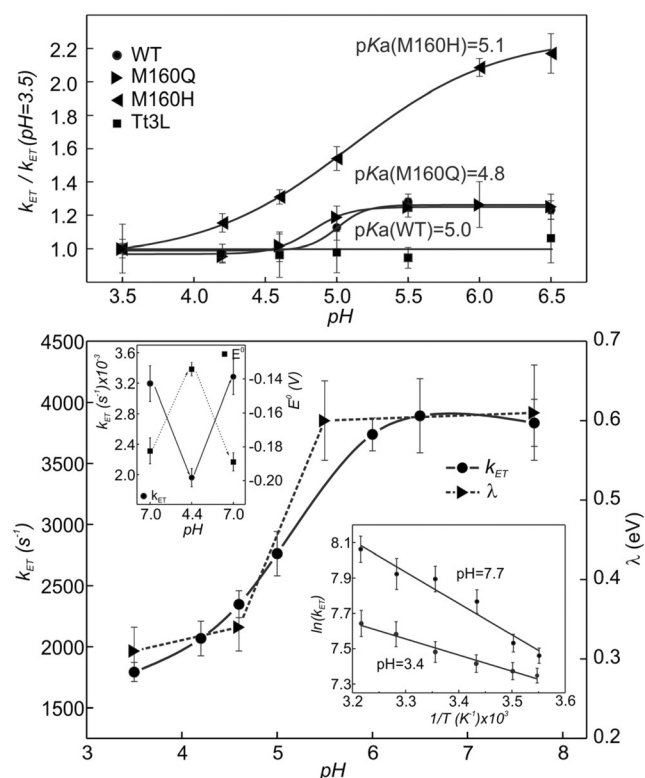
Rate constants of heterogeneous ET at zero driving force ( $k_{ET}$ ;  $\Delta G^\circ=0$ ) were obtained using Laviron's working curve<sup>[14]</sup> (Figures S2–S5) and ET activation enthalpies,  $\Delta H^\ddagger$  were estimated from Arrhenius plots in the temperature range 5–40 °C using a nonisothermal cell. Simple Boltzmann considerations show that the  $\sigma_u^*/\pi_u$  relative populations do not change significantly within this range (Figure S6). Considering that the entropic contribution in redox proteins is usually very small,<sup>[15]</sup> one can approximate  $\Delta H^\ddagger \approx \Delta G^\ddagger = \lambda/4$  for  $\Delta G^\circ=0$ .<sup>[16]</sup>

As shown in Figure 1 and S7, we observe a pH-dependent transition of  $k_{ET}$  for M160H with a pK<sub>a</sub> of about 5.0 and a two-fold increase from low to high pH values. This is paralleled by a transition of the activation parameters. At low pH we obtain  $\lambda = 0.34$  eV ( $\Delta H^\ddagger = 8.2$  kJ mol<sup>-1</sup>), a value similar to those reported for *Tt*-Cu<sub>A</sub> and M160Q, with

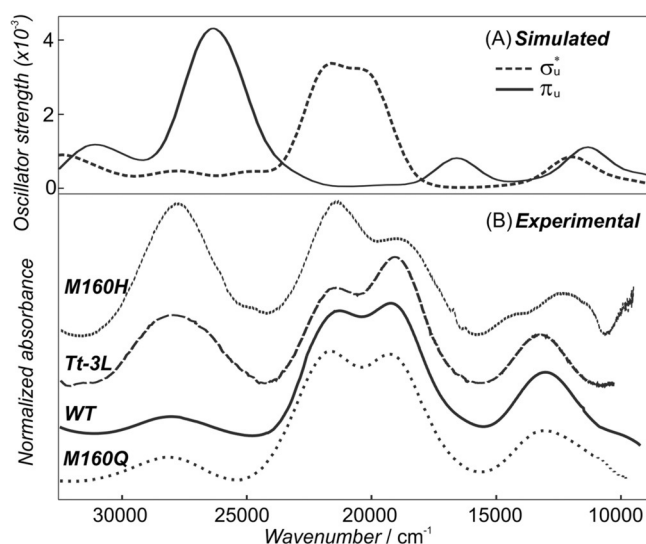
a  $\sigma_u^*$  redox-active GS.<sup>[10]</sup> At high pH, instead, the measured values ( $\lambda = 0.60$  eV,  $\Delta H^\ddagger = 15.5$  kJ mol<sup>-1</sup>) are similar to *Tt*-3L, a variant with a  $\pi_u$  GS,<sup>[12]</sup> thus suggesting a  $\sigma_u^*/\pi_u$  transition. These results may appear to contradict ET theory at first glance. Upon activation of the  $\pi_u$  GS as the redox-active one,  $k_{ET}$  is expected to decrease due to its higher  $\lambda$  value. In fact, Gorelsky and coworkers proposed a larger  $\lambda$  value in the  $\pi_u$  GS rendering it inefficient for ET.<sup>[9]</sup> Surprisingly, we observe the opposite trend for the M160H mutant, that is, the pH-dependent increase of  $\lambda$  concurs with an unexpected rise of  $k_{ET}$ . In the context of Marcus theory, this observation can only be rationalized in terms of a about 7.5-fold enhancement of the electronic coupling towards the metal electrode that overrides the larger  $\lambda$ . The observed transition is fully reversible with pH (Figure 1). Moreover, from the peak areas of the CVs, it is estimated that less than 10% of the adsorbed protein is lost during the process of changing pH back and forward.

The  $k_{ET}$  values for WT and M160Q are almost identical, and both exhibit a small variation with pH of about 20%. This indicates that the single point mutation of the internal residue M160 exerts no appreciable effect on the protein/SAM interactions. The activation parameters of WT *Tt*-Cu<sub>A</sub> and M160Q are also identical ( $\lambda = 0.29$  eV;  $\Delta H^\ddagger = 7.0$  kJ mol<sup>-1</sup>), independent of pH and consistent with the value expected for a  $\sigma_u^*$  GS.<sup>[9,10,17]</sup> While small rearrangements of protein and interfacial water molecules can account for the mild pH kinetic effect observed for WT and M160Q,<sup>[18,19]</sup> they cannot explain the much larger effect observed for M160H. First one has to consider that the structure and main spectroscopic features are largely preserved upon mutation, as determined by UV/Vis, NMR, RR, EXAFS, EPR, and QM/MM calculations.<sup>[10,20]</sup> Second and most important, reorientation effects are not expected to produce a two-fold increase of  $\lambda$ , as observed for M160H. Therefore we conclude that the enhanced coupling observed in M160H at high pH arises from the activation of the  $\pi_u$  GS, which exhibits a different electronic density distribution compared to the  $\sigma_u^*$  GS, as suggested by previous pathways and QM calculations and NMR experiments.<sup>[10]</sup> In contrast, the small variation of  $k_{ET}$  for WT and M160Q is most likely the result of minor reorientations due to protonation/deprotonation of surface residues at the protein/SAM binding site, consistent with the pH-dependence of the SPR signals (Figure S8). The results obtained for *Tt*-3L are in good agreement with this interpretation. In this variant the 3 loops that constitute the putative binding site to the SAM are mutated, exhibiting slightly higher and pH insensitive  $k_{ET}$ , which probably denotes a marginally different coarse orientation in the adsorbed state. The activation parameters of *Tt*-3L are insensitive to pH and are in the range reported for a  $\pi_u$  redox-active molecular orbital ( $\lambda = 0.60$  eV;  $\Delta H^\ddagger = 15.5$  kJ mol<sup>-1</sup>).<sup>[12]</sup>

In order to unveil whether the alternation of the active GS could account for the observed electrochemical response, we studied the effect of pH on the different protein variants in solution by UV/Vis, resonance Raman (RR), EPR, and NMR spectroscopy. For the mixed-valence WT and M160Q variants, the UV/Vis spectra are dominated by two intense S<sub>Cys</sub>→Cu charge transfer (CT) bands at 21 270 and 18 700 cm<sup>-1</sup>, N<sub>His</sub>→



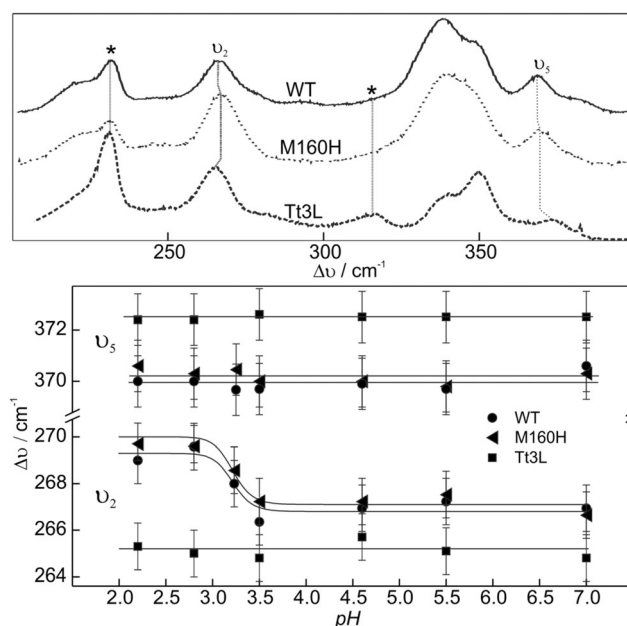
**Figure 1.** A) Variation of  $k_{ET}$  as a function of pH. The values shown are normalized by  $k_{ET}$  at pH 3.5. B) Increase of  $k_{ET}$  and  $\lambda$  as a function of pH for M160H. Inset 1: Reversibility of the pH-induced changes on  $E'$  and  $k_{ET}$ . Inset 2: Arrhenius plots at pH 3.4 and 7.7 from which  $\lambda$  is obtained.



**Figure 2.** A) TD-DFT calculations of the oscillator strength for the electronic transitions of  $\sigma_u^*$  and  $\pi_u$  GSs of Cu<sub>A</sub> centers. B) Experimental UV/Vis spectra of WT, M160Q, Tt-3L-Cu<sub>A</sub> and M160H acquired at pH 7 and 25 °C.

Cu CT transitions at 28000 and 25800 cm<sup>-1</sup> and a  $\psi \rightarrow \psi^*$  intervalence band at 12650 cm<sup>-1</sup> (Figure 2). These features correspond to a  $\sigma_u^*$  GS, as previously determined from TD-DFT calculations.<sup>[10]</sup> These calculations also show that the UV/Vis spectrum of the  $\pi_u$  GS is characterized by a blue shift of the S<sub>Cys</sub>→Cu band to about 28000 cm<sup>-1</sup>, partially overlapping with the N<sub>His</sub>→Cu transitions, whereas the intervalence band appears at 12100 cm<sup>-1</sup>.<sup>[10]</sup> In the M160H and Tt-3L mutants, due to the higher population of the  $\pi_u$  state, the UV/Vis spectra are a convolution of the spectroscopic features of both GS (Figure 2). The intensity ratio of the CT bands at 28000 and 18500 cm<sup>-1</sup> provides an estimate of the  $\sigma_u^*/\pi_u$  relative populations that, as shown in Figure S9, correlate well with the  $\Delta E_{\sigma_u^*/\pi_u}$  values obtained by NMR.

The RR spectra of WT Tt-Cu<sub>A</sub>, M160Q and M160H acquired under 514 nm excitation, that is, in resonance with the S<sub>Cys</sub>→Cu CT of the  $\sigma_u^*$  GS, are all very similar, while Tt-3L presents some small differences (Figure 3 and Figure S10). In all cases they are dominated by normal modes arising from distortions of the Cu<sub>2</sub>S<sub>2</sub> diamond core.<sup>[21,22]</sup> Interestingly, the position of the  $\nu_2$  band (ca. 260 cm<sup>-1</sup>) of WT, M160H and M160Q is slightly sensitive to pH and exhibits an apparent pK<sub>a</sub> of 3.1, while for Tt-3L the spectra are pH-independent. This is in agreement with our recent report of an acid-base transition in WT Tt-Cu<sub>A</sub> with a pK<sub>a</sub> of 3.5 attributed to a small pH-induced effect in the protein matrix, which does not impact on the  $\sigma_u^*/\pi_u$  populations but affects the rate of proton exchange of the equatorial ligand H157.<sup>[23]</sup> Remarkably, the  $\nu_2$  band corresponds to an A<sub>1g</sub> vibrational mode that presents significant contributions of Cu–N, Cu–S and Cu–Cu stretching<sup>[21,22]</sup> and, therefore, is expected to be sensitive to perturbations in H157 through the Cu–N stretching component. Given that the 514 nm laser line selectively excites the  $\sigma_u^*$  GS, the observed changes do not directly reflect changes of  $\sigma_u^*/\pi_u$  populations. Instead, they are most likely related to subtle perturbations of the site in the  $\sigma_u^*$  GS, which in turn

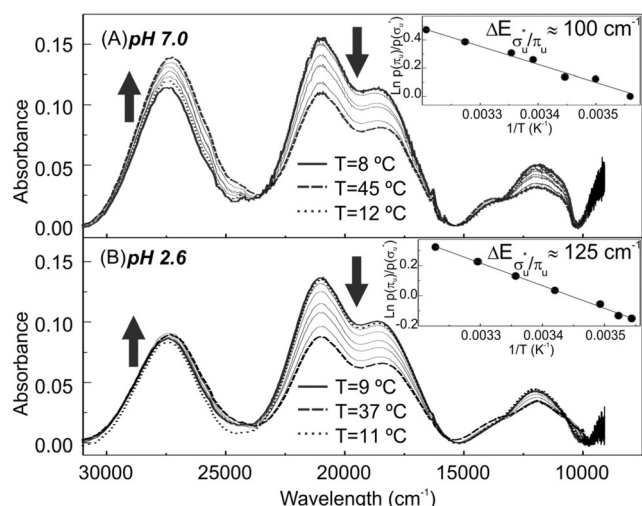


**Figure 3.** Top: RR spectra acquired with 514 nm excitation at pH 5.5 for Cu<sub>A</sub> WT, Tt-3L and M160H. Bottom: pH dependency of the  $\nu_2$  and  $\nu_5$  bands for the studied species. WT and M160H exhibit a transition with an apparent pK<sub>a</sub> of 3.2 for  $\nu_2$ , while for Tt-3L it remains constant within experimental error.

may affect the size of the energy gap in M160H (see below). Consistently, the band  $\nu_5$  (ca. 360 cm<sup>-1</sup>), which corresponds to a normal mode involving almost exclusively the Cu and S atoms that are not solvent exposed, is not affected by pH in any protein variant (Figure 3 and Figure S10). The fact that the RR spectra of the  $\sigma_u^*$  GS for all the protein variants are very similar and vary only slightly with pH for M160H do not allow to describe the changes in the ET rates based on the assumption of a perturbed  $\sigma_u^*$  state. Indeed, a perturbation of the  $\sigma_u^*$  GS eliciting a two-fold increase of  $\lambda$  is expected to result in about 10 cm<sup>-1</sup> upshift of most RR bands that is not observed in the present study.<sup>[24]</sup> We are then left with the possibility that the  $\pi_u$  GS is the redox-active state in the M160H mutant at high pH.

In contrast to the other variants, the electronic absorption spectrum of M160H varies with pH (Figure 4 and S11). Specifically, we observe a reversible variation of the 28000 cm<sup>-1</sup>/18500 cm<sup>-1</sup> relative intensities. The temperature dependencies of the UV/Vis spectra at pH 2.4 and 7, that is, before and after the transition revealed by RR spectroscopy, show that the population of the  $\pi_u$  state increases with pH, thus suggesting a about 25 % smaller  $\sigma_u^*/\pi_u$  energy gap that, according to the electrochemical response, is sufficient to render the  $\pi_u$  GS as the only redox active at high pH, and *vice versa*. EPR spectra show that the  $g_{\parallel}$  value is lowered from 2.219 to 2.210 at low pH, while the mixed valence features are preserved (Figure S12 and Table S2). This change can be interpreted as an increase in the energy gap for the vertical transition from the  $\sigma_u^*$  GS to the  $\pi_u$  excited state (Table S2). These results prompted us to study the energy gap between both GSs by NMR spectroscopy, which (despite being challenging under these extreme pH conditions) is the most





**Figure 4.** Top: UV/Vis spectra of M160H at pH 7.0 (A) and 2.6 (B) at increasing temperatures. After obtaining spectra at the maximum temperatures, the samples were cooled down to acquire new spectra and rule out protein denaturing. Insets:  $\sigma_u^*/\pi_u$  populations and energy gaps obtained from the ratio of the  $S_{\text{Cys}}$  CT bands at  $28\,000\text{ cm}^{-1}$ / $18\,500\text{ cm}^{-1}$  as a function of temperature.

adequate technique for this purpose.  $^1\text{H}$  NMR spectra show perturbed chemical shifts for the four  $\beta$  Cys protons at pH 2.5. The observed shift perturbation at low pH faithfully reproduces that induced by temperature changes that correspond to a larger population of the  $\sigma_u^*$  (Figure S13), that is consistent with a larger energy gap between the two GS at low pH.

Overall, UV/Vis, EPR, and NMR spectroscopy concur in supporting a larger population of the  $\pi_u$  state at high pH in variant M160H. On the other hand, RR shows that there are no perturbations of the  $\sigma_u^*$  state induced by pH changes, allowing us to rule out the existence of a conversion from a symmetric to an asymmetric mixed valence  $\sigma_u^*$  state, as reported for an artificial  $\text{Cu}_A$  site engineered into the blue copper protein azurin ( $\text{Azu-Cu}_A$ ).<sup>[24–26]</sup> Such a conversion involves distinct changes of the vibrational and electronic spectra and a about 150 mV up-shift of the formal potential, that are not observed in the present work. Notably, the energy gap of  $Tt$ -3L is as small as for the M160H mutant, but in this case the UV/Vis and RR spectra are pH-independent. Apparently, substitution of the three loops necessary to create  $Tt$ -3L, which results in replacement of L155, G156, and Q158 in the vicinity of H157, alters the metal site environment to render it insensitive to pH in the studied range.<sup>[12]</sup>

Both the electrochemical and spectroscopic results are consistent with an acid-base equilibrium that modulates the  $\Delta E_{\sigma_u^*/\pi_u}$  value of M160H. The apparent pKas obtained for the adsorbed proteins, however, are upshifted relative to solution values. This is not unexpected as, most likely, it reflects differences of local pH, dielectric constants, and solvent accessibilities of relevant amino acids in the two experimental situations.<sup>[27–29]</sup> Indeed, we determined a similar apparent upshift of the isoelectric point of  $\text{Cu}_A$  upon adsorption that reveals a lower interfacial pH compared to the bulk solution (Figure S14).

In summary, we present compelling evidence of what, to the best of our knowledge, constitutes the first example of a (biological) redox center that can undergo pathways switching resulting from the reversible activation of two alternative ground states. Compared with recent reports from our group,<sup>[10,12]</sup> here we have been able to probe the redox properties of the two states in the same molecular species, the M160H mutant of  $Tt$ - $\text{Cu}_A$ . The switching could not be directly observed for the WT protein with the present experimental approach, presumably due to the larger  $\Delta E_{\sigma_u^*/\pi_u}$  that appears to render it insensitive to pH variations. However, DFT calculations indicate that the nearly degenerate states may be populated with minimal structural perturbations.<sup>[10]</sup> Moreover, the present results show that both states are redox active and capable of participating in heterogeneous ET reactions with comparable efficiency. The increase of  $k_{\text{ET}}$  for the  $\pi_u$  state in spite of the higher reorganization energy suggests that, as a consequence of the GS switching, the preferred electronic pathways are alternated due to redistribution of the electronic density. This hypothesis has been challenged on the basis of theoretical and spectroscopic results for  $\text{Azu-Cu}_A$ .<sup>[30]</sup> This model system has proven very useful for studying structural, electronic and redox features of the  $\text{Cu}_A$  site, as it constituted one of the few robust and accessible  $\text{Cu}_A$  centers.<sup>[9,24,25,31–34]</sup> Recent investigations, however, demonstrated that several properties, including energy gap modulation and pH susceptibility, are markedly different between native and engineered  $\text{Cu}_A$  sites.<sup>[20,23]</sup> Certainly, the study of native and engineered sites provides complementary views of this dinuclear site.

The finding that both GS can actually be redox active in the same protein variant strongly suggests that their thermal equilibrium may constitute the basis of the puzzling efficiency and directionality of electron entry and exit in  $\text{Cu}_A$  sites, as the enhanced superexchange coupling of the  $\pi_u$  state may render it suitable for participating in the ET reaction, even if poorly populated.<sup>[10]</sup> In addition, it puts forward the idea that the features of the  $Tt$ - $\text{Cu}_A$  site, namely its stability and transistor-like properties for the ET reaction, may be exploited for the development of protein-based molecular electronic devices.

## Experimental Section

**Protein preparation:** WT and mutant  $\text{Cu}_A$ -soluble fragments from subunit II of the cytochrome  $ba_3$  from *T. thermophilus* were produced as described previously<sup>[35,36]</sup> and stored in 100 mM phosphate buffer (pH 6.0; 100 mM KCl).

**Electronic absorption spectra:** UV/Vis spectra were acquired with a Thermo Scientific Evolution Array spectrophotometer employing 1 cm or 0.1 cm path length as required. Measurements were performed in either a 10 mM acetate buffer (500 mM  $\text{KNO}_3$ ) or 10 mM phosphate buffer (200 mM  $\text{KNO}_3$ ) as required.

**Resonance Raman spectroscopy:** RR spectra were acquired in backscattering geometry by using Jobin Yvon XY 800 Raman microscope equipped with a CCD detector. See the Supporting Information for further details.

**Cyclic voltammetry:** CV experiments were performed with a Gamry REF600 potentiostat using a water jacketed non-isothermal cell contained into a Faraday cage (Vista Shield). See the Supporting Information for further details.

NMR spectroscopy: NMR experiments were carried out on a Bruker Avance II Spectrometer operating at 600.13 MHz ( $^1\text{H}$  frequency). See the Supporting information for further details.

EPR spectroscopy: X-band CW-EPR measurements were performed on a Bruker EMX-Plus spectrometer. See the Supporting Information for further details.

SPR spectroscopy: Surface Plasmon Resonance experiments were carried out using a SPR Navi 210A instrument at 670 nm. See the Supporting Information for further details.

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