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Dramatic electronic perturbations of Cu_A center via subtle geometric changes

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Abstract

Cu_A is a binuclear copper site acting as electron entry port in terminal heme-copper oxidases. In the oxidized form, Cu_A is a mixed valence pair whose electronic structure can be described using a potential energy surface with two minima: σ_u^* and π_u , that are variably populated at room temperature. We report that mutations in the first and second coordination spheres of the binuclear metallocofactor can be combined in an additive manner to tune the energy gap and, thus, the relative populations of the two lowest-lying states. A series of designed mutants span σ_u^*/π_u energy gaps ranging from 900 to 13 cm⁻¹. The smallest gap corresponds to a variant with an effectively degenerate ground state. All engineered sites preserve the mixed-valence character of this metal center and the electron transfer functionality. An increase of the Cu-Cu distance less than 0.06 Å modifies the σ_u^*/π_u energy gap by almost two orders of magnitude, with longer distances eliciting a larger population of the π_u state. This scenario offers a stark contrast to synthetic systems, as model compounds require a lengthening of 0.5 Å in the Cu-Cu distance to stabilize the π_u state. These findings show that the tight control of the protein environment allows drastic perturbations in the electronic structure of Cu_A sites with minor geometric changes.

Graphical Abstract

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Supporting Information.

EXAFS fittings for oxidized and reduced Cu_A variants, complete ¹H and ¹³C NMR signals assignment, X-band EPR spectrum, UV-Vis spectra deconvolutions and protein film voltammograms. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Cu_A is a binuclear copper center present in cytochrome *c* oxidase (CcO) and N₂O reductase, involved in highly efficient long-range intra- and intermolecular electron transfer processes.^{1,2} This high efficiency has been attributed to its unusual coordination features, which give rise to a rigid site and a unique electronic structure.^{3,4} The two copper ions are bridged by the thiol groups of two cysteine ligands, defining a rigid Cu₂S₂ core (Figure 1). The coordination sphere of the site is completed by two equatorial histidines and two distal, weak axial ligands. Since one copper is bound to a methionine, and the other is bound to a backbone carbonyl, the axial ligands induce a slight asymmetry in the metal core. The oxidized state of the center is paramagnetic, with an unpaired electron delocalized between the two copper ions giving rise to a Type III mixed valence pair, formally Cu^(+1.5) - Cu^(+1.5),^{3,5,6} while the reduced state is a diamagnetic Cu⁽⁺¹⁾ - Cu⁽⁺¹⁾ center.

The electronic structure of oxidized Cu_A is best described using a double-well adiabatic potential energy surface, where the two minima are of different orbital symmetry, σ_u^* and π_u . In all known Cu_A sites, the σ_u^* minimum is at lower energy than the π_u minimum.⁷⁻¹⁰ However, the small σ_u^*/π_u energy gap allows the π_u state to be partially populated at room temperature, rapidly interconverting with the σ_u^* state.^{11,12} Moreover, the σ_u^*/π_u energy gap can be modulated by different perturbations that preserve the mixed valence nature of the center. These perturbations can be induced by replacing the weak axial Met ligand with other residues,^{13,14} through modifications of the secondary coordination sphere,¹⁵ or by pH changes.¹⁶

The σ_u^* and π_u states have been associated with Cu-Cu distances of 2.5 Å and 3.0 Å, respectively. In this way, the Cu-Cu distance has been proposed as the principal reaction coordinate defining the σ_u^*/π_u energy gap, and therefore the relative populations of these levels.^{7,8} The short distance attributed to the low energy σ_u^* state has been substantiated by crystal structures and EXAFS data in several proteins. Structural¹⁷⁻¹⁹ and spectroscopic characterization of the π_u state, however, has traditionally been determined through the study of biomimetic synthetic complexes for which only the π_u level is populated.^{20,21} Surprisingly, perturbed Cu_A centers in proteins with larger contributions of the π_u state do not present features consistent with such a long Cu-Cu distance.^{13,15}

Here we report the rational design of a mixed valence Cu_A center with degenerate σ_u^* and π_u states using the Cu_A fragment from the ba_3 oxidase from *Thermus thermophilus* ($Tt\text{CuA}$). A combination of axially perturbed variants on the $Tt\text{CuA}$ protein, and in a chimeric variant in which three protein loops surrounding the Cu_A site were replaced by those present in the Cu_A domain of the human aa_3 oxidase ($T\beta\text{L}$),¹⁵ allows the analysis of a series of engineered systems in which the σ_u^*/π_u energy gap spans two orders of magnitude. The combined use of spectroscopic techniques at ambient and cryogenic temperatures provides a complete description of the relative populations of the two lowest lying electronic states, demonstrating that minor changes in the Cu-Cu distance have a dramatic effect in the relative population of the σ_u^* and π_u levels. As a consequence, the π_u state stabilized by the cupredoxin scaffold does not display a long Cu-Cu distance, in stark contrast to model compounds. This finding is consistent with the requirement of a rigid metal site for efficient long-range electron transfer, enabling variable electronic structures and suggests possible roles of the π_u in electron transfer.

RESULTS

Rational Engineering of the Cu_A center

Mutation of the equatorial Cys or His copper ligands in the Cu_A site significantly alters its electronic structure, generally disrupting the mixed valence nature of the center.^{22,23} By contrast, mutating the axial Met160 ligand impacts the σ_u^*/π_u energy gap while maintaining the mixed valence character of the metal site.¹³

Effecting a Met160Gln substitution widens the σ_u^*/π_u energy gap from 600 to 900 cm^{-1} , thus diminishing the room temperature thermal-averaged population of π_u ground-state from *ca.* 5% exhibited by WT Cu_A to *ca.* 1%. The Met160His variant exhibits the opposite behavior: its σ_u^*/π_u energy gap is 200 cm^{-1} , corresponding to a room temperature π_u population of *ca.* 28%.¹³ Second-sphere mutations also modify the relative population of these states. A chimeric variant in which the three loops surrounding the Cu_A site in the bacterial protein were replaced by its eukaryotic homologues without modifying the identity of the metal ligands ($Tt\beta\text{L}$, Figure 1) exhibits a σ_u^*/π_u energy gap of 240 cm^{-1} corresponding to a room temperature π_u population of *ca.* 25%.¹⁵

The combination of first and second sphere mutations has been shown to elicit additive effects on the reduction potentials of copper sites in proteins.^{24,25} We reasoned that similar additive effects could be operative in defining the relative energies of the two lowest lying states in the Cu_A site. To test this hypothesis, we introduced mutations Met160Gln and Met160His within the scaffold of $T\beta\text{L}$.

First and second sphere mutants have additive effects on low-lying state energetics but preserve the mixed valence features

Figure 2 shows the UV/Vis absorption spectra of oxidized Cu_A variants obtained by introducing mutations Met160Gln and Met160His on the scaffolds of wild type $Tt\text{CuA}$ and $T\beta\text{L}$. These spectra display the two S(Cys)-Cu ligand-to-metal charge transfer (LMCT) bands at *ca.* 21,000 and 18,000 cm^{-1} characteristic of Cu_A . Additionally, the spectra contain

d-d and intervalence bands around 12,000 cm⁻¹ that are also typical of Cu_A. A simultaneous Gaussian deconvolution of the spectra facilitated by also obtaining circular dichroism spectra (Figures S1 and S2) shows that the same transitions occur in all spectra (Table S1). However, a key point of distinction between the spectra are the intensities of the S(Cys)–Cu LMCT bands, which decrease across the series *T*3L Met160Gln > *T*3L > *T*3L Met160His. An increase in an absorption feature near 27,000 cm⁻¹ attends the decrease of the 21,000 and 18,000 cm⁻¹ band intensities. The 27,000 cm⁻¹ band has been assigned as a LMCT originating from the π_u state.¹⁶ Therefore, these intensity changes diagnose changes in the relative population of the σ_u^* and π_u states. This trend reproduces that reported in the series *T*CuA Met160Gln > *T*CuA > *T*CuA Met160His (Figure 2),¹³ revealing that the first and second shell mutations have an additive effect over this energy gap. Unfortunately, the oxidized *T*3L Met160Gln variant was insufficiently stable to allow NMR spectroscopic characterization.

The ψ - ψ^+ intervalence band in the near-IR region reports on the direct electronic coupling between the Cu ions, as shown by Solomon and coworkers.²⁶ For the Cu_A variants under analysis, this band is shifted across the series from 12,570 (*T*3L) to 12,000 cm⁻¹ (*T*3L Met160His). The coupling energy H_{AB} (estimated as half of the ψ - ψ^+ intervalence band energy) decreases as the π_u population increases (Table 1). *T*3L Met160His shows the most red-shifted transition, suggesting the highest room-temperature population of the π_u state.

The continuous wave X-band EPR spectrum of *T*3L Met160His obtained at 77 K confirms that the mixed valence character is maintained (Figure S3). This variant displays a $g_{//}$ value of 2.237, the lowest in this series of mutants (Table 1).

The reduction potential of *T*3L Met160His at neutral pH is 74 mV, *i.e.* 187 mV lower than *T*CuA, 5 mV lower than Met160His and 168 mV lower than *T*3L, thus showing the additivity of the effect of first and second sphere mutations on the redox potentials²⁷. Protein film voltammetry experiments (Figure S4) yield a reorganization energy for the heterogeneous electron transfer reaction of 0.6 ± 0.1 eV, which is consistent with a π_u redox active molecular orbital, as predicted by quantum mechanical calculations^{7,13} and experimentally verified for the other Cu_A variants.^{13,15,16}

Probing the σ_u^*/π_u population at cryogenic temperatures

The absorption spectrum of *T*3L Met160His was sensitive to temperature changes in the 277–313 K range (Figure S5). This series of spectra show three isosbestic points at 23,500, 16,000 and 13,000 cm⁻¹, consistent with an equilibrium between the two low-lying excited states. To better probe this thermal equilibrium, we recorded UV/Vis absorption spectra at 77 K for all variants (Figure 3 and S6, Table S2). The spectral features of two species (*T*CuA Met160Gln and *T*CuA) are similar at 77 K and at room temperature, while the spectra of the three other variants (*T*3L, *T*CuA Met160His and *T*3L Met160His) are significantly affected by temperature. Indeed, the spectra of *T*CuA Met160Gln, *T*CuA, *T*3L and *T*CuA Met160His (that differ at 298 K) are effectively identical at 77 K, suggesting that this feature represents the low energy σ_u^* state common to all variants. This is not the case for the most perturbed mutant, *T*3L Met160His, which is the only one showing an intense absorption band at 27,000 cm⁻¹, *i.e.*, exhibiting a significant population

of the π_u state at 77 K, suggesting a considerable decrease of the σ_u^*/π_u energy gap. A subtle blue shifting of the $\psi-\psi^+$ intervalence band also supports this trend. These data unequivocally support a resting electronic structure for oxidized Cu_A in which two low-lying electronic states are variably populated following Boltzmann statistics.

Relative population of the σ_u^* and π_u states

The fast equilibrium between the two lowest lying electronic states in resting, oxidized Cua sites can be probed by NMR.^{11,12,28,29} This technique allows a direct estimation of the energy gap between the two states at room temperature, since the observed resonances are located at chemical shift values that correspond to the Boltzmann-averaged shifts of the σ_u^* and π_u states for each nucleus.

Homo- and heteronuclear NMR spectra were recorded, and assignments were carried out via a previously reported strategy (see Experimental Section).¹² The ¹H NMR spectrum of T3L Met160His shows three groups of hyperfine-shifted signals (Figure S7). The four broad signals between 300 and 100 ppm correspond to the Cys C_β protons. Resonances from the His imidazole protons are located between 35 and 11 ppm. Finally, two upfield additional shifts are observed between 0 and -10 ppm that correspond to the Gly115 amide NH proton and the Cys149 H_α.¹²

The ¹³C NMR spectrum obtained for the T3L Met160His variant (Figure 4) shows a distribution of the hyperfine-shifted signals similar to those exhibited by other Cu_A variants. These resonances can be clustered in three groups: (1) the upfield region, including the largely invariant signals from the Cys C_β's, (2) the 400–200 ppm region, comprising mostly the ¹³C resonances from the Cu-bound His imidazole ligands and (3) the 1000–500 ppm region. The latter region contains the signal corresponding to the Cys153 C_α in all variants, and the Cys149 C_α in select cases. These resonances show a large variability in their spectral positions among the different variants, particularly the Cys149 C_α. Consequently, these resonances have been proposed as diagnostic of the relative population of the σ_u^* and π_u states due to the different electron spin density on this carbon in either low-lying state.¹² The Cys149 C_α signal shifts to higher chemical shift values as the π_u population increases, while the Cys153 C_α signal follows the opposite trend. For the Tt3L Met160His mutant, the peak assigned to Cys149 C_α appears at the highest shifts (935 ppm, Table 1) in comparison with previously reported Cu_A containing variants. On the other hand, Cys153 C_α appears at the lowest frequency (613 ppm, Table 1) reported.

The assignment of several hyperfine-shifted signals to the engineered His160 ligand (Table S3) confirms the presence of net electron spin density in this axial ligand, confirming an inner-sphere Cu–N(His) interaction. This phenomenon was also found for the single mutant TtCuA Met160His.¹³

The ¹H and ¹³C chemical shifts from oxidized Cu_A sites show anomalous temperature dependence, i.e., deviating from Curie magnetization law.^{28,30} This behavior has been accounted for the variable population of the σ_u^* and π_u states following a Boltzmann distribution. Analysis of this temperature behavior according to a two-state model provides an estimate of the σ_u^*/π_u energy gap. Global fitting of the temperature-dependent data

afforded energy gap of $13 \pm 8 \text{ cm}^{-1}$ for *T3L* Met160His, revealing that this variant has effectively degenerate σ_u^* and π_u states.

Cu-Cu distance of the variants

Cu K-edge X-ray absorption spectra (XAS) including extended X-ray absorption fine structure (EXAFS) were obtained for the five stable variants: *TiCuA* Met160Gln, *TiCuA*, *TiCuA* Met160His, *T3L* and *T3L* Met160His. Figure S8, S9 and Table S4 show the results of fitting the EXAFS data corresponding to oxidized samples of Cu_A variants. For *TiCuA* and *T3L*, an extra S-atom was added for the fitting to account for the Met ligand. This scatterer was replaced by an N/O-atom to fit the spectra of mutants Met160Gln and Met160His. Improvements to the fits suggesting that the introduced axial ligands bind the metal center without inducing profound changes to the overall structural unit. Data analysis shows that the primary coordination spheres about each Cu are largely unaltered, with differences less than 3% for Cu-S and Cu-N distances among the variants. As expected, Cu_A sites in reduced samples reveal *ca.* 2% longer distances for Cu-S and Cu-N bonds when compared to distances obtained from fitting data corresponding to the oxidized samples.

A key finding of these measurements is that the Cu-Cu distance of the different variants changes less than 0.06 Å across the series. Despite these small changes, larger Cu-Cu distances correlate with larger σ_u^* - π_u energy gaps (Table 1). Indeed, the σ_u^*/π_u energy gap shows a decreasing exponential dependence with the Cu-Cu distance (Figure S10), disclosing a net trend.

DISCUSSION

Primary- and second-sphere variants were produced of the Cu_A site in subunit II from the *ba3* oxidase from *Thermus thermophilus*. This series of Cu_A variants possess energy gaps between the two lowest lying electronic states, σ_u^* and π_u , spanning almost two orders of magnitude: 900 to 13 cm^{-1} . This translates to variants where the room-temperature π_u population is *ca.* 1% to a variant *T3L* Met160His, where the two states are effectively equally populated. The primary- and secondary coordination sphere mutations have an additive effect on the relative energies of the alternative ground states σ_u^* and π_u in Cu_A .

The experimentally determined reorganization energy measured for *T3L* Met160His agrees well with the value expected for a redox reaction taking place exclusively from the π_u ground state, despite the fact that the π_u and σ_u^* states are equally populated at room temperature, and that the reorganization energy for the σ_u^* state is only 0.3 eV.^{7,13}

Several spectroscopic parameters can be used as independent probes of the relative population of the σ_u^* and π_u states, that are summarized in Table 1. The availability of constructs with distinct energy gaps facilitates evaluation of these parameters as this energy gap changes. The ratio of LMCT bands distinctive of each state ranges from a 3:1 to a 1:3 ratio in the series of mutants (Table 1), providing an estimate of the population ratio at room temperature of both states. The $g_{\parallel}$ value in the EPR spectra reflects the energy difference between the σ_u^* ground state and a π_u Frank-Condon excited state, in accord with the model proposed by Solomon and coworkers.⁷ This energy calculated for the whole series of

mutants follows the trend displayed by the σ_u^*/π_u energy gap determined by NMR, as shown in Table 1, giving strong support to the double-well description of the electronic structure of Cu_A (Figure S11).

Boltzmann statistics predict that only *T3L* Met160His should exhibit a net population of the π_u state at 77 K. Indeed, this is the only variant showing an intense band at 27,000 cm⁻¹. The observed π_u population based on the ratio of the LMCT bands is *ca.* 20%. This corresponds to an energy gap of 50 cm⁻¹, in very good agreement with the 13 cm⁻¹ gap determined by NMR at room temperature.

The Cu–Cu distance has been proposed as the main reaction coordinate dominating the relative population of the two ground states, with energy minima corresponding to 2.5 Å (σ_u^*) and 3.0 Å (π_u).^{7,26} However, experimental information supporting these distances were retrieved from structures of Cu_A sites in proteins and in model compounds, respectively. Our present data show that the population of these two levels can be modulated significantly with a change in Cu–Cu distance less than 0.06 Å. Although EXAFS distances have an error of *ca.* ±0.02 Å, there remains a correlation between the intermetallic distance and the energy gap, with longer Cu–Cu bonds favoring a population of the π_u level. However, a key point emerges: dramatic geometric changes are not required to effect substantial changes to electronic properties governing reactivity. In the present case, the electronic structure of Cu_A sites in proteins can be drastically modulated with minor structural changes, such as those that might be observed when redox partners bind to the protein. The amplification of minute structural perturbations contributed by environment and allostery as profound changes in electronic structure may be one more key means by which biology controls electron flow and merits further inquiry.

EXPERIMENTAL SECTION

Protein expression and purification

Met160Gln and Met160His mutants of *T3L* were obtained by PCR-based quick-change mutagenesis method over the *T3L* DNA encoding sequence cloned into a pET9a vector. The plasmid sequences were confirmed via DNA sequencing.

All Cu_A-soluble fragments from subunit II of the *ba₃* oxidase from *Thermus thermophilus* were expressed from pET9a (KanR) series transformed in *E. coli* BL21 or Overexpress C41(DE3) strains, growing in either rich LB medium or M9 minimal medium supplemented with labeled or unlabeled ammonium sulfate (1.2 g/L, 99% ¹⁵N when labeled) and glucose (4 g/L, 99% ¹³C when labeled), respectively, according to the desired labeling scheme. Typical yields were approximately 20 mg L⁻¹ and 35 mg L⁻¹ for labeled and unlabeled samples, respectively. Purification of the proteins from cell lysates was done by ammonium sulfate precipitation followed by a Superdex-75 gel filtration step, as described previously.^{12,31,32} Recombinant protein identity was verified by ESI-MS, obtaining a unique peak at 14071±2 Da, coherent with the predicted molecular weight for this mutant: 14070.7 Da. Protein samples for NMR experiments were prepared and stored in phosphate buffer (100 mM, pH 6) with KCl (100 mM) in either 10% or 100% D₂O as required for each experiment, and concentrated to 250–500 mL of 0.6–2 mM protein concentration.

Optical spectroscopy

Room temperature electronic spectra were acquired with a Jasco V-670 spectrophotometer employing 1 cm or 0.1 cm path length as required, placed into a jacketed cell-holder for temperature control through a circulation thermostat. Measurements were performed in phosphate buffer.

The variations of the populations of the alternative ground-states were estimated by considering that $S_{\text{cys} \rightarrow \text{cu}}$ LMCT absorption bands are centered at 27,000 and 21,000 cm^{-1} for the π_u and σ_u^* ground states, respectively. For the calculations we corrected by relative molar absorptivity and by the contribution of the overlapping LMCT $N_{\text{His} \rightarrow \text{cu}}$ band.²⁷ Thus, the probability of populating the π_u ground-state (P_π) for the different variants at different conditions were calculated as:

$$P_\pi = \frac{A_{27000} - 0.32 \cdot A_{21000}}{A_{27000} + A_{21000}}$$

Circular dichroism spectra were acquired with a Jasco J-810 spectrophotometer employing 1 cm or 0.1 cm path length as required, placed into a jacketed cell-holder for temperature control through a circulation thermostat. Measurements were performed in phosphate buffer.

For low temperature electronic spectra acquisition, lyophilized protein samples were resuspended in water, in order to regenerate the saline phosphate buffer, and glycerol was added up to 40%. Spectra were obtained in glass 5 mm-diameter NMR tubes, by using a Cary 60 spectrophotometer adapted to accommodate a glass liquid nitrogen dewar, at 77 K. Four spectra were obtained for each sample, measured over the same point, and averaged for report in this work.

Electronic spectra were deconvoluted by automatic simultaneous fitting of electronic and circular dichroism spectra with gaussian bands.

NMR spectroscopy

NMR experiments were carried out on a Bruker Avance II Spectrometer operating at 600.13 MHz (^1H frequency). ^1H NMR spectra were acquired with a triple-resonance (TXI) probe head using presaturation, PASE and SuperWEFT schemes on spectral windows of 48 – 360 kHz (total recycle times around 300 and 40 ms respectively). ^1H , ^{13}C HMQC experiments were acquired on spectral widths of approximately 50 kHz in the ^1H dimension (1024 points) and approximately 100 kHz in the indirect dimension (32 points). The delay for coherence transfer was set to 2 ms, and the relaxation delay was set to approximately 30 ms. Saturation Transfer Difference (STD) and NOE experiments were performed on samples prepared in 100% D_2O buffer, either completely oxidized (NOE) or partially reduced (STD; ~10% reduced protein, by addition of sodium ascorbate). Signals were irradiated for ~50 ms at a power of ~5 mW.

^{13}C NMR spectra were acquired with a broadband observe (BBO) probehead tuned at the proper frequency, using an excitation pulse of 6.9 μs at 12.6 W and applying inverse gated

decoupling. For ^{13}C NMR experiments, the carrier frequency was set to 800, 200, or -450 ppm depending on which signals were being studied. The spectral window and delays also varied in each experiment, which resulted in total recycle times of approximately 20 ms for the acquisition of signals around 1000 and -450 ppm, and approximately 100 ms for those near the diamagnetic region. No further signals were detected when the carrier was moved to different frequency offsets.

Analyses of the chemical shifts and their temperature dependences were performed as described previously.²⁸

X-Ray Absorption Spectroscopy

X-Ray absorption spectra were acquired in Stanford Synchrotron Radiation Lightsource (SSRL) under 3.0 GeV and 500 mA ring energy. Cu-K edge EXAFS measurements were collected using beamline 7-3. Samples were maintained at 10 K a liquid helium cryostat. A Si(220) double-crystal monochromator was used for energy selection, and a Rh-coated mirror (set to an energy cutoff of 13 keV) was used for harmonic rejection. Internal energy calibration was performed by assigning the first inflection point of an Fe foil spectrum to 8980.3 eV. Data were collected in fluorescence mode (windowed onto Cu $K\alpha_1$) using a Canberra 30-element Ge solid-state detector perpendicular to the incident beam. Elastic scatter into the detector was attenuated using a Soler slit with an upstream Ni filter. After acquisition, EXAFS were fitted using EXAFSPAK³³ with input scattering paths calculated and FEFF7 calculations.^{34,35} The selected algorithm allowed distance and Debye-Waller factors variations as fixes the coordination numbers.

Electrochemical determinations

Cyclic voltammograms of protein solution samples were acquired with a Gamry REF600 potentiostat in a cell equipped with a mercaptohexanol-coated polycrystalline gold electrode, a platinum wire counter electrode and an Ag/AgCl Dri-Ref (3 M KCl) reference electrode. Electrodes were positioned in a non-isothermal reduced-volume electrochemical cell in order to minimize the distance among them and maintaining a constant geometry. Protein samples were concentrated to *ca.* 0.3 mM (10 mM phosphate buffer, 500 mM KNO_3 , pH 7) before measurements.

The electron transfer reorganization energy was determined by protein-film voltammetry using Au electrodes coated with self-assembled monolayers of 1-mercapto-6-hexadecanol and 1-hexanethiol in 6:4 proportion. Electrodes were incubated in a 100 mM protein solution (phosphate buffer 10 mM, pH 7.0) for 2 hours. Voltammograms were acquired in 10 mM phosphate buffer, pH 7.0, with 200 mM KNO_3 as support electrolyte.

Heterogeneous ET rate constants at zero over-potential were determined using the Laviron's method.³⁶ This method employs a working curve to determine the rate constant from separation of anodic and cathodic peaks as a function of the scan rate. From the widths at half-height of the voltammetric peaks we obtained a charge transfer coefficient of 0.57, which is within the range required for the method. Rate constants were determined as a function of temperature between 5 and 40 °C, and the reorganization energy λ was extracted

from Arrhenius plots, under the usual approximation of negligibly small activation entropies, *i.e.* $G^* \approx H^* \approx \lambda/4$.

EPR spectroscopy

EPR spectrum was obtained from a sample of oxidized *Tf3L* Met160His in phosphate buffer (100 mM) at pH 6 with KCl (100 mM). Spectra were measured on a Bruker EMX-Plus spectrometer equipped with an X-band microwave bridge (microwave frequency, 9.4 GHz) and a continuous-flux cryostat Oxford. EPR parameters: sample temperature: 20 K; microwave frequency: 9.39 GHz; microwave power: 2 mW; field modulation: 100 kHz; modulation amplitude: 4 G. The EPR data were analyzed and simulated with fitting toolbox EasySpin based on MATLAB.³⁷

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(Left) The Cu_A site from *Thermus thermophilus* ba₃ oxidase (*TtCuA*, PDB ID: 2CUA). The loops replaced in the *Tt3L* variants are colored. (Right) Protein sequence alignment for Cu_A variants. Metal ligands are highlighted and color code is preserved.

Figure 2.
Absorption spectra for *TtCuA* (center left), *Tt3L* (center right), and their Met160Gln (top) and Met160His (bottom) mutants.

Figure 3.
Experimental absorption spectra of *TtCuA* and *T3L*, and their mutants *TtCuA* Met160Gln, *TtCuA* Met160His and *T3L* Met160His, recorded at room temperature (right) and low temperature (left).

Figure 4. ^{13}C NMR spectra of *TtCuA*, *Tt3L*, *TtCuA* Met160Gln, *TtCuA* Met160His and *Tt3L* Met160His mutants. The location of the $\text{C}\alpha$ signal from Cys149 is highlighted for all the variants.

Table 1.

Summary of spectroscopic parameters for Cu_A variants.

Cu _A Variant	E (NMR), (cm ⁻¹) ^a	$\sigma_u^*:\pi_u$ RT Pop. ^b	g//	E (EPR), cm ⁻¹ ^c	H _{AB} , cm ⁻¹	LMCT band ratio ^d	$\sigma_u^*:\pi_u$ RT Pop. ^e	$\delta\text{Cys149C}_\alpha$, ppm	$\delta\text{Cys153C}_\alpha$, ppm	Cu-Cu distance (Å) ^f
TCuA Met160Gln	900±300	99:1	2.190	4,810	6,375	3	97:3	153	ND	2.45±0.02
TCuA	600±200	95:5	2.195	4,690	6,325	1	96:4	295	881	2.46±0.02
TT3L	240±87	75:25	2.210	4,390	6,285	0.8	81:19	539	832	2.47±0.02
TCuA Met160His	200±50	72:28	2.230	4,126	6,050	0.3	68:32	830	647	2.47±0.02
TT3L Met160His	13±8	51:49	2.237	3,840	6,000	0.3	51:49	935	613	2.50±0.02

^a $\sigma_u^*:\pi_u$ energy gap at room temperature (RT) estimated by the temperature dependence of the NMR chemical shifts.^b Calculated by assuming a two-state Boltzmann distribution with the $\sigma_u^*:\pi_u$ estimated by NMR.^c $\sigma_u^*:\pi_u$ energy gap estimated from g// values.^d A27000/A21000 ratio, obtained from the absorption spectra.^e Estimated from the UV-Vis band ratio (see Experimental Section).^f Obtained from the EXAFS fittings. Expected errors in EXAFS distances are ±0.02 Å.