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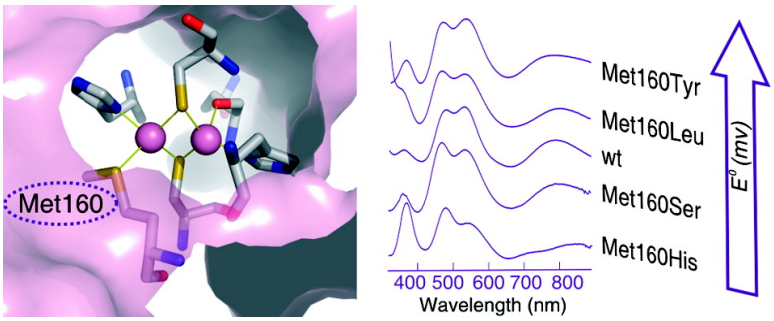

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The Met Axial Ligand Determines the Redox Potential in Cu_A Sites

Gabriela N. Ledesma,[†] Daniel H. Murgida,[‡,§] Hoang Khoa Ly,[‡] Hainer Wackerbarth,[¶] Jens Ulstrup,[¶] Antonio J. Costa-Filho,[#] and Alejandro J. Vila*,[†]

Instituto de Biología Molecular y Celular de Rosario-CONICET, Universidad Nacional de Rosario, Suipacha 531, S2002LRK, Rosario, Argentina, Max Volmer Laboratory of Biophysical Chemistry, Institute of Chemistry, Technical University of Berlin, Strasse des 17. Juni, D-10623, Berlin, Germany, INQUIMAE-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria C1428EHA, Buenos Aires, Argentina, Department of Chemistry and NanoDTU, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark, and Instituto de Física de São Carlos, Universidade de São Paulo, C.P. 369, CEP 13560-950, São Carlos, SP, Brazil

Received May 3, 2007; E-mail: vila@ibr.gov.ar

Copper ions in proteins are broadly essential in biological electron-transfer chains.[1−4] The crucial importance of the protein environment in poising the metal ions for electron transfer is clear, but the details for optimal control are still largely unknown. Comprehensive efforts addressing blue single Cu centers have notably unraveled weakly bound axial ligands as a key feature in redox potential fine-tuning.[3−8] Instead, the role of weak metal ligands in tuning the redox potential of the Cu_A site (the electron entry port in cytochrome *c* oxidases) is not clear. This site contains two copper ions bound to two bridging Cys, two terminal His, and two weakly bound axial ligands: a Met and a backbone carbonyl (Figure 1).[9−12] In most cases, mutagenesis of the Cu_A site has disclosed major structural perturbations even to the extent that the integrity of the metal center is jeopardized.[11,13] Replacement of the axial Met by hydrophobic residues in the oxidases from *Rhodobacter sphaeroides*[14,15] and *Pseudomonas denitrificans*[16] increased the redox potential of this center, impairing intramolecular electron transfer. However, a thorough mutagenesis study in Cu_A−azurin revealed no influence of the Met ligand in the redox potential.[17] In order to resolve this intriguing difference, we have studied a series of axial Met ligand mutants of the soluble subunit II from *Thermus thermophilus* ba_3 oxidase[18,19] and we found that they lead to significant changes in the redox potential of this dimetallic center even by minor electronic structure perturbations.

The electronic absorption and EPR spectra of wild-type (wt) Cu_A and four mutants in position 160 are shown in Figure 2. The spectra show the same essential optical features of native Cu_A centers: two intense $S_{cys} \rightarrow$ Cu charge-transfer (CT) bands at 480 and 530 nm; a near-IR band centered at 790 nm assigned to a class III mixed valence Cu−Cu $\psi \rightarrow \psi^*$ transition, and a weaker band around 365 nm attributed to a His → Cu CT.[20,21] The most significant variations among the spectra are the relative intensity of the CT peaks and the maximum of the near-IR band. Introduction of a Gln,[19] Ser, or Tyr residue replacing Met160 does not perturb significantly the near-IR band. Instead, M160H and M160L mutants display 64 and 15 nm red shifts, respectively, compared to the wt center. Similar red shifts have been interpreted as a lengthening of the Cu−Cu bond in the Cu_2S_2 core.[19,22] In the case of M160H, this phenomenon can be attributed to the introduction of a more strongly bound metal ligand in the coordination sphere of one of the copper ions. M160H also gives a 4-fold more intense band at 366 nm, suggesting that at least one of the equatorial Cu−His interactions has been perturbed by this mutation. A similar effect has been reported for the Met123Glu mutant in Cu_A−azurin.[17] The smaller effect in M160L can be due to a steric perturbation in the side chain surroundings.

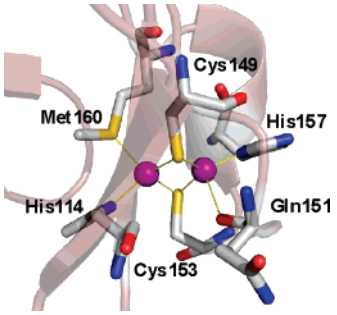

Figure 1. Copper ligands of the purple Cu_A site (PDB 2CUA).

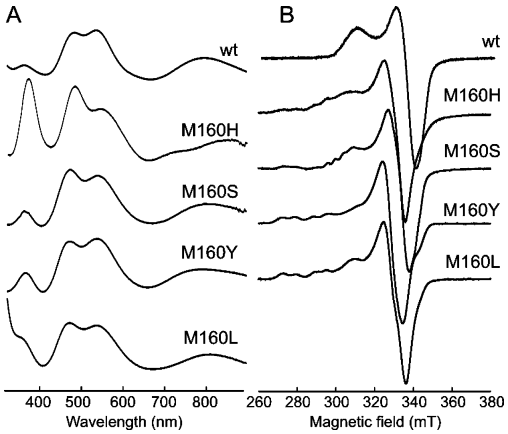

Figure 2. (A) UV−visible absorption spectra and (B) X-band EPR spectra recorded at 77 K of wt Cu_A and its Met160 mutants.

The X-band EPR spectra of wt Cu_A and the four axial ligand mutants (M160H, M160S, M160Y, and M160L) are also shown in Figure 2. The EPR spectrum of the wt protein shows a typical Cu_A signal ($g_\perp = 2.010$, $g_{//} = 2.195$), with no resolved Cu hyperfine structure in the $g_{//}$ region as previously observed.[19,21,23] Simulation of the EPR spectra from the four axial mutants (Tables 1 and S1) reveals that a mixed valence form with $g_\perp \sim 2.010$ and $g_{//} \sim 2.200$ is preserved (Table S2 and Figure S3), in agreement with the conservation of the optical features. Moreover, small but significant changes in the $g_{//}$ values for M160H and M160L mutants compared to the values determined for the wt spectrum are observed (Table 1). This suggests a larger perturbation by the axial mutation in these mutants. In addition, as observed in other mutagenesis studies, some valence localized species are formed, as revealed by the presence

[†] Universidad Nacional de Rosario.
[‡] Technical University of Berlin.
[§] Universidad de Buenos Aires.
[¶] Technical University of Denmark.
[#] Universidade de São Paulo.

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Table 1. X-Band EPR Parameters and Reduction Potential (in mV) of Cu_A Center from *Thermus thermophilus* Oxidase Subunit II and its M160 Mutants

	EPR parameters			
Mutant	$A_{//}$	$g_{\perp}$	$g_{//}$	E°
wt	3.1[a]	1.99[a]	2.17[a]	293 ± 6[b]
		2.00[a]		297[c]
		2.010[b]	2.195[b]	
M160H[b]	ND[d]	2.020	2.230	148 ± 9
M160S[b]	3.9	2.009	2.195	209 ± 10
M160Y[b]	ND[d]	1.99	2.185	348 ± 2
M160L[b]	ND[d]	2.000	2.220	346 ± 5
M160Q	4.2[a]	2.02	2.19[a]	158 ± 2[b]
		2.02[a]		

[a] From ref 19. [b] This work. [c] From ref 24. [d] ND = not detected.

of typical $A_{//}$ for T2 centers (Table S2).[13,17,19] This could be induced by structural instability of the metal site caused by the mutations. M160S Cu_A was the only mutant showing a clearly defined seven-line hyperfine structure ($A_{//}$ = 3.9 mT) in its EPR spectrum. Overall, the spectroscopic data show that the mixed valence center is preserved in all variants, with only minor perturbations.

Reduction potentials of wt Cu_A and the M160 mutants (including M160Q[18,19]) were determined either by CV or by UV−vis spectroelectrochemistry (Table 1 and Figures S1 and S2). In all cases, we observe well-behaved almost reversible electrochemical responses. CVs exhibit peak widths and scan rate dependences characteristic of a one-electron transfer process in a protein monolayer, suggesting a single electrochemically active Cu_A in each studied case. Introduction of His, Ser, and Gln residues in the axial position decreases the redox potential compared to the wt value, while M160Y and M160L mutations cause an increase of >50 mV. The largest potential change was a 145 mV decrease for M160H, in line with the larger electronic structure perturbation in this mutant. This behavior contrasts with that reported for Cu_A−azurin, where mutagenesis of the Met ligand by Asp, Glu, or Leu in this protein slightly alters the reduction potential (±16 mV; Table S3).[17] The much larger effects reported in this work show that the axial ligand plays a key role in tuning the redox potential in Cu_A. Indeed, the present results strongly support the mutagenesis studies in whole oxidases that revealed an increase of 120 mV in the redox potential by introduction of Leu or Ile residues in this position.[14−16] The apparent discrepancy with the results in Cu_A−azurin can be accounted for by the different Cu−S(Met) distances in these proteins (2.98/3.16 Å in Cu_A−azurin[25] vs 2.46 Å in the *Thermus* fragment[9]). Inspection of the structures of the full oxidases reveals that this parameter ranges from 2.36 Å in the *Rhodobacter* enzyme[26] to 2.61 Å in the *Thermus* enzyme,[27] suggesting this fragment is an appropriate model for the whole oxidases (cf. Table S4). Cu_A−azurin displays the shortest Cu−Cu distance among all Cu_A sites (2.42/2.35 Å), thus representing a more robust site,[28] being less sensitive to changes in a farther Met ligand.

A conserved Met residue as weakly bound axial ligand in the most exposed copper ion is a robust feature of natural Cu_A sites. In contrast, there are native blue copper sites with a Met, Gln, or even no axial ligand at all. This diversity has been interpreted as a way to tune the redox potential to match the functional needs of each system that spans a wide range of partners. Instead, Cu_A sites are only present in cytochrome *c* oxidases, N_2O, and NO reductases,[29,30] with conserved electron-transfer partners. This suggests that a Met residue has been selected by nature as the optimal ligand in this position. While still preserving the mixed valence nature of the center, other residues could thus induce redox potentials that would not match their biochemical roles. More studies are needed to address the impact of replacing the Met ligand in the reorganization energy of the Cu_A center.[14−16,31]

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Supporting Information Available: Table of UV−vis and X-band EPR parameters, CVs and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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