Mutations of the Weak Axial Ligand in the *Thermus* Cu_A Center Modulates Its Electronic Structure

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Here we report the first stable, axial ligand mutations,[1] Met160Gln and Met160Glu, of a Cu_A center in the *Thermus thermophilus* ($TtIICu_A$) fragment of cytochrome ba_3.[2a] The binuclear, delocalized ($S = {}^1/_2$) Cu_A center serves as the initial electron acceptor in cytochrome *c* oxidases (COX) and nitrous oxide reductase (N_2OR).[3] The Cu_A domains and the type 1 (T1) blue copper centers are members of the cupredoxin superfamily[4] of electron transfer (ET) proteins.[5] The Cu_A centers have two bridging Cys thiolates, two terminal His imidazole ligands, and one weak axial ligand to each copper, a Met thioether or the peptide carbonyl of Glu (Figure 1). The weak axial ligands give each Cu a distorted tetrahedral geometry,[6] similar to the T1 centers.[6e]

Molecular orbital calculations[7] have suggested a rational for the conservation of the Met ligand in the T1 centers despite a long bond distance, 2.9 to 3.1 Å; namely, that it modulates the spin density and reduction potential (E°). Replacements of Met 121 in *Pseudomonas aeruginosa* azurin[8] have substantiated the prediction that stronger axial ligands should decrease E°s; however, mutants such as Met121Gln, an analogue to stellacyanin, have higher E°s than expected, suggesting that other factors are also significant. Gamelin *et al*.[9] have proposed that the weak ligand can also modify the important ET characteristics of the binuclear Cu_A center. Specifically, stronger axial ligand interactions should favor larger Cu−Cu distances and a distortion of the Cu−S core that would alter the spin density distribution, reduction potential, reorganization energy, and ultimately, the ET rate.

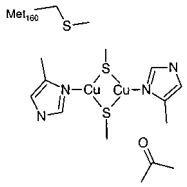

Figure 1. Cu_A center.

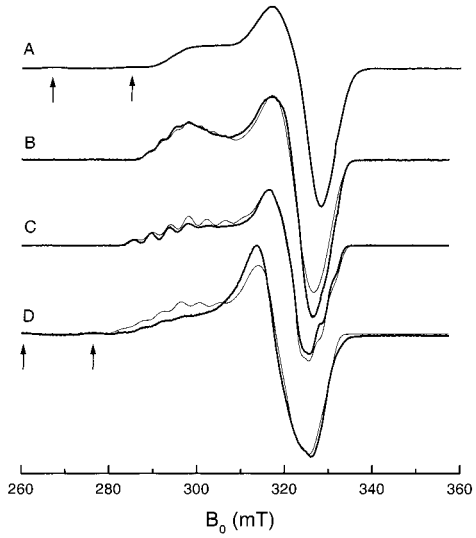

Figure 2. X-band (9.13 GHz) EPR spectra recorded at 11 K of (A) wt, (B) T9wt, (C) Met160Gln, and (D) Met160Glu. Solid lines are experimental data, and thin lines are simulated spectra. Arrows indicate the T2 signal. (100 mM potassium phosphate/200 mM KCl (A) and 50% glycerol (B and C), pH 8.0 or 100 mM sodium acetate/200 mM KCl, pH 4.2 (D)).

These ideas have been difficult to verify experimentally. Stable weak ligand mutations in Cu_A fragments have been elusive; in an engineered Cu_A center constructed in a soluble fragment of the *Escherichia coli* quinol oxidase (CyoA), the Met118Gly mutation prevents formation of the Cu_A site.[2f] Consequently, the only data available are from the larger and more complicated N_2-OR and COX proteins.[10]

Figure 2 shows the EPR spectra of the wild-type (wt) $TtIICu_A$ soluble fragment characterized previously[2a] and mutated Met160Gln and Met160Glu centers. In addition, the spectrum of a soluble fragment which is 10 amino acids shorter (T9wt) than the original fragment[2a] is shown in trace B. Absent from the T9wt fragment is a His residue which is a ligand in a type 2 (T2) center present in preparations of the longer *Thermus* fragment[11] (trace 2A). Unlike wt, the T9wt spectrum shows resolved Cu hyperfine structure in the g_z region and no detectable T2 center. This

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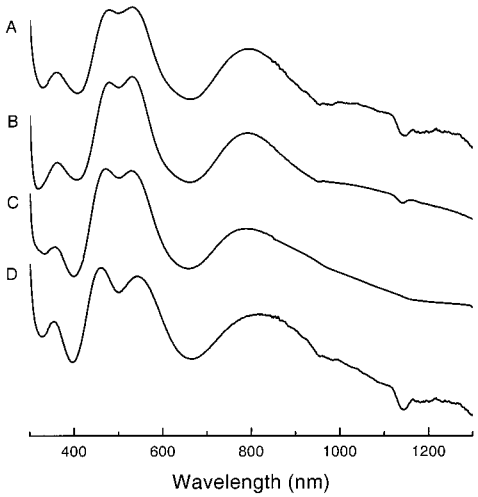

Figure 3. Absorption spectra of (A) wt, (B) T9wt, (C) Met160Gln, and (D) Met160Glu in 100 mM potassium phosphate/200 mM KCl buffer, pH 8.0.

Table 1. X-band EPR Paramenters of Characterized Cu_A Centers: A_z, the z-Component of the Cu Hyperfine Interaction; the *g*-Values and Symmetry

fragment	A_z (mT)	g_z	g_y	g_x	symmetry
T9wt $TtIICu_A$	3.1	2.17	2.00	1.99	axial
M160Q ($TtIICu_A$)	4.2	2.19	2.02	2.02	axial
M160E ($TtIICu_A$)	4.2	2.20	2.05	2.00	rhombic[14]
$PdIICu_A$[12d]	3.1	2.19	2.03	1.99	axial
$BsIICu_A$[2e]	3.82	2.18	2.03−1.99	2.03−1.99	axial
purple azurin[2d,12b]	5.5	2.17	2.06	2.06	axial
purple amicyanin[2e]	3.24	2.18	1.99−2.02	1.99−2.02	axial
purple CyoA[12d]	6.8:5.3	2.20	2.02	2.00	axial[a]
PsN_2OR[3]	3.83	2.18	2.03	2.03	axial

[a] Slightly valence trapped.

indicates that the loss of resolution in the g_z region of wt is due to overlap with the T2 signal. Simulations of the T9wt spectrum (Figure 2B), where the two coppers were taken as magnetically equivalent,[13] yield an A_z value of 3.1 mT that is in agreement with a previously published multifrequency EPR characterization of ^{65}Cu-labeled wt TtIICu.[11a]

The mutations affect the EPR spectra, particularly the g_z region, where the Cu hyperfine becomes more resolved and larger in both mutants. Simulations using the program described in ref 13 of these spectra give the EPR parameters listed in Table 1. Interestingly, A_z of this limited series of Cu_A centers span the range of A_z found in most characterized Cu_A centers. Only two engineered Cu_A centers, purple azurin and CyoA, have larger hyperfine coupling constants (A_z > 5.0 mT) than these mutants (A_z = 4.2 mT). Additionally, the EPR spectrum of Met160Glu is more rhombic than that of wt.[14] Similar strong axial ligand mutants resulting in rhombic EPR spectra have been reported for the T1 centers.[15] A survey of published X-ray structures and EPR data, shows that T1 centers with rhombic EPR spectra have strong axial ligands (2.6 Å) and a Cu(II) about 0.3 Å out of the plane of the equatorial ligands. Axial EPR spectra correlate with weak axial ligands (2.9 to 3.1 Å) and a smaller Cu(II) displacement (0.1 Å). Given the structural similarity of the T1 and Cu_A sites, we expect a similar correlation in this series of purple centers.

Table 2. Optical Absorption Band Maxima for Characterized Cu_A Sites[a]

fragment	source	absorption maxima (nm)				Cu−Cu distance (Å)
wt and T9wt $TtIICu_A$	*T. thermophilus*[2a]	363	480	530	790	2.43[12a]
M160Q ($TtIICu_A$)	*T. thermophilus*	355	471	529	789	
M160E ($TtIICu_A$)	*T. thermophilus*	354	463	542	815	
$PdIICu_A$	*P. denitrificans*[2b]	354	480	530	808	2.6[6a]
$BsIICu_A$	*B. subtilis*[2c]	365	480	530	790	2.44[12a]
purple azurin	*P. aeruginosa*[2d]	350	485	530	765	2.39[12b]
purple amicyanin	*T. versutus*[2e]	360	483	532	790	
purple CyoA	*E. coli* [2f,5b]	358	475	536	765	2.48[6d]
AcN_2OR	*A. cycloclastes*[2g]	350	481	534	780	
PsN_2OR	*P. stutzeri* [2h]	350	480	540	780	2.44[12c]

[a] Cu−Cu distances are from X-ray structures where available and EXAFS data.

The Met160Glu and Met160Gln optical absorption spectra (Figure 3) exhibit changes in the S−Cu charge transfer (CT) doublet[16] centered near 500 nm and the near infrared (IR) transition at 790 nm (Table 2). Both mutants show a greater intensity and a blue shift of the higher energy CT partner of the doublet relative to wt. The maxima of this band are 480, 471, and 463 nm for wt, Met160Gln, and Met160Glu, respectively. In the T1 centers, the blue shift of the S−Cu CT band in Met121Gln is consistent with a sulfur-to-oxygen substitution and a stronger ligand field.[15,17] Met160Glu also shows a pronounced red shift of the near-IR transition relative to wt, from 790 to 815 nm. The unusually close Cu−Cu distances found in the Cu_A sites compared to those in the synthetic compounds produce a distinctive higher energy d−d band in the protein sites.[9,18] λ_{max} of this transition is larger than that (808 nm) observed in the $PdIICu_A$ center (Table 2), suggesting that the Cu−Cu distance of Met160Glu is ≥2.6 Å. Gamelin *et al.*[9] have noted that the orbital splitting should be very sensitive to geometry changes in the Cu_A core. This prediction is particularly pertinent to the Met160Glu mutant where an elongated Cu−Cu distance would alter the Cu−S−Cu angle of the core.

These EPR and optical characteristics suggest that the order of increasing axial ligand strength should be wt < Met160Gln < Met160Glu. Significantly, stronger axial ligand interactions shift more spin from the ligands—mainly the sulfur ligands—onto the copper nuclei. A_z increases from 3.1 mT in wt to 4.2 mT in Met160Gln and Met160Glu. Thus, the optical and EPR differences support the view that the axial ligands are plausible modulators for altered spin density distribution and core geometry.

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