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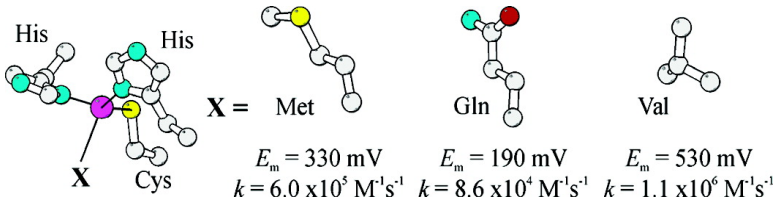

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Reduction Potential Tuning at a Type 1 Copper Site Does Not Compromise Electron Transfer Reactivity

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Abstract: Type 1 (T1) copper sites promote biological electron transfer (ET) and typically possess a weakly coordinated thioether sulfur from an axial Met [Cu(II)−S^{δ} ∼ 2.6 to 3.3 Å] along with the conserved His_2Cys equatorial ligands. A strong axial bond [Cu(II)−$O^{\epsilon 1}$ ∼ 2.2 Å] is sometimes provided by a Gln (as in the stellacyanins), and the axial ligand can be absent (a Val, Leu or Phe in the axial position) as in ceruloplasmin, Fet3p, fungal laccases and some plantacyanins (PLTs). Cucumber basic protein (CBP) is a PLT which has a relatively short Cu(II)−S(Met89) axial bond (2.6 Å). The Met89Gln variant of CBP has an electron self-exchange (ESE) rate constant (k_{ese}, a measure of intrinsic ET reactivity) ∼7 times lower than that of the wild-type protein. The Met89Val mutation to CBP results in a 2-fold increase in k_{ese}. As the axial interaction decreases from strong $O^{\epsilon 1}$ of Gln to relatively weak S^{δ} of Met to no ligand (Val), ESE reactivity is therefore enhanced by ∼1 order of magnitude while the reduction potential increases by ∼350 mV. The variable coordination position at this ubiquitous ET site provides a mechanism for tuning the driving force to optimize ET with the correct partner without significantly compromising intrinsic reactivity. The enhanced reactivity of a three-coordinate T1 copper site will facilitate intramolecular ET in fungal laccases and Fet3p.

Introduction

Type 1 (T1) copper sites are the mononuclear redox-active cofactors present in the cupredoxins; a large family of single domain electron transfer (ET) proteins.[1,2] Cupredoxin domains are also found in a number of copper-containing enzymes including the multi-copper oxidases and cytochrome *c* oxidase (in the latter case binding a binuclear Cu_A center). A T1 copper site always involves strong coordination of the metal by two His residues and the thiolate sulfur of a Cys.[1] The copper ion is usually displaced in the direction of a Met ligand that is located approximately 2.6 to 3.3 Å away, resulting in a distorted tetrahedral geometry (see Figure 1). In the cupredoxin azurin a unique active site structure involves a fifth interaction provided by a backbone carbonyl oxygen (2.6 Å from the copper) giving a trigonal bipyramidal geometry.[7] A corresponding carbonyl group is present at all T1 copper sites but is usually much more distant from the metal ion. The axial Met ligand at a T1 copper site is not conserved and in the stellacyanins (STCs) a Gln coordinates[3,8] with a Cu(II)−$O^{\epsilon 1}$ bond length of ∼ 2.2 Å (see Figure 1). The STCs along with the plantacyanins (PLTs) and the uclacyanins make up the phytocyanins[9] which possess an altered cupredoxin fold.[3,4,8,10] The PLTs usually have an axial Met ligand with the bond lengths among the shortest observed[4,10] for T1 copper sites [the Cu(II)−S(Met89) distance in cucumber basic protein (CBP) is 2.6 Å]. Two PLTs have been identified in tomato[2,11] and lily[12] which have a Val and Leu, respectively, in the axial position [the PLT from tobacco has an axial Gln ligand[2,11]]. As the side chains of Val and Leu are noncoordinating the T1 sites in these proteins must be three-coordinate. Various multi-copper oxidases also possess trigonal T1 copper

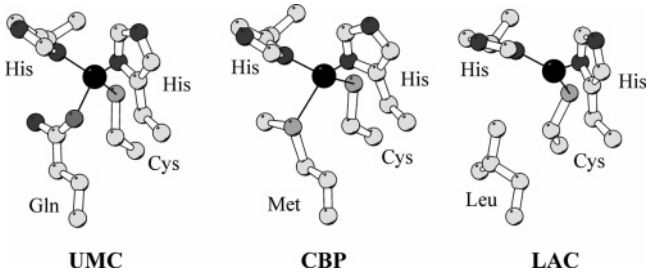

Figure 1. T1 Cu(II) site structures of umecyanin (UMC, the STC from horseradish roots, PDB entry 1X9R[3]), CBP (PDB entry 2CBP[4]), and laccase (LAC) from *Melanocarpus albomyces* (PDB entry 1GM0[5]) drawn with MOLSCRIPT.[6] The copper ions are shown as black spheres, and from UMC to LAC the strength of the axial interaction decreases.

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sites, with a Leu in the axial position in domain 6 of ceruloplasmin (mammalian),[13] a Phe or Leu in fungal laccases[5,14] (see Figure 1) and a Leu in Fet3p (yeast).[15]

Mutagenesis studies have demonstrated that alterations to the axial ligand at a T1 copper site can influence the reduction potential (E_m) over a range of ~300 mV within a particular protein fold.[16−18] Other factors are important as the E_m's of T1 copper sites cover a range from ~200 to 800 mV [this excludes the three-coordinate T1 site of ceruloplasmin which cannot be oxidized even by H_2O_2[19] giving a lower limit of ~1 V for its E_m], including the fixed location of the ligating residues provided by the constrained β-barrel arrangement of a cupredoxin domain.[20,21] The T1 copper site environment also ensures minimal changes upon redox interconversion and thus the reorganization energy (λ) is much lower than that for small molecule copper complexes.[20,22] A low λ is important for function, as the typical driving forces for physiological reactions are small and the distances involved are relatively long. The influence of natural T1 site (axial ligand) variations on intrinsic ET reactivity is not well appreciated and has not been compared within the same protein fold. In this study we analyze the effect of the physiologically relevant axial ligand alterations on the ET reactivity of CBP [a PLT was chosen as they naturally possess Met, Gln, and Val/Leu in the axial position[2,11,12]].

Materials and Methods

Design and Assembly of the CBP Artificial Coding Region. Oligonucleotides were obtained from MWG Biotech AG. pET11a vector and *E. coli* ORIGAMI B(DE3) cells were purchased from Novagen. Restriction enzymes were purchased from New England Biolabs, *Pfu* DNA polymerase and pGEM-T vector were obtained from Promega, and *Taq* DNA polymerase was purchased from Sigma. The sequences of both strands of all PCR products were determined. An artificial coding region for the 96 amino acid residues of CBP was synthesized using a method described previously.[23] Five overlapping oligonucleotides, ranging in length from 61 to 79 bases (P1−5, Table S1 in the Supporting Information), were designed.[23] PCR was carried out, initially with oligonucleotides P1−5.[23] The product of this reaction was amplified using P6 and P7 (complementary to the outermost 5′ sequences of both strands, Table S1 in the Supporting Information). The resulting PCR product was purified and ligated into pGEM-T, creating pGEMCBP1. *Nde*I and *Bam*HI reaction sites were introduced at the 5′ and 3′ ends, respectively, by PCR using primers P8 and P9 (Table S1 in the Supporting Information) to yield pGEMCBP2. The *Nde*I/*Bam*HI fragment was cloned into pET11a, to create pETCBP (this construct has a start codon at the 5′ end of the coding strand).

Overexpression and Purification of Recombinant CBP. *E. coli* ORIGAMI B (DE3) cells were transformed with pETCBP, and protein expression was induced in cells grown at 30 °C to an OD_{600} of ~0.6−0.8 with 50 μM isopropyl-β-D-thiogalactopyranoside (the expression levels in this *E. coli* strain can be controlled by the concentration of inducer and 50 μM resulted in the largest yields of soluble protein i.e., not in inclusion bodies). $Cu(NO_3)_2$ (0.5 mM) was added to the culture, and the cells were grown for a further 12 h (at 30 °C) before harvesting. The cells from 4 L of LB culture were suspended in 50 mM Tris pH 7.5, disrupted by sonication, centrifuged, and diluted (to 10 mM Tris). The supernatant plus copper (25 μM) was incubated with carboxymethyl (CM) sepharose (Pharmacia), equilibrated with 10 mM Tris pH 7.5, for 60 min at 4 °C with stirring. The bound CBP was eluted with 10 mM Tris pH 7.5 plus 500 mM NaCl, diluted with 10 mM Tris pH 7.5 and loaded onto a CM-sepharose column equilibrated with the same buffer. Elution was achieved with a gradient of 0−200 mM NaCl in the same buffer. CBP with an A_{278}/A_{595} ratio of 6.0 gave a single band on a 15% SDS-PAGE gel and a mass of 10 420 [determined by electrospray ionization mass spectrometry] consistent with the calculated mass of 10 424 for the holo-protein without the N-terminal Met residue.

Mutants. Met89Gln and Met89Val variants of CBP were prepared as described previously[24] and were isolated and purified using the same procedure as wild type (WT) CBP. Met89Gln and Met89Val CBP have masses of 10 416 and 10 388 compared to calculated values of 10 421 and 10 392, respectively, for the copper proteins.

UV/vis Spectrophotometry. UV/vis spectra were acquired at 25 °C on a Perkin-Elmer λ35 spectrophotometer with samples usually in 10 mM phosphate buffer. The molar absorption coefficients (ϵ values) were determined as described previously.[23] Azide binding (in the range of ~30 μM to 120 mM) was analyzed[25] for Met89Val CBP (26 μM) in 20 mM Tris pH 7.5 at 25 °C.

EPR Spectroscopy. X-band EPR spectra were recorded on a Bruker EMX spectrometer at −196 °C, and 2,2-diphenyl-1-picrylhydrazyl was used as the reference. Samples typically contained 2 mM protein in 40% glycerol and 25 mM Hepes at pH ~7.5. The spectra were simulated using the program SimFonia (Bruker).

Protein Electrochemistry. Measurement of the E_m of proteins (at $I = 0.10$ M) was carried out at ambient temperature (21 ± 1 °C) using an electrochemical setup and approach described previously.[26] Measurements were carried out at scan rates of typically 20 mV/s. All reduction potentials are referenced to the normal hydrogen electrode (NHE), and voltammograms were calibrated using the $[Co(phen)_3]^{3+/2+}$ couple (370 mV vs NHE). The gold working electrode was modified using a saturated solution of 4,4-dithiodipyridine.[26] A pH-jump method was used for the electrochemistry samples by diluting the protein (10-fold) with 20 mM buffer [$I = 0.10$ M (NaCl)]. Stock protein solutions (~1 mM) were stored in 1 mM buffer [$I = 0.10$ M (NaCl)]. For studies in the pH range 5.0−5.5, acetate buffer was used while Mes buffer was utilized in the 5.5−6.9 range and Tris was used in the pH 7.0−9.0 range. The final pH of the sample after mixing was checked.

NMR Spectroscopy. 1D, 2D, and paramagnetic ^{1}H NMR spectra of proteins were acquired in 10 mM phosphate in 99.9% D_2O and typically at pH* 8.0 (pH* indicates a pH meter reading uncorrected for the deuterium isotope effect) on a JEOL Lambda 500 spectrometer as described previously.[27] Saturation transfer experiments on 1:1 mixtures of Cu(I) and Cu(II) proteins were performed in 35 mM phosphate pH* 8.0 (WT CBP), 10 mM phosphate pH* 8.0 (Met89Gln CBP), and 10 mM phosphate pH* 7.4 (Met89Val CBP) all in 99.9% D_2O. For electron self-exchange (ESE) rate constant (k_{ese}) measurements, proteins were in 99.9% deuterated phosphate buffer (usually at $I = 0.10$ M). For WT CBP, 35 mM phosphate at pH* 8.0 and also 35

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Table 1. Properties of WT, Met89Gln, and Met89Val CBP and of WT UMC and the Gln95Met UMC Variant

parameters	WT CBP	Met89Gln CBP	Met89Val CBP	WT UMC[a]	Gln95Met UMC[a]
		UV/vis[b]			
λ_1 (nm) [ϵ_1 (M^{-1} cm^{-1})]	595 [3200]	595 [4100]	613 [5000]	606 [4300]	601 [4100]
λ_2 (nm) [ϵ_2 (M^{-1} cm^{-1})]	442 [1810]	447 [1170]	445(sh) [380]	464 [500]	453 [870]
ϵ_2/ϵ_1	0.57	0.28	0.08	0.12	0.21
LF transitions centered at (nm)	~770	~800	~740	~860	~750
		EPR[c]			
g_x	2.018	2.018	2.038	2.033	2.025
g_y	2.059	2.100	2.050	2.057	2.060
g_z	2.205	2.260	2.214	2.320	2.240
A_z (gauss)	56	32	77	33	47
		Paramagnetic ^{1}H NMR[d]			
δ (ppm) Asn40 $C^{\alpha}H$	17.2	16.8	20.1	13.7	16.0
		Diamagnetic ^{1}H NMR[e]			
δ (ppm) His39 $C^{\epsilon 1}H$	6.50	6.93	6.00		
		Electrochemistry			
E_m (mV)[f]	328	189	526	290	423
		ET reactivity			
k_{ese} (M^{-1} s^{-1})[g]	6.0×10^5	8.6×10^4	1.1×10^6	1.8×10^4	1.0×10^5

[a] The data for WT umecyanin (UMC, the STC from horseradish roots) are taken from refs 23, 29, and 30 while that for Gln95Met UMC are from refs 24 and 29. [b] pH 7.9 for WT CBP, pH 7.8 for Met89Gln CBP, and pH 7.6 for Met89Val CBP, WT UMC, and Gln95Met UMC. sh, shoulder. The bands at ~600 and ~450 nm in WT CBP have been assigned as the S(Cys) $\pi \rightarrow$Cu(II) $3d_{x^2-y^2}$ and S(Cys) pseudo-$\sigma \rightarrow$Cu(II) $3d_{x^2-y^2}$ LMCT transitions, respectively.[28] [c] All data at ~pH 7.5. [d] Asn40 is adjacent to the His39 ligand of CBP and experiences a large isotropic shift due to its backbone amide group hydrogen bonding to the thiolate sulfur of the Cys79 ligand.[4] Paramagnetic ^{1}H NMR spectra of oxidized proteins were acquired at pH* 8.0 (25 °C) for WT and Met89Gln CBP and pH* 7.4 for Met89Val CBP. For WT and Gln95Met UMC, spectra were acquired at pH* 7.5 (40 °C), and the δ values listed are for the $C^{\alpha}H$ resonance of Asp45 which is adjacent to the His44 ligand and whose backbone amide hydrogen bonds to the thiolate sulfur of the Cys85 ligand.[3] [e] Acquired at 25 °C and at pH* 8.0 for WT and Met89Gln CBP and at pH* 7.4 for the Met89Val variant. [f] For measurements at 21 ± 1 °C in 20 mM Tris pH 7.0 (I = 0.10 M). [g] Electron self-exchange rate constants all measured at I = 0.10 M and at pH* 8.0 for WT CBP, pH* 7.8 for Met89Gln CBP, pH* 7.4 for Met89Val CBP, and pH* 7.5 for WT and Gln95Met UMC. The data for WT, Met89Gln, and Met89Val CBP were acquired at 25 °C while the values for WT and Gln95Met UMC were obtained at 40 °C.

mM phosphate plus 400 mM NaCl at pH* 8.0 (I = 0.50 M) were used. For Met89Gln and Met89Val, CBP measurements were made in 36 mM phosphate pH* 7.8 and 38 mM phosphate pH* 7.4, respectively. The concentration of Cu(II) protein in the samples was determined as described previously[27] using the main absorption band at around 600 nm (see Table 1). k_{ese} values were determined at 25 °C by measuring the influence of increasing concentrations of Cu(II) protein (approximately <10% of the total protein concentration) on the relaxation rates of active site resonances (mainly $C^{\delta 2}H$ and $C^{\epsilon 1}H$ resonances of the His ligands) in the Cu(I) protein.[27] T_1^{-1} and T_2^{-1} values of resonances in normal 1D spectra and T_2^{-1} values of signals in super-WEFT spectra ($T_{2,WEFT}^{-1}$)[27] were determined.

Results

UV/vis, EPR, and Paramagnetic NMR. The UV/vis spectra of WT, Met89Gln, and Met89Val CBP are shown in Figure S1 (in the Supporting Information), and the positions and intensities of the two main bands, assigned as S(Cys)→Cu(II) ligand to metal charge transfer (LMCT) bands for the WT protein,[28] are listed in Table 1. The intensity of the band at ~450 nm decreases while that at ~600 nm increases on going from WT to Met89Gln to Met89Val CBP. The positions of these two bands are hardly altered by the mutations made except in the case of Met89Val CBP. Also included in Table 1 are the positions of the broad features above ~680 nm which are due to ligand field (LF) transitions[18,28] and which increase in energy along the series Met89Gln, WT, and Met89Val CBP. The hyperfine coupling in the g_z region (A_z) of the EPR spectrum decreases (and g_z increases) upon making the Met89Gln mutation to CBP and increases in the Met89Val variant (see Figure S2 in the Supporting Information and Table 1). The paramagnetic NMR spectra (see Figure 2) of WT, Met89Gln, and Met89Val CBP have been assigned (see Table 2). One of the $C^{\gamma}H$ signals of the axial Met89 ligand in WT CBP is observed at ~32 ppm (peak d).[33] This signal is absent in the spectra of the two Met89

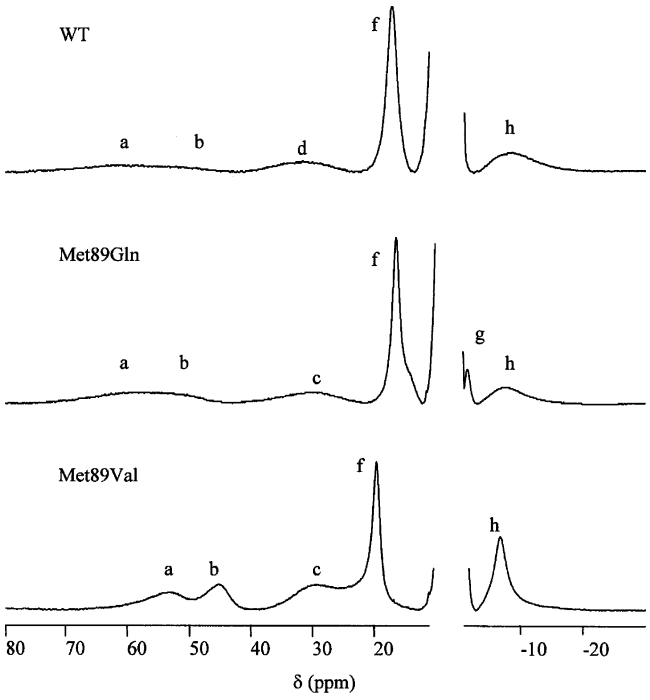

Figure 2. ^{1}H NMR spectra (25 °C) of WT and variants of CBP in 99.9% deuterated 10 mM phosphate at pH* 8.0 for WT and Met89Gln CBP and pH* 7.5 for Met89Val CBP.

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Table 2. Hyperfine Shifted Resonances in the ^{1}H NMR Spectra of WT, Met89Gln, and Met89Val CBP, and Their Diamagnetic Counterparts in the Cu(I) Variants[a]

	WT	Met89Gln		Met89Val		
resonance	δ_{obs} (ppm) in Cu(II) protein[b]	δ_{obs} (ppm) in Cu(II) protein	δ_{dia} (ppm) in Cu(I) protein[c]	δ_{obs} (ppm) in Cu(II) protein	δ_{dia} (ppm) in Cu(I) protein[c]	assignment
a	~60	~59	7.07	54	7.25 (7.22)	His84 $C^{\delta 2}H$
b	~50	~52	7.24	46	7.20 (7.17)	His39 $C^{\delta 2}H$
c	no[d]	~30	6.96/7.45 (6.93/7.44)	~30	5.97/no[d] (6.00/7.43)	His39/84 $C^{\epsilon 1}H$
d	~32					Met89 $C^{\gamma}H$
e	23.7	23.6	no[d]	32.1	no[d]	His39 $N^{\epsilon 2}H$
f	17.2	16.8	4.66	20.1	4.52	Asn40 $C^{\alpha}H$
g	no[d]	−1.3	no[d]	no[d]		His39/84 $C^{\beta}H$
h	−8.7	−7.9	5.40	−6.3	no[d]	Cys79 $C^{\alpha}H$

[a] Data recorded at 25 °C in 99.9% deuterated 10 mM phosphate buffer at pH* 8.0 for WT and Met89Gln CBP and at pH* 7.4 for Met89Val CBP. Resonance e was observed at pH 4.7 for WT and Met89Gln CBP and at pH 5.3 for Met89Val CBP (90% H_2O/10% D_2O). The observed shifts (δ_{obs}) for the Cu(II) proteins are made up of shifts in an analogous diamagnetic system (δ_{dia}) plus the pseudo-contact (δ_{pc}) and Fermi-contact (δ_{Fc}) contributions. The δ_{pc} values are small for Cu(II) cupredoxins,[31,32] and so δ_{iso} (the isotropic shift, $\delta_{iso} = \delta_{obs} - \delta_{dia}$) provides a good estimate of δ_{Fc} which is a measure of spin density on a particular proton. [b] Saturation transfer from the paramagnetic signals to their diamagnetic counterparts in Cu(I) WT CBP was not observed under a variety of conditions probably due to the very broad nature of the Cu(II) signals. The assignments made are based on those for the two CBP variants included here and also from work on other cupredoxins.[30−33] [c] The hyperfine shifted resonances were irradiated and saturation transfer observed to their diamagnetic counterparts in the Cu(I) form using a 1:1 mixture of Cu(II) and Cu(I) proteins at 25 °C for resonances a and b and at 10 °C for all other signals in Met89Gln CBP and at 5 °C for all resonances in Met89Val CBP (chemical shifts at 25 °C are given in parentheses). The assignments for the Cu(I) proteins are obtained from 2D (TOCSY and NOESY) spectra, and other active site resonances have been assigned: Cu(I) WT CBP (25 °C); 7.39 and 7.19 ppm (His84 $C^{\epsilon 1}H$ and $C^{\delta 2}H$ respectively), 7.12 and 6.50 ppm (His39 $C^{\delta 2}H$ and $C^{\epsilon 1}H$ respectively), 7.15, 6.79, 6.73 ppm (all Phe13) and −0.16 ppm (Met89 $C^{\epsilon}H_3$), Cu(I) Met89Gln CBP (25 °C); 7.23, 7.02, 6.86 ppm (all Phe13), Cu(I) Met89Val CBP (25 °C); 0.16 and −0.83 ppm (Val89 $C^{\gamma}H_3$), 1.68 ppm (Val89 $C^{\beta}H$), 3.52 ppm (Val89 $C^{\alpha}H$) and 7.15 and 6.83 ppm (Phe13). [d] Not observed.

variants, but the overlapping signals from the $C^{\epsilon 1}H$ protons of the two His ligands are observed at around 30 ppm due to significant resonance sharpening, particularly in Met89Val CBP (these signals are broadened beyond detection in the spectrum of the WT protein). Peak f arises from the $C^{\alpha}H$ proton resonance of Asn40[30−32] (adjacent to the His39 ligand) whose backbone amide group hydrogen bonds to the thiolate sulfur of the Cys79 ligand.[4] The shift of this signal provides a relative indication of the spin density distribution on the Cys ligand. The δ_{iso} (isotropic shift) for this resonance is 12.6 ppm in WT CBP [$\delta_{iso} = \delta_{obs} - \delta_{dia}$, where δ_{obs} is the observed chemical shift and δ_{dia} is the shift in an analogous diamagnetic system such as the Cu(I) protein (δ_{dia} is ~4.6 ppm for the Cu(I) forms of CBP; see Table 2)] and 12.1 and 15.6 ppm for Met89Gln and Met89Val CBP, respectively.

The addition of azide to Met89Val CBP results in a decrease in absorption at 613 nm in the UV/vis spectrum with a band at 407 nm increasing in intensity. These spectral alterations are almost identical to those observed upon addition of azide to the Met121Gly/Ala/Val/Leu azurin variants[34] and are indicative of azide binding in the axial position.[35] A K_d value of 0.10 M for azide binding to Met89Val CBP has been determined which is ~3 times larger than that for the Met121Val variant of azurin.

Diamagnetic NMR. The diamagnetic ^{1}H NMR spectra of Cu(I) WT, Met89Gln, and Met89Val CBP are very similar. Active site resonances have been assigned (see Table 2) including the $C^{\epsilon 1}H$ resonance of the His39 ligand which is particularly influenced by the mutations that have been made. This proton points directly at the phenyl ring of Phe13 in the structure of WT CBP[4] which explains the upfield position for its resonance (6.50 ppm). His84 is also situated close to Phe13, and a number of dipolar connectivities are observed between aromatic resonances of Phe13 and the imidazole ring signals of both His ligands. Furthermore, NOEs are observed between the $C^{\epsilon}H_3$ signal of the axial Met89 ligand and the $C^{\delta 2}H$ and $C^{\epsilon 1}H$ resonances of His39 and also the phenyl ring resonances of Phe13 [all consistent with the structure of WT CBP[4]]. The His39 $C^{\epsilon 1}H$ resonance experiences a considerably smaller ring current effect in Met89Gln CBP (see Tables 1 and 2), and NOEs between the imidazole ring proton signals of the His ligands and Phe13 resonances are much weaker, indicating subtle structural alterations in this region. In the Met89Val CBP variant the His39 $C^{\epsilon 1}H$ resonance experiences a larger upfield shift than in the WT protein (see Tables 1 and 2). The side-chain resonances of Val89 have been assigned in the spectrum of Met89Val CBP and a complex NOE pattern among the $C^{\gamma}H_3$ signals (which are upfield shifted due to the ring current effects of Phe13), the phenyl ring resonances of Phe13, and signals from the two His ligands is observed (NOEs are also found between the imidazole resonances and the phenyl signals of Phe13).

Reduction Potentials. The behavior of all three proteins [anodic and cathodic peaks of equal intensity, a peak separation of ~60 mV at a scan rate of ~20 mV/s and with peak currents proportional to (scan rate)$^{1/2}$ in the range of ~5 to 200 mV/s] indicate good quasi-reversible electrochemistry at a modified gold electrode. The E_m of WT CBP is 328 mV at pH 7.0 ($I = 0.10$ M). Upon replacing the axial Met ligand with a Gln, the E_m decreases by ~140 mV [to 189 mV at pH 7.0 ($I = 0.10$ M)]. The removal of the axial ligand results in an almost 200 mV increase in E_m [to 526 mV for Met89Val CBP at pH 7.0 ($I = 0.10$ M)]. In all cases the E_m values exhibit very little and similar dependencies on pH in the range 5.0 to 9.0.

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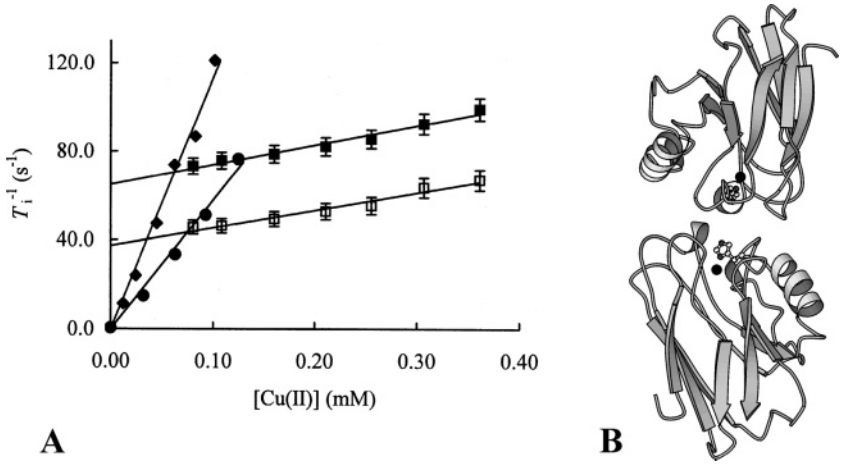

Figure 3. Plots (25 °C) whose slopes provide the k_{ese} values (A). For WT CBP a plot of T_1^{-1} (●) against [Cu(II)] for the His39 $C^{\epsilon 1}H$ resonance in 35 mM phosphate pH* 8.0 ($I = 0.10$ M) is shown while for the Met89Gln variant plots of $T_{2,WEFT}^{-1}$ against [Cu(II)] for the His84 $C^{\epsilon 1}H$ (□) and His39 $C^{\delta 2}H$ (■) resonances in 36 mM phosphate pH* 7.8 ($I = 0.10$ M) are presented. The data included for Met89Val CBP are for the [Cu(II)] dependence of T_1^{-1} (◆) of the His39 $C^{\epsilon 1}H$ resonance in 38 mM phosphate pH* 7.4 ($I = 0.10$ M). The error bars for the T_1^{-1} data are smaller than the symbols. The proposed ESE homodimer structure for cupredoxins (B). The arrangement of adjacent azurin molecules in the crystal structure [PDB entry 4AZU[36]] in which two molecules interact via their surface hydrophobic patches through which the exposed His ligands, which are in the purported ET pathway for ESE, protrude. The copper ions in the two monomers are shown as black spheres.

Electron Self-Exchange Reactivity. The k_{ese} value provides a measure of the intrinsic ET reactivity of a redox system. This reaction has no driving force and is therefore ideal for the assessment of the influence of active site mutations on ET (especially for proteins such as those studied herein where the physiological partners are not known). k_{ese} values measured by ^1H NMR spectroscopy (see Figure 3A) using T_1^{-1} (rather than T_2^{-1}) values of resonances are more precise,[27] and so most of the k_{ese} values quoted have been determined in this way (using His ligand signals). For Met89Gln CBP, resolved resonances in the conventional 1D spectrum of the Cu(I) protein suitable for ESE studies are not available and an alternative approach, using the super-WEFT pulse sequence,[27] has been applied. This method relies on the determination of T_2^{-1} values of Cu(I) resonances which are selected in super-WEFT spectra of partially oxidized samples (such resonances will be close to the active site and thus are suitable for k_{ese} determinations).

The His39 $C^{\epsilon 1}H$ resonance is well resolved in the NMR spectrum of Cu(I) WT CBP, and the dependence of its T_1^{-1} value on the concentration Cu(II) protein could be readily determined (see Figure 3A). The slope of this plot gives a k_1 value {k_1 is the slope of a plot of T_1^{-1} against [Cu(II)] while k_2 is obtained from the T_2 data} of 6.0×10^5 M^{-1} s^{-1} at pH* 8.0 ($I = 0.10$ M). The k_2/k_1 ratio for this resonance is 1.2 (the dependence of T_2^{-1} on [Cu(II)] is not shown) signifying that it does belong to the slow exchange regime [a k_2/k_1 ratio close to 1 is observed for such signals[27,37]], and therefore the slopes of these plots do provide k_{ese}. To check the appropriateness of the super-WEFT approach for measuring the k_{ese} of CBP, measurements were made with the WT protein and values ranging from 5.0×10^5 to 7.4×10^5 M^{-1} s^{-1} were obtained from a variety of active site resonances (giving an average of 5.8×10^5 M^{-1} s^{-1}) at pH* 8.0 ($I = 0.10$ M). The reactivity of WT CBP was also determined at higher ionic strength ($I = 0.50$ M) giving a k_{ese} value of 7.9×10^5 M^{-1} s^{-1} (this is the k_1 value determined using the His39 $C^{\epsilon 1}H$ signal, and a k_2/k_1 ratio of 1.2 is obtained). The k_{ese} of WT CBP is large for a cupredoxin,[38] and the absence of a sizable *I* dependence indicates that the basic patch at the opposite end of the β-barrel to the active site[4] does not have a significant influence on protein association.

The dependence of $T_{2,WEFT}^{-1}$ for the His84 $C^{\epsilon 1}H$ and His39 $C^{\delta 2}H$ resonances of Met89Gln CBP on [Cu(II)] are shown in Figure 3A. The slopes of these plots yield k_{ese} values of 7.9×10^4 M^{-1} s^{-1} and 8.9×10^4 M^{-1} s^{-1} respectively. Data for a number of other active site resonances were analyzed, and all fall within this range giving an average value (25 °C) of 8.6×10^4 M^{-1} s^{-1} at pH* 7.8 ($I = 0.10$ M). For Met89Val CBP, the His39 $C^{\epsilon 1}H$ and His84 $C^{\epsilon 1}H$ proton signals are well resolved in the spectrum of the Cu(I) protein allowing the dependence of T_1^{-1} on [Cu(II)] for both to be analyzed (the data for the His39 $C^{\epsilon 1}H$ resonance is shown in Figure 3A) giving k_1 values of 1.2×10^6 M^{-1} s^{-1} and 1.0×10^6 M^{-1} s^{-1}, respectively. The very fast rate of exchange observed for Met89Val CBP makes the measurement of T_2^{-1} values of His ligand resonances in the Cu(I) form difficult once a small amount of Cu(II) protein has been added, and thus $T_{2,WEFT}^{-1}$ values were determined giving $k_{2,WEFT}/k_1$ ratios of close to 1 for both signals. From an average of the k_1 values a k_{ese} (25 °C) of 1.1×10^6 M^{-1} s^{-1} is obtained for Met89Val CBP at pH* 7.4 ($I = 0.10$ M).

Discussion

The spectroscopic attributes of a distorted T1 copper center, such as that found in WT CBP,[18,28,39] are increased absorbance at ~450 nm in the UV/vis spectrum (a large ϵ_2/ϵ_1 ratio; see Table 1) and a rhombic EPR signal (large separation between g_x and g_y). These features have been assigned[18,28] to a coupled angular movement of the Cys and Met ligands resulting from an increased axial interaction [the Cu(II)−S(Met) bond length is 2.6 Å in CBP[4]] and a weaker Cu−S(Cys) bond giving a more flattened tetragonal site as compared to T1 centers with classic

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properties [lower absorption at ~450 nm and an axial EPR spectrum]. Upon making the Met89Gln and Met89Val mutations to CBP the band at ~450 nm lowers in intensity while that at ~600 nm increases. The increased g_z and decreased A_z observed in the Met89Gln variant give values analogous to those for STCs (see Table 1[23] and refs 17 and 40) and other Met→Gln T1 variants,[41−44] indicative of axial O$^{\epsilon 1}$ coordination.[3,8,30,41,42] The spectroscopic properties of cucumber STC have been assigned[28] to a stronger axial interaction resulting in tetrahedral distortion relative to the classic T1 site structure which must also be the case for Met89Gln CBP. The lower energy of the LF transitions, and the diminished shift of the Asn40 C$^{\alpha}$H resonance in Met89Gln CBP as compared to the WT protein point to the increased axial interaction resulting in decreased covalency of the interactions of Cu(II) with the equatorial ligands.[17,18,28,45] The UV/vis and EPR spectral properties of Met89Val CBP are analogous to those of naturally occurring[18,46] and engineered[17,34,47] three-coordinate T1 sites. The increased energy of the LF transitions[17,18] and the significantly increased δ_{iso} for the C$^{\alpha}$H resonance of Asn40 indicate that the copper moves into the plane of the equatorial ligands, and the interaction with these (mainly Cys) increases upon removal of the axial ligand. The increased A_z signifies that the enhanced equatorial interactions do not fully compensate for the loss of the axial ligand[17] [in the azurin variants which replace the axial Met with a noncoordinating residue A_z hardly changes[16,34] presumably due to an increased Cu−O(carbonyl) interaction]. Confirmation that the axial position at the T1 site of Met89Val CBP is vacant is obtained from the titration with azide, which demonstrates that the site is accessible;[34,35] although the relatively low affinity ($K_d \approx 0.10$ M) highlights that it is well protected. In conclusion the axial interaction is strongest in Met89Gln CBP, weaker in the WT protein, and absent in the Met89Val variant (see Figure 1). As the axial interaction decreases, the interaction with the equatorial ligands increases.

The NMR spectra of the Cu(I) proteins demonstrate that the overall active site architecture of CBP undergoes only subtle structural changes as a consequence of the mutations made. The His39 C$^{\epsilon 1}$H resonance experiences different upfield ring current effects from the phenyl moiety of Phe13 which are largest in Met89Val CBP and smallest in the Met89Gln variant. The shift of this resonance therefore reflects the strength of the equatorial interaction, and as the axial contact decreases, the copper moves into the His_2Cys plane and the Phe13-His39 ring current effect increases.

The influence of the axial ligand on the E_m values of T1 copper sites is well documented.[16−18,20,21,29,41−49] The types of effects that we have observed upon mutation of this residue are similar to those seen previously, and the magnitude of the changes resulting from swapping axial residues is analogous to studies with cucumber STC[17] and umecyanin (UMC, the STC from horseradish roots) [see Table 1[29]] which are also phytocyanins. The axial ligand at a T1 site can tune the E_m over a range of ~350 mV in CBP with a stronger interaction giving a lower value. It has been suggested that alterations in the effective nuclear charge as a consequence of the differing axial and equatorial [mainly Cu−S(Cys)] interactions are the major cause of these effects.[17,18] A number of other features play a significant role in controlling the E_m values of T1 copper sites[20,21,50] including additional attributes of their constrained active sites and the electrostatic environment of the protein.

ET reactivity is governed by a number of factors[51−54] including, for a bimolecular reaction such as ESE, the association of partners [ESE is activation controlled,[55] and thus $k_{ese} = K_a \times k_{et}$, where K_a is the association constant for homodimer formation and k_{et} is the rate constant for ET]. ESE in cupredoxins has been shown[56,57] to involve the association of the two molecules via their hydrophobic patches which surround the exposed His ligand (see Figure 3B). Changes to the axial residue at the active site should not influence the hydrophobic patch structure of CBP and thus K_a for ESE will be unaffected by the mutations made, and the observed differences in k_{ese} values must arise from alterations in k_{et}. The k_{et} value for ESE depends on the electronic coupling matrix element (H_{DA}) which is influenced by the donor (D) to acceptor (A) distance and the reorganization energy (λ) which is made up of inner sphere (ligand, λ_i) and outer sphere (remainder of protein and solvent, λ_o) components (the absence of a driving force for this reaction means that changes in E_m will not have any influence). The distance for ET should not be significantly altered by the mutations made, and paramagnetic NMR studies (see Figure 2 and Table 2) indicate that the spin density on the His ligand is almost unaffected and this feature should not influence H_{DA}. It is therefore probable that the 7-fold decrease in the k_{ese} of CBP upon making the Met89Gln mutation is due to an increased λ. Given the buried nature of the T1 site of CBP [although more exposed in the phytocyanin fold than in other cupredoxins[3,4,8,10]], and the ~ 5.5-fold increase in the k_{ese} of UMC (UMC and CBP possess almost identical phytocyanin folds) upon making the Gln95Met mutation [see Table 1[29]], it is most likely that this effect is due to an increased λ_i. The Met→Gln axial ligand mutation in azurin[41] and amicyanin,[42] both with slightly altered cupredoxin topologies as compared to phytocyanins (which never naturally possesses an axial Gln ligand), result in 63- and 12-fold decreases in k_{ese} (the sizable effect in the case of azurin is due to a large structural change upon reduction which gives a two-coordinate site). Structural studies of Cu(II) and Cu(I)

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WT UMC indicate that this STC exhibits larger active site alterations upon redox interconversion than a cupredoxin with an axial Met ligand.[3] Thus a center with an axial Gln ligand seems to possess a λ_i value which is larger than that for a site with an axial Met ligand [λ values for T1 copper sites with axial Met ligands are ~0.7 eV[7,20,22,58] with λ_i values as low as ~0.3 eV[59,60]]. This conclusion is consistent with the fact that the k_{ese} of UMC at elevated I (where any influence of its acidic patch[3] or other surface charge features on homodimer formation are minimized) is low for a cupredoxin (data not shown), whereas that for CBP is one of the largest reported.[38] Furthermore, intramolecular ET studies on STC indicated a 1.4-fold larger λ than for azurin,[61] although this difference was attributed to λ_o, due to the more exposed nature of the active site of the phytocyanin.

The Met89Val CBP variant has a k_{ese} value almost twice that for the WT protein. As argued above this effect is most likely due to an enhanced k_{et} which can only really be a consequence of a decreased λ, and an altered λ_i would appear to be the most likely cause (the spin density on the His ligands actually decreases in this variant; see Figure 2 and Table 2). Previous studies of the ET reactivity of three-coordinate T1 copper site variants have always been complicated by the influence of the mutations on driving force and H_{DA} as well as λ.[45,58,62−64] Our studies indicate that the λ_i value of a T1 copper site seems to be proportional to the number of coordinated atoms,[59] with a three-coordinate site having the smallest value. Crystal structures of the Cu(II) and Cu(I) forms of the same three-coordinate T1 copper site have not been determined [in the ceruloplasmin structure the high potential T1 site in domain 6 is probably in the Cu(I) state,[19,65] but the resolution is very low and a Cu(II) structure is not available].

As the axial interaction decreases at a T1 copper site from strong $O^{\epsilon 1}$ of a Gln to the weak thioether sulfur of a Met to no ligand, the intrinsic ET reactivity increases. It has been shown previously, particularly by resonance Raman studies,[17,66] that alterations at the axial position tune the strength of the Cu−S(Cys) bond (this is also the case for the studies described herein). This is a very important feature as the Cu−S(Cys) bond is thought to be the major contributor to λ_i.[59] It was recently demonstrated[17] that weakening the axial interaction at the active site of cucumber STC by making the Gln99Met and Gln99Leu mutations results in an elevated contribution of the Cu−S(Cys) bond to λ_i. However, this is thought to be offset by a reduced contribution of the axial ligand, giving all sites reasonably low λ_i values.[17] The results that we have obtained demonstrate that intrinsic ET reactivity is decreased at a site with an axial Gln ligand and is enhanced at a trigonal T1 site. These effects are most likely due to altered λ values, and thus differences in the Cu−S(Cys) reorganization are outweighed by the contribution of the residue in the axial position to λ_i. The superior reactivity of a site with no axial ligand poses the question as to why all T1 copper centers have not evolved to be three-coordinate. However, the lowest E_m value for such a site is ~450 mV.[46,67] Almost all naturally occurring cupredoxins have E_m values below 400 mV, and the removal of the axial Met ligand, although favorable for intrinsic reactivity, would have an adverse influence on the driving force for their reductive ET reactions. Additionally, the enhanced Cu−S(Cys) covalency which occurs upon removal of the axial ligand may not be beneficial for the ET reactions of cupredoxins (by enhancing H_{DA}) as these proteins all utilize the exposed C-terminal His ligand (a decreased axial interaction will facilitate ET via this ligand, but the effect is not expected to be so large) as the route for ET with physiological partners.[68−70] An axial Gln ligand allows relatively low E_m values to be achieved, and although λ is increased the effect is not sufficiently large to be detrimental to physiological reactivity. Therefore, the axial ligand at a T1 copper site can tune E_m to ensure a favorable driving force exists for reaction with a particular partner without significantly compromising ET. As cupredoxins are involved in bimolecular interactions, this usually ensures that their reactions are diffusion controlled with their surface features facilitating k_{on} (the rate of association) and ensuring specificity. For cupredoxin domains in multi-copper oxidases a balance between the oxidizing capability of their T1 sites and the ability to transfer electrons from the T1 center to the trinuclear copper site (Cu_3), where oxygen is bound and reduced, has to be maintained. A three-coordinate T1 center (trigonal sites are mainly found in the multi-copper oxidases) will promote ET to the Cu_3 site by both reducing λ and enhancing H_{DA} as the Cys ligand is in the proposed ET pathway.[71]

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Supporting Information Available: A table showing the primers used to synthesize and amplify the artificial coding region for CBP, and figures showing comparisons of UV/vis and EPR spectra of WT, Met89Gln, and Met89Val CBP. This material is available free of charge via the Internet at http://pubs.acs.org.

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