Spectroscopic and Magnetic Studies of Human Ceruloplasmin: Identification of a Redox-Inactive Reduced Type 1 Copper Site†

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ABSTRACT: Ceruloplasmin is unique among the multicopper oxidases in that in addition to the usual copper stoichiometry of one Type 1 copper site and a Type 2/Type 3 trinuclear copper cluster, it contains two other Type 1 sites. This assignment of copper sites, based on copper quantitation, sequence alignment, and crystallography, is difficult to reconcile with the observed spectroscopy. Furthermore, some chemical or spectroscopic differences in ceruloplasmin have been reported depending on the method of purification. We have studied the resting (as isolated by a fast, one-step procedure) and peroxide-oxidized forms of human ceruloplasmin. Using a combination of X-ray absorption spectroscopy, a chemical assay, magnetic susceptibility, electron paramagnetic resonance spectroscopy, and absorption spectroscopy, we have determined that peroxide-oxidized ceruloplasmin contains one permanently reduced Type 1 site. This site is shown to have a reduction potential of ∼1.0 V. Thus, one of the additional Type 1 sites in ceruloplasmin cannot be catalytically relevant in the form of the enzyme studied. Furthermore, the resting form of the enzyme contains an additional reducing equivalent, which is distributed among the remaining five copper sites as expected from their relative potentials. This may indicate that the resting form of ceruloplasmin in plasma under aerobic conditions is a four-electron oxidized form, which is consistent with its function in the four-electron reduction of dioxygen to water.

The multicopper oxidases are a broad class of enzymes that couple the four-electron reduction of dioxygen to water with the four sequential one-electron oxidations of substrate (*1*). They are widely distributed in nature, occurring in bacteria, fungi, plants, and animals. They contain copper ions of the following types: at least one blue copper or Type 1 site (T1)[1] characterized by an absorption band of ∼5000 M^{-1} cm^{-1} at ∼600 nm and narrow parallel hyperfine splitting [$A_{\|} = (43-95) \times 10^{-4}$ cm^{-1}] in the electron paramagnetic resonance spectrum (EPR), a normal or Type 2 site (T2) characterized by the lack of intense absorption bands and normal EPR features [$A_{\|} = (158-201) \times 10^{-4}$ cm^{-1}], and a Type 3 copper pair (T3) characterized by an absorption band of ∼5000 M^{-1} cm^{-1} at ∼330 nm and strong antiferromagnetic coupling leading to the lack of an EPR signal. The T2 and T3 sites form a trinuclear cluster, which is the site for dioxygen reduction (*2*−*5*). Spectroscopic studies have shown that a fully reduced T2/T3 trinuclear cluster is the minimum structural unit required for reaction with dioxygen (*1*). The function of the T1 site is to transfer electrons from substrate to the trinuclear cluster and thus is the site for substrate oxidation. Therefore, the multicopper oxidases operate via a ping-pong mechanism (*1, 6*):

$$4S + Cu(II)_4 \rightarrow 4S^+ + Cu(I)_4 \quad (1)$$

$$4H^+ + O_2 + Cu(I)_4 \rightarrow 2H_2O + Cu(II)_4 \quad (2)$$

The best studied members of this class are *Rhus vernicifera* laccase and ascorbate oxidase. Crystal structures exist for ascorbate oxidase (*5*), human ceruloplasmin (*7*), and a fungal laccase (*8*). Other members of the multicopper oxidase family include the fungal laccases, ceruloplasmin, FET3, and phenoxazinone synthase, among others. The laccases are characterized by their low substrate specificity (*9*−*12*): a variety of *o*-diphenols are possible physiological substrates (*13*−*20*). In contrast, ascorbate oxidase has a high specificity for L-ascorbate (*21*−*22*).

The only multicopper oxidase found in humans is ceruloplasmin (Cp) [ferroxidase, iron(II):dioxygen oxidoreductase, EC 1.16.3.1]. The physiological role of Cp is still somewhat in dispute; however, a consensus is emerging that it is a plasma ferroxidase. A variety of experiments on iron metabolism provide strong evidence for this hypothesis (*23*−*27*). Extensive in vitro studies have demonstrated that Cp can efficiently catalyze the oxidation of Fe(II) to Fe(III) under

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[1] Abbreviations: BSA, bovine serum albumin; Cp, ceruloplasmin; EPR, electron paramagnetic resonance; MES, 2-(*N*-morpholino)ethanesulfonic acid; 1,2-MeIm, 1,2-dimethylimidazole; MePY2, methylbis-(2-pyridylethyl)amine; MOPS, 2-(*N*-morpholino)propanesulfonic acid; NHE, normal hydrogen electrode; SQUID, superconducting quantum interference device; T1, Type 1; T1PR, Type 1 permanently reduced; T2, Type 2; T3, Type 3; XAS, X-ray absorption spectroscopy.

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close to physiological conditions (*28*−*34*). Kinetic and equilibrium dialysis studies demonstrate a high affinity for divalent metal ions (K < 10 mM) (*28*, *30*−*32*, *35*), and two putative labile divalent cation-binding sites have been identified by recent crystallographic work (*36*). Although the ferroxidase activity appears to be the primary function of Cp, a role in copper transport has also been proposed, mostly based on studies of intravenously injected radiolabeled copper (*37*−*40*). Studies on the genetic disorder aceruloplasminemia shed further light on the function of Cp. Patients who have aceruloplasminemia show extensive deposition of iron in a number of organs, particularly the liver and brain, providing substantial evidence that ceruloplasmin is involved in iron metabolism (*41*−*46*). However, there is no evidence for disruption of copper metabolism, such as copper loading in the liver, thus casting doubt on the role of Cp in copper transport.

Ceruloplasmin is unique among the multicopper oxidases in that it contains additional copper sites beyond the four required for oxidase activity. Huber and Frieden were among the first to establish that human Cp contains six coppers per molecule, with an additional labile copper-binding site that does not alter the oxidase activity (*32*). Ortel et al. (*47*) and later Messerschimdt and Huber (*48*) examined the sequence homology among the various multicopper oxidases and plastocyanin and noted that there are three putative T1-binding sites, one with a leucine in place of methionine. The crystal structure of Zaitseva et al. confirmed this basic stoichiometry of six integral coppers (a trinuclear cluster and three coppers bound to the three T1-binding sites) and a seventh labile copper distributed between the two cation-binding sites (*7*, *36*).

However, it has been difficult to reconcile the basic stoichiometry of six copper ions per molecule in mammalian Cp with the observed spectroscopy and chemistry. Different authors have reported that 40−50% of the copper is paramagnetic by EPR double integration (*49*−*52*), which is inconsistent with the 67% expected for six Cu(II), two of which (the T3 copper pair) are antiferromagnetically coupled. Also, Deinum and Vänngård reported that the number of electrons accepted by the protein equaled the number of coppers, implying that all of the copper was oxidized (*51*). Furthermore, Calabrese and co-workers have purified Cp from humans, sheep, and other mammalian sources by a rapid, one-step affinity column (*53*). The mammalian Cp they obtain is pure and still contains the six integral copper ions (no labile copper), but has even less paramagnetic copper (as little as 32%) (*54*), a lower absorption intensity (8000 M^{-1} cm^{-1}) (*53*), and a variable but consistently low amount of T2 Cu as detected by the low-field hyperfine line in EPR (*54*, *55*). They propose that their rapid, minimally destructive isolation procedure yields protein which differs from that obtained by the longer, traditional means, and which has a magnetic interaction between the T2 and T3 sites not seen in the other multicopper oxidases that reduces the T2 Cu signal in EPR. Since only four coppers are required for the four-electron reduction of dioxygen, it is important to understand the role of the additional copper centers in Cp's physiological function.

This study is intended to reconcile the spectroscopic and magnetic data on human Cp with the stoichiometry of six coppers per molecule as determined by copper quantitation and X-ray crystallography, and to elucidate the possible functions of the additional copper sites. In our earlier studies on laccase, it was observed that much of the confusion surrounding the spectroscopy of that enzyme was due to reduced copper which could be oxidized by the addition of H_2O_2 (*56*−*59*). We have followed a similar strategy with Cp, focusing on two different forms of the enzyme: a form of the enzyme as isolated by the method of Calabrese and co-workers (*53*), which we designate as the "resting form"; and a "peroxide-oxidized form", which is the resting form treated with 20−30-fold excess of H_2O_2 for 1−3 h.[2] We have used copper K-edge X-ray absorption spectroscopy (XAS) and a chemical assay to quantitate the amount of reduced copper in these samples, Curie slope SQUID magnetic susceptibility and EPR double integration to quantitate the amount of paramagnetic copper, and EPR simulations and absorption intensities to probe the distribution of reducing equivalents among the copper sites.

EXPERIMENTAL PROCEDURES

The chloroethylamine used in the derivatization of Sepharose 4B was recrystallized from hot ethanol. Water was purified to a resistivity of 15.5−17 MΩ cm^{-1} using a Barnstead Nanopure deionization system. $[Fe(bipy)_3]_2[SO_4]_3$ and $[Fe(bipy)_2(CN)_2]NO_2$ were synthesized by literature procedures (*60*, *61*). $[Cu(1,2\text{-MeIm})_2](PF_6)$ and $[(MePY2)Cu(CH_3CN)](ClO_4)$ were kindly provided by Professor Ken Karlin at Johns Hopkins University. All other chemicals used were reagent grade and were used without further purification.

Human Cp from plasma withdrawn the previous day was purified by the method of Calabrese et al. (*53*), with the following modifications. The conductivity and the pH were not adjusted. After loading Cp onto the derivatized Sepharose 4B, the column was washed with a small amount of MES buffer (pH 7.0, 0.1 M) containing 0.1 M 6-aminohexanoic acid, to remove nonspecifically bound proteins. The column was then washed with MES buffer (pH 7.0, 0.1 M) containing 30−40 mM $CaCl_2$, until the eluent showed an absorbance at 280 nm of less than 0.05. This was then repeated with MES buffer containing 50−60 mM $CaCl_2$. This removed other proteins that adhere to the derivatized Sepharose 4B. The column was then washed with MES buffer to remove the $CaCl_2$. Finally, pure Cp was eluted with potassium phosphate buffer (pH 7.0, 0.2 M).[3] The 610/280 ratio of resting purified Cp was generally ∼0.034.[4] All samples were used immediately or kept frozen at −80 °C until use and only used or frozen and thawed once. Protein concentration was determined by the microbiuret assay (*62*). Total copper concentration was determined by the biquinoline assay (*63*). Cu(I) concentration was determined by the biquinoline assay as modified by McMillin and co-workers (*64*). Average total copper content was 6.12 ± 0.12 coppers per molecule. Oxidase activity on a few samples was measured spectrophotometrically by the *o*-dianisidine assay

[2] Some samples were additionally reacted with a 50-fold excess of $[Fe(CN)_6]^{3-}$; these samples showed no differences as compared to those oxidized with H_2O_2 alone.

[3] The use of $CaCl_2$ to remove other proteins bound to the derivatized Sepharose 4B was developed by Musci and Calabrese.

[4] The low 610/280 ratio is due to partial reduction of the T1 copper (vide infra). The peroxide-oxidized form has a 610/280 ratio of ∼0.045, in agreement with what has previously been reported.

(*65*) and found to be consistent with previously reported values. Gel electrophoresis was run on representative samples using a Mini-PROTEAN II cell, 7.5% Tris-HCl gels, and protein standards obtained from Bio-Rad. This showed a pair of strong bands at ~132 kDa, and a series of weak bands which increased in intensity if the protein was stored at 4 °C for long periods of time; thus, the protein was pure but contained a small amount of proteolytically cleaved enzyme, as seen previously. Peroxide-oxidized Cp was prepared by incubation of resting Cp with a 20−30-fold excess of H_2O_2 for 1−3 h at 4 °C. This was either left in or removed by passage through a G-25 column and reconcentrated using Centricon concetrators. No differences were observed in fresh samples prepared by either method; in all of the SQUID susceptibility samples, the H_2O_2 was removed. A few samples (specifically the XAS and Q-band EPR samples) also contained 50-fold excess of $[Fe(CN)_6]^{3+}$; these samples showed no differences as compared to those oxidized with H_2O_2 alone. Protein concentrations were in the range of 0.05−0.10 mM, except for SQUID, XAS, and some EPR experiments, in which protein concentrations were 0.5−1.0 mM. All samples were run in potassium phosphate buffer (pH 7.0, 0.2 M), except for samples prepared for SQUID susceptibility. Since potassium phosphate buffer contains unacceptable levels of ferric impurities, Cp samples for SQUID susceptibility were exchanged into deuterated MOPS buffer (pH 7.0, 0.1 M). Cp samples in MOPS buffer showed no differences from those in phosphate buffer.

Absorption spectra were recorded on a Hewlett-Packard HP8452A diode array spectrophotometer in a 1-cm path length cuvette. X- and Q-band EPR spectra were obtained with a Bruker ER 220-D-SRC spectrometer. For X-band EPR, sample temperatures were maintained at 77 K using a liquid nitrogen finger dewar. For Q-band EPR, sample temperatures were maintained at 96 K using a nitrogen flow system. EPR spectra were spin-quantitated using a 1.0 mM aqueous copper standard run in the same tube as the sample where possible (*66*). EPR spectra were simulated using SIM15 obtained from the Quantum Chemistry Program Exchange and modified to allow for anisotropic line widths.

Magnetic susceptibility data were taken on a Quantum Design Model MPMS SQUID magnetometer. A palladium standard was used as a calibrant. Protein samples were loaded in polycarbonate capsules sealed with a drop of acetone and with a small hole in the top in order to flush the space above the sample. These were then loaded into a plastic drinking straw attached to the end of the magnetometer drive rod. Samples were evacuated and flushed with helium gas, and then loaded into the probe maintained at 10 K. After loading, the samples were heated to 125 K and maintained at that temperature for 10 min to removed any condensed oxygen. Susceptibility data were collected versus $1/T$ in the temperature range of 5.25−115 K and at a field of 3.5 T. Sample volumes were 150 μL. To account for dissolved oxygen, trace metal impurities, and other extraneous paramagnetic signals in the data, several approaches were used. Either a blank sample of aerobic bovine serum albumin (BSA) at the same mg mL^{-1} concentration was run in the same buffer and under the same conditions as the Cp samples, or both the Cp sample and a blank sample of either BSA in buffer or just buffer were degassed by pump-purging at ~0 °C under argon and run under the same conditions.

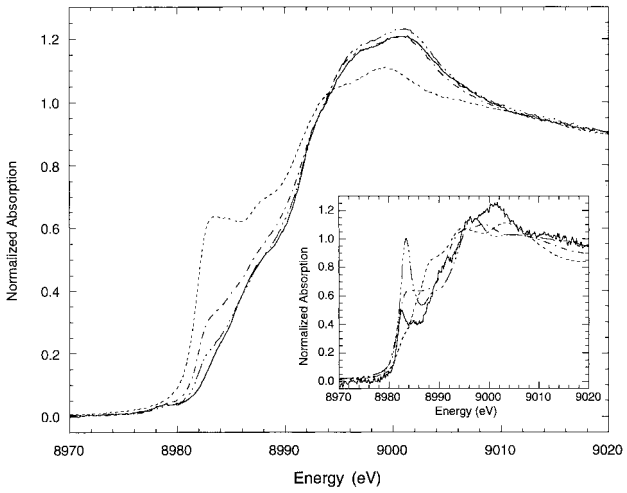

FIGURE 1: Normalized copper K-edge XAS of the fully oxidized model spectrum (—), peroxided-oxidized Cp (—···—), resting Cp (—·—), and reduced Cp (···). Inset: Renormalized XAS of the T1PR copper site generated by subtraction of the oxidized T1Hg laccase XAS and two oxidized plastocyanin edges from the peroxide-oxidized Cp XAS (—); the normalized XAS of known two-coordinate ($[Cu(1,2\text{-MeIm})_2]PF_6$) (—···—), three-coordinate (T1Hg laccase, *4*) (—·—), and four-coordinate Cu(I) ($[(MePY2)Cu(CH_3CN)]ClO_4$) (···) sites are shown for comparison.

Differences between these methods of background subtraction were minimal.

XAS data were measured at the Stanford Synchrotron Radiation Laboratory on unfocused 8-pole wiggler beamline 7-3 under dedicated conditions (3.0 GeV, 60−100 mA). Monochromatic radiation was obtained using a Si(220) double-crystal monochromator which was detuned 50% for harmonic rejection. The beam height was defined to be 1 mm. The fluorescence signals were measured with an argon-filled ionization chamber detector equipped with Soller slits and a Ni filter (*67, 68*). Internal energy calibration was performed by simultaneous measurement of the absorption of a copper foil placed between the second and third ionization chambers. The first inflection point of the copper foil spectrum was assigned to 8980.3 eV. The samples were loaded into 2 mm Lucite XAS cells with 63.5 μm Mylar windows, frozen immediately, and kept under liquid nitrogen prior to measurements. The samples were maintained at a constant temperature of 10 K throughout the measurements by an Oxford Instruments CF1208 continuous-flow liquid helium cryostat. The data represent an average of four scans for each Cp sample.

RESULTS

I. Detection and Quantitation of Cu(I) by XAS and Biquinoline Assay. To obtain direct information on the amount of reduced copper in different samples of human Cp, we used copper K-edge XAS. Cu(I) exhibits an intense feature at ~8984 eV by XAS, corresponding to an electric dipole-allowed 1s → 4p transition (*59*). The shape and intensity of this feature can be used to determine the coordination number of Cu(I) sites in proteins. The XAS spectra for three different samples of Cp are shown in Figure 1. The reduced protein sample was generated by addition of ~100-fold excess of ascorbate. It showed a complete loss of the absorption features at 330 and 610 nm and no EPR signal. As expected, the XAS of this sample has a large

Table 1: Quantitation of Reduced Copper, Paramagnetic Copper, and Absorption Intensities in Resting and Oxidized Cp

method	resting	oxidized
% reduced Cu by XAS	33.2 ± 5.0	12.1 ± 5.0
% reduced Cu by biquinoline assay	32.7 ± 0.5	20.3 ± 1.0
% paramagnetic Cu by magnetic susceptibility	40.2 ± 6.2	51.7 ± 6.2
% paramagnetic Cu by EPR	35.8 ± 5.9	47.6 ± 2.2
molar absorptivity at 610 (M^{-1} cm^{-1})	7320 ± 630	9590 ± 680
molar absorptivity at 330 (M^{-1} cm^{-1})	4470 ± 170	4950 ± 300

feature at 8983 eV relative to the normalized Cu K-edge. The resting sample also shows significant intensity at 8983 eV. This indicates that there is a substantial amount of reduced copper in Cp isolated by the one-step method. The XAS spectrum of peroxide-oxidized Cp (Figure 1) shows that there is still some residual intensity at 8983 eV. Therefore, a portion of the copper in the sample was not oxidized, even by a large excess of a powerful oxidant.

To quantitate the amount of reduced copper present in the resting and peroxide-oxidized samples described above, a fully oxidized reference is required as a base line. This was constructed from the XAS of the T1Hg derivative of laccase, in which the Type 1 copper was replaced with mercury, leaving just the oxidized trinuclear cluster (*4*), and the XAS of oxidized plastocyanin (*69*), a blue copper protein. Appropriately weighted (50% oxidized T1Hg laccase and 50% oxidized plastocyanin), summed, and renormalized, this model spectrum was used as a fully oxidized Cp base line along with the fully reduced Cp sample to quantitate the amount of reduced copper in resting and peroxide-oxidized Cp (Figure 1). The intensity difference at 8983 eV between the peroxide-oxidized sample and the fully oxidized model spectrum relative to the reduced protein is 12.1 ± 5%. For the resting sample, the intensity difference is 33.2 ± 5% relative to the reduced protein (Table 1). This corresponds to 0.73 Cu(I) per molecule in the peroxide-oxidized sample and 2.0 Cu(I) per molecule in the resting sample.

To further evaluate the above results, the biquinoline assay as modified by McMillin and co-workers (*64*) was run on both resting and peroxide-oxidized samples of Cp. This is a spectrophotometric assay for the amount of Cu(I) in protein samples. This assay showed that 32.7 ± 0.5% of the copper in the resting sample is reduced, consistent with the result by XAS. Both methods show that two of the six copper sites in Cp as isolated by the procedure of Calabrese and co-workers are Cu(I). The chemical assay yields 20.3 ± 1.0% Cu(I) in the peroxide-oxidized protein samples. Although consistently higher than the result by XAS, the chemical assay confirms that roughly one of the six coppers stays reduced even in the presence of large quantities of oxidant.

II. Quantitation of Paramagnetic Copper by SQUID Magnetic Susceptibility and EPR. SQUID magnetic susceptibility is a bulk technique for the detection of the amount of paramagnetic centers; as such, it can detect paramagnetic sites which do appear in EPR. The slope of the magnetic susceptibility versus $1/T$ corresponds to the number of copper sites per molecule which are paramagnetic with $S = 1/2$ multiplied by the theoretical Curie−Weiss constant for one Cu(II). This constant was calculated using a g_{ave} of 2.1152 that was derived from g values determined by EPR simulation (vide infra). The Curie slope SQUID magnetic susceptibility data for resting and peroxide-oxidized Cp are shown in Figure 2, along with theoretical lines for two, three, and four paramagnetic copper sites per molecule for comparison. The y-intercept corresponds to the diamagnetic correction and was set equal to zero. The error bars show the standard deviation in the slope of the experimental data. From the slope of the line of best fit, 40.2 ± 6.2% of the copper in the resting enzyme is paramagnetic by SQUID magnetic susceptibility. In the peroxide-oxidized enzyme, 51.7 ± 6.2% of the copper is paramagnetic (Table 1). There was no evidence for the population of a spin quartet state within the temperature range used in these experiments (5.25−115 K).

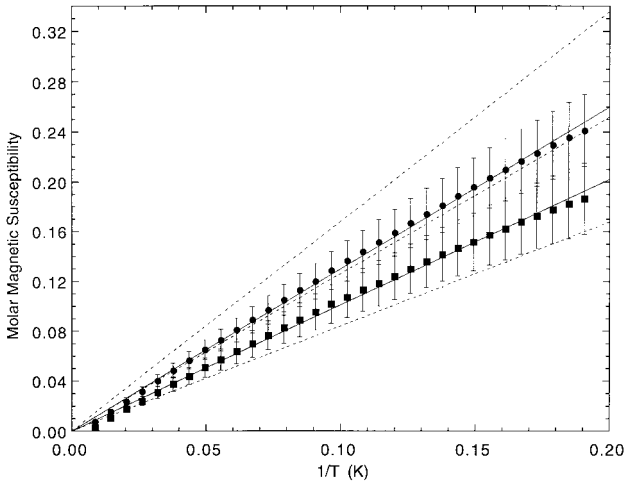

FIGURE 2: Molar magnetic susceptibility data for resting (■) and peroxide-oxidized (●) Cp, along with the least-squares lines of best fit (—), error bars at one standard deviation unit, and theoretical lines for two, three, and four paramagnetic sites per molecule (···).

The X-band EPR spectra of resting and peroxide-oxidized Cp at 77 K are shown in Figure 3A,B. The amount of paramagnetic copper in resting and peroxide-oxidized Cp was determined by double integration of the X-band spectra relative to a copper standard. By EPR, the amount of paramagnetic copper was 35.8 ± 6.2% in resting Cp and 47.6 ± 2.2% in peroxide-oxidized Cp (Table 1). The larger error bars in the amount of paramagnetic copper by EPR in resting Cp as compared to peroxide-oxidized Cp may reflect some heterogeneity in resting Cp from different batches, as previously reported (*54*).

These results are consistent with the amount of paramagnetic copper that was observed previously by EPR quantitation (*49*−*52*, *54*). Furthermore, these data are consistent within experimental error with the values obtained by SQUID magnetic susceptibility. Thus, all of the paramagnetic copper in human Cp is EPR-detectable. This rules out the possibility that there is some coupling between the copper sites that might lead to a fraction of paramagnetic copper not appearing in the EPR spectrum, due to fast relaxation or very large line widths. The observation that ∼50% of the copper is paramagnetic in peroxide-oxidized Cp indicates that no fraction of the 1 reducing equiv, as observed by XAS and the modified biquinoline assay, is distributed on the T3 copper pair, since that would have led to more than 50% of the copper being paramagnetic. Thus, of the six coppers per molecule in peroxide-oxidized Cp, three are Cu(II) with

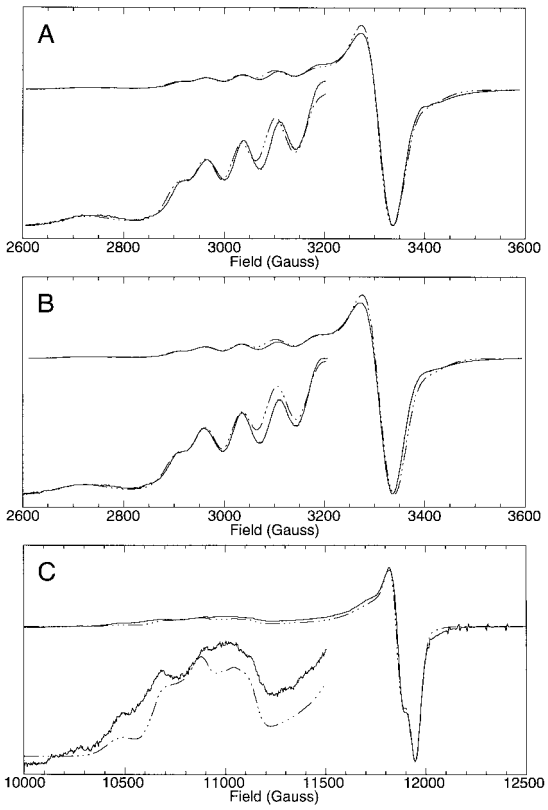

FIGURE 3: Experimental (—) and simulated (—···—) EPR spectra of (A) resting Cp, X-band (microwave frequency, 9.498 GHz); (B) peroxide-oxidized Cp, X-band (microwave frequency, 9.497 GHz); and (C) peroxide-oxidized Cp, Q-band (microwave frequency, 34.08 GHz). Experimental conditions: microwave power, 20 mW; modulation amplitude, 16 G; modulation frequency, 100 kHz; time constant, 0.5 s. Spin Hamiltonian parameters from the simulations are listed in Table 2. The fraction of each site that was reduced as determined by the simulation of the resting Cp spectrum is described in the text and summarized in Table 3.

Table 2: EPR Simulation Parameters of Oxidized Cp at X- and Q-Band Frequencies

Cu site	*g* value		*A* value ($\times 10^{-4}$ cm^{-1})		line width, X-band ($\times 10^{-4}$ cm^{-1})		line width, Q-band ($\times 10^{-4}$ cm^{-1})	
	$g_{\|\|}$	$g_\perp$	$A_{\|\|}$	$A_\perp$	$L_{\|\|}$	$L_\perp$	$L_{\|\|}$	$L_\perp$
T1A	2.2620	2.0520	62.5	10.0	42.0	39.0	85.0	53.0
T1B	2.2130	2.0410	74.0	12.0	42.0	39.0	75.0	53.0
T2	2.2650	2.0555	180	32.0	104	85.0	140	160

$S = 1/2$, two are an antiferromagnetically coupled Cu(II) pair with $S_{tot} = 0$, and one is Cu(I) with $S = 0$. Likewise, the decreased amount of paramagnetic copper present in the resting samples indicates that not all of the additional reducing equivalent is distributed on the T3 copper pair.

III. Distribution of Reducing Equivalents among the Copper Sites. The experimental X-band and Q-band (run at 96 K) EPR spectra of peroxide-oxidized Cp are shown in Figure 3B,C. These spectra were simulated in order to obtain a reasonable estimate of the spin Hamiltonian parameters for the different copper sites. The simulated spectra are shown in Figure 3B,C, and the parameters are listed in Table 2. The experimental spectra could be adequately simulated with one T2 Cu site and two T1 Cu sites with different parameters, but equal double integrated intensity (i.e., equal amounts of the three different copper sites). This indicates that the one reduced copper seen in the XAS corresponds uniquely to one of the three T1 sites. These parameters were then used to simulate the resting Cp EPR spectrum to determine the distribution of reduced copper over the T2 and two redox-active T1 sites (Figure 3A). From this simulation, we determine that the fraction of reduced copper in resting Cp was 0.20 Cu(I), 0.36 Cu(I), and 0.29 Cu(I) in sites T1A, T1B, and T2 respectively (Table 3). From the difference in the amount of paramagnetic copper in resting Cp as determined by EPR double integration and the amount of reduced copper by XAS and the biquinoline assay, the amount of reduced T3 coppers in resting Cp is 0.15 (Table 3).

Table 3: Distribution of Reduced Copper in Resting Cp

copper site	by EPR	by absorption
redox-active T1	0.56	0.47
T1PR	1.00	1.00
T2	0.29	0.33[a]
T3	0.15[a]	0.19
total	2.00	2.00

[a] By difference.

The molar absorptivities of the 330 and 610 nm bands of resting and peroxide-oxidized Cp are listed in Table 1. The molar absorptivities of the 330 and 610 nm bands in peroxide-oxidized Cp are 4950 ± 300 and 9590 ± 680 M^{-1} cm^{-1}. These values are consistent with what has been previously observed for one oxidized T3 copper pair in laccase and ascorbate oxidase ($\epsilon_{330} \approx 5000$ M^{-1} cm^{-1}) and two oxidized T1 copper sites ($\epsilon_{600} \approx 5000$ M^{-1} cm^{-1} for one oxidized T1 site) in laccase, ascorbate oxidase, and blue copper proteins. From this, the change in band intensities in going from the resting to the peroxide-oxidized form can be correlated with an increase in the amount of oxidized T3 and T1 copper sites. Given negligible absorption intensity in the reduced enzyme and using the molar absorptivities observed above for the peroxide-oxidized samples, $\Delta\epsilon_{610} = 2270$ M^{-1} cm^{-1} and $\Delta\epsilon_{330} = 480$ M^{-1} cm^{-1} correspond to the oxidation of 0.47 T1 Cu and 0.19 T3 Cu. Thus, this indicates that the remaining reduced copper in resting Cp, 0.33 Cu, is on the T2 copper site (Table 3). These values are consistent with the results from EPR simulations: most (~0.5) of the additional reducing equivalent in resting Cp is distributed on the T1 sites, and only a small amount (<0.2) is on the T3 copper pair.

Combining the data from all of the different methods used, it is clear that one of the six coppers present in peroxide-oxidized Cp is reduced and localized on a single T1 site, which we designate as the T1 permanently reduced site (T1PR). We can estimate the XAS K-edge spectrum for this unique Cu(I) site by subtraction of the oxidized T1Hg laccase XAS (i.e., oxidized trinuclear cluster) and two oxidized plastocyanin edges (appropriately normalized) from the peroxide-oxidized Cp XAS shown in Figure 1. This is shown in the insert in Figure 1. The shape and intensity of the peak at 8983 eV compared to those of known two-, three-, and four-coordinate Cu(I) sites indicate that this Cu(I) is best described as three-coordinate (*59*). Three-coordinate Cu(I) species typically have a peak maximum at 8983.5 ± 0.4 eV with an normalized amplitude of 0.63 ± 0.05.

IV. Attempts To Oxidize the T1PR Cu Site. The different spectroscopic and magnetic studies discussed above dem-

Table 4: Oxidants Used in Attempts To Oxidize T1PR Cu

oxidant	$E°$ vs NHE (V)	x-fold excess	minimum $E°$ of T1PR Cu (V)	ref
H_2O_2	1.349	30	*a*	*69*
$S_2O_8^{2-}$	2.01	15	*a*, *b*	*70*
$Fe(CN)_6^{3-}$	0.433	500	0.708	*71*
$Fe(bipy)_2(CN)_2^{+}$	0.781	12	0.960	*72*
$IrCl_6^{2-}$	0.867	10	1.077	*73*
$Fe(bipy)_3^{3+}$	1.074	9	*b*	*74*

[a] Two-electron oxidant. [b] Caused significant protein degradation.

onstrate that one of the T1 copper sites (T1PR) stays reduced in Cp even in the presence of 20−30 equiv of H_2O_2 and 50 equiv of $[Fe(CN)_6]^{3-}$. We tried to oxidize the T1PR copper sites with larger amounts of oxidant and with several more powerful oxidants (Table 4). Since much greater than 30-fold excess of H_2O_2 caused significant protein degradation, we also tried $S_2O_8^{2-}$, a powerful two-electron oxidant, at up to 15-fold excess. Although it caused some protein degradation, no change in the 610 nm absorption band or in the EPR spectrum is observed. Since H_2O_2 and $S_2O_8^{2-}$ are two-electron inner sphere oxidants, we also tried a series of one-electron outer sphere oxidants: $[Fe(CN)_6]^{3-}$ ($E°$ vs NHE = 0.433 V) (*72*), $Fe(bipy)_2(CN)_2^{+}$ (0.781 V) (*73*), $IrCl_6^{2-}$ (0.867 V) (*74*), and $Fe(bipy)_3^{3+}$ (1.074 V) (*75*). $Fe(bipy)_3^{3+}$ caused large amounts of protein degradation, even at low concentrations; however, the others did not at up to the number of equivalents listed in Table 4. In none of these experiments was there any evidence of copper oxidation either by absorption, by EPR quantitation, or by the appearance of new EPR features, even after an incubation of several hours with the oxidant.

There are two possible explanations for the inability to oxidize this copper site: either a very high reduction potential, or the lack of any good electron-transfer pathway from the surface of the enzyme to the copper site. This latter possibility will be addressed later. Assuming that there is a pathway for electron transfer to this copper site, one can estimate a lower limit for the potential for this site. Using the data for $Fe(bipy)_2(CN)_2^{+}$ and assuming that oxidation of up to 10% might be missed in either the absorption or the EPR spectrum, the Nernst equation was used to calculate a minimum potential for the T1PR site of 0.960 V versus NHE (Table 4). Applying the same calculation to $IrCl_6^{2-}$ yields a minimum potential of 1.077 V versus NHE for the T1PR. Therefore, we conclude that the minimum potential of the T1PR site is ∼1.0 V. Since this is in the range of the redox potentials for tyrosine residues, this explains why more powerful oxidants, such as $Fe(bipy)_3^{3+}$, cause protein degradation but do not oxidize this copper site.

DISCUSSION

A naturally occurring T1 copper site which cannot be oxidized at physiological pH is highly unusual. Several factors contribute to the potential of a metal site in a protein, most notably the contribution of the electronic structure of the oxidized and reduced states and the dielectric properties of the protein matrix. Contributions of electronic structure to the reduction potential of blue copper sites have been examined in detail (*76*). This work showed that the axial ligand is a key factor in modulating the potential of blue copper sites. The long axial copper−thioether bond present in plastocyanin destabilizes the oxidized state more than the reduced state, therefore contributing to the higher reduction potentials seen among blue copper sites relative to normal copper. Elimination of the axial ligand altogether would cause a further significant increase in the potential, since the lower coordination number favors Cu(I). Experimentally this has been observed in azurin, where replacement of the axial methionine ligand with a noncoordinating hydrophobic residue can increase the potential by as much as 138 mV (*77*). Among wild-type blue copper sites, the fungal laccases lack an axial methionine ligand by comparison of their primary amino acid sequences with those of other blue copper sites. Indeed, some of the fungal laccases have the highest potentials previously observed for T1 copper sites (∼0.780 V), although the range of potentials is quite large (as low as 0.450 V) (*12*, *78*). A nonoxidizable blue copper site has been previously observed in the low-pH form of plastocyanin, in which one of the histidines is protonated and thus no longer bound to the copper, which moves further into the plane of the remaining three ligands (*79*−*81*); however, this site has a very different ligand set (N_{His}, S_{Met}, S_{Cys}) from the putative three-coordinate T1 site of the fungal laccases (2 N_{His}, S_{Cys}). From this, it is clear that the lack of an axial ligand in the T1PR site could at least partly explain an extremely high potential. From examination of the amino acid sequence and crystal structure of Cp, we assign the T1PR copper to be the T1 site in domain 2, in which the usual axial methionine is replaced by a noncoordinating leucine residue. Other possible factors which could contribute to the very high potential of this site are the dipole environment of the protein matrix and specific interactions with nearby residues. For example, Lindley et al. have noted that two of the cation-binding residues found in domains 4 and 6, namely, a histidine and an aspartate, have been replaced by a tyrosine and an asparagine in domain 2, respectively (*36*).

Another possible contribution to the redox inactivity of the T1PR copper site is that there is no good electron-transfer pathway from the surface. However, examination of the crystal structure of Cp shows that there is a solvent-exposed asparagine (Asn 323) adjacent to one of the histidines ligated to the copper (His 324) (Figure 4). This would provide an approximately 10 Å, all σ-bond electron-transfer pathway from the surface to the copper. This is roughly the same distance as from the labile cation-binding sites to the T1 sites in domains 4 and 6, and, indeed, Asn323 is at the same sequence position in domain 2 as the cation-binding residues in domains 4 and 6 (*36*). For comparison, the distance from bound ascorbate to the T1 site in ascorbate oxidase is ∼7 Å (*5*). Thus, although 10 Å is relatively long for electron transfer and histidine provides poor coupling with the copper (this bond is only 2% covalent in plastocyanin) (*82*−*84*), it is clear that a lack of accessibility for electron transfer cannot be the dominant reason for the inability of this site to be oxidized by exogenous oxidants, although it may contribute to some extent. Thus, a very high potential, due to some combination of electronic structure and protein matrix contributions, appears to play the key role in why this copper site is permanently reduced.

In resting Cp, the form as isolated by the rapid one-step procedure, an additional one copper per molecule is reduced.

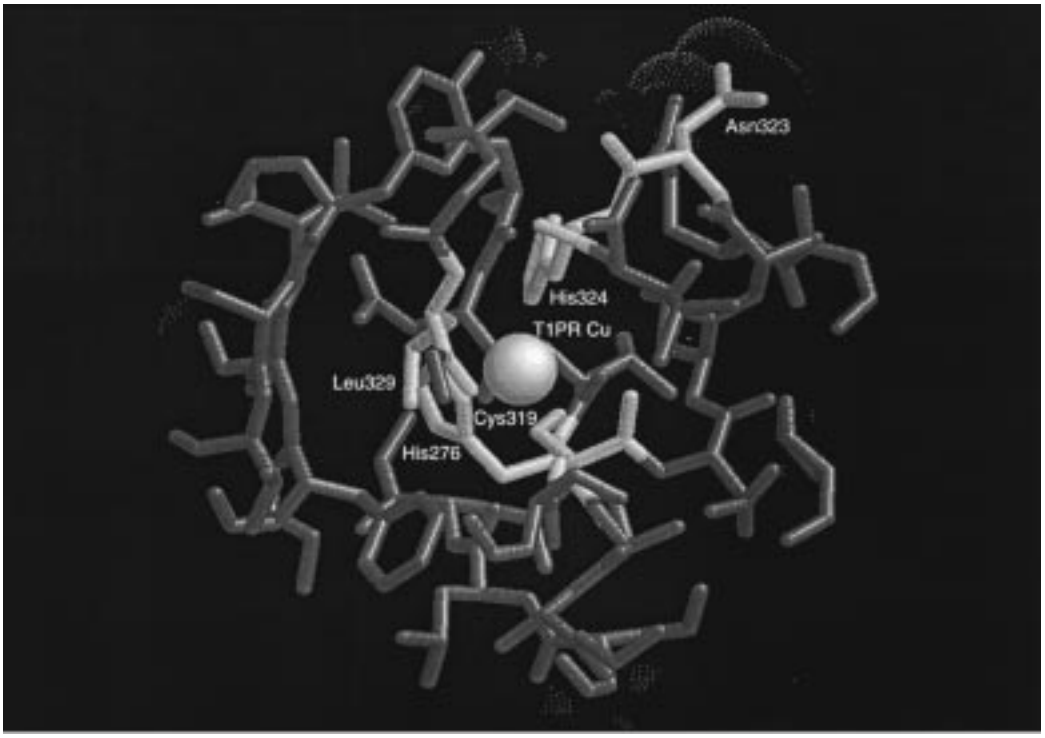

FIGURE 4: View of the crystal structure of human Cp within 10 Å of the T1PR Cu(I) in domain 2. The copper ion, its three ligands (Cys 319, His 276, and His 324), Leu529, and the solvent-exposed Asn 323 are shown in white. A Connelly surface showing the regions of the protein which are solvent-exposed is also shown in white. This figure was created using crystallographic coordinates taken from the Brookhaven Protein Data Bank (*82*), entry code 1KCW.

Unlike the one reduced copper per molecule in peroxide-oxidized Cp, in the resting enzyme, this 1 equiv is distributed among the remaining five copper sites. The observation that resting Cp is a four-electron oxidized form of the enzyme is interesting, since it is consistent with the stoichiometry of dioxygen reduction, which is a four-electron process. Thus, this may indicate that under turnover conditions, one-fifth of the five redox-active coppers stays reduced. The observed distribution of the additional reducing equivalent [$\geq$0.5 T1 Cu(I), $<$0.2 T3 Cu(I)] is roughly what is expected given the relative reduction potentials of the copper sites at pH 7.0 in phosphate buffer: the redox-active T1 coppers have the highest potentials, and the T3 copper pair has the lowest potential (*85*, *86*).

The identification of a unique permanently reduced T1 site in Cp raises the question of its possible functional significance. These results would seem to indicate that the T1PR copper site is not involved in the ferroxidase activity of Cp, since its potential is beyond the range that would be physiologically accessible. Furthermore, the crystal structure shows that while the other two T1 sites still have an adjacent labile divalent metal-binding domain, the T1PR site lacks this structure. Thus, with respect to the physiological role of the two additional T1 sites in Cp, one of them appears not to be catalytically relevant. This does not preclude the possibility that some allosteric effector might dramatically perturb the reduction potential, bringing it into the range in which it could be oxidized under turnover conditions. However, there is no evidence which suggests that this occurs. Also, this does not preclude the possibility that the T1PR site might have some other functional significance. Three explanations for the existence of a permanently reduced, noncatalytic copper site in Cp seem possible. (1) Structural. A structural role for metal sites in biology is a common theme; thus, it is possible that the T1PR site adds to the structural integrity of the enzyme. However, previous studies have shown that the Cu(I) state is generally more labile in blue copper sites than the Cu(II) state, so it would be unusual if this site had a major structural role. (2) Copper transport. This has long been proposed as a possible function of Cp, so if correct, then a non-redox-active copper might still be functionally significant; however, Cp's role in copper transport has been recently questioned (vide supra). (3) Evolutionary vestige. It has been previously proposed that Cp arose from the duplication of an ancestral blue copper gene, since its six domains show such a large amount of internal homology (*47*, *48*). It is possible that Cp has retained a blue copper-binding motif in domain 2 that is only partially functional; i.e., it still binds copper but has lost its axial methionine and can no longer be oxidized, thus eliminating any physiological role.

In summary, we have established that one of the three T1 copper sites in Cp is permanently reduced. The inability of this site to be oxidized is due predominantly to a very high potential and not the lack of a suitable electron-transfer pathway to the surface. In the absence of any data suggesting an allosteric regulation of this site's potential, one must then conclude that this site is not involved in the enzyme's ferroxidase activity. The spectroscopic and magnetic differences observed between the resting and peroxide-oxidized enzyme are due to an additional reducing equivalent which is distributed among the five redox-active copper sites roughly according to their relative reduction potentials. This suggests that the physiological form of Cp under aerobic conditions might be a four-electron oxidized form, consistent with its function in the four-electron reduction of dioxygen. Given this, the role of the other additional T1 site remains to be examined.

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