Spectroscopic Studies and Electronic Structure Description of the High Potential Type 1 Copper Site in Fungal Laccase: Insight into the Effect of the Axial Ligand

Amy E. Palmer,‡ David W. Randall,† Feng Xu,‡§ and Edward I. Solomon‡

Contribution from the Department of Chemistry, Stanford University, Stanford, California 94306, and Novo Nordisk Biotechnology, Davis, California 95616

Received April 5, 1999. Revised Manuscript Received June 9, 1999

Abstract: A variety of spectroscopic techniques combined with density functional calculations are used to describe the electronic structure of the nonaxially ligated, trigonal planar type 1 copper site in three fungal laccases with substantially different type 1 copper reduction potentials. These methods are also applied to a mutant of the high-potential Polyporus pinsitis laccase in which the nonligating axial phenylalanine (Phe) is changed to methionine (Met). Optical absorption, circular dichroism, and magnetic circular dichroism spectroscopies of all three fungal laccases reveal that, relative to the classic blue copper protein plastocyanin, the ligand field strength at the type 1 Cu center and the oscillator strength of the charge-transfer transitions increase. Resonance Raman spectra show that the envelope of Cu–S(Cys) stretching bands is shifted to higher energy in the fungal laccases, imposing a stronger Cu–S(Cys) bond. Differences in the EPR spectra of the fungal laccases and plastocyanin are found to result from the increased ligand field and decreased 4s mixing into the Cu d10,1,2 half-filled, highest occupied molecular orbital (HOMO). All three fungal laccases display similar spectroscopic properties despite their differing reduction potentials. Electronic absorption, circular dichroism (CD), magnetic circular dichroism (MCD), resonance Raman, and EPR spectroscopies show significant perturbation of the electronic structure of the fungal laccase type 1 copper site upon mutation of the axial Phe to Met, consistent with the site becoming more like that in plastocyanin, which has an axial Met ligand; the ligand field decreases, covalency of the Cu–S(Cys) bond decreases, and the Raman shifts of the Cu–S stretching bands decrease. Density functional calculations on the fungal laccase site provide insight into the origin of the experimentally observed increase in covalency and ligand field strength. These calculations show that it is the elimination of the Met ligand donor interaction that leads to an increase in the donor strength of the S(Cys). The contribution of the axial ligand to the reduction potential is discussed.

Introduction

Laccase (p-diphenol: dioxygen oxidoreductase, EC 1.10.3.2) belongs to the family of multicopper oxidases that includes ascorbate oxidase, ceruloplasmin, and FET3.1,2 Functionally, all multicopper oxidases couple four one-electron substrate oxidations with the four-electron reduction of dioxygen to water. Spectroscopic studies, sequence alignments, and crystal structure comparisons reveal that all multicopper oxidases contain at least one type 1 (T1), one type 2 (T2), and one type 3 (T3) Cu center.1–4 These different Cu sites are defined by the spectroscopic properties they exhibit in the oxidized (Cu2+) state. The T1, or blue, Cu site is distinguished by an intense (ε ≈ 5000 M–1 cm–1) absorption (abs) feature around 600 nm and small (<100 × 10–4 cm–1) parallel hyperfine coupling in electron paramagnetic resonance (EPR). The T2, or normal, Cu site displays parallel hyperfine coupling (>160 × 10–4 cm–1) typical of normal tetragonal Cu and does not exhibit intense features in the visible absorption or CD spectrum. The T3, or coupled binuclear, Cu site is comprised of two Cu2+ ions that are antiferromagnetically coupled through a bridging hydroxide.5 The resulting diamagnetic (Stotal = 0) T3 Cu site lacks an EPR signal, but it displays an absorption feature around 330 nm (ε ≈ 5000 M–1 cm–1). Laccase is the simplest of the multicopper oxidases, containing one of each type of Cu for a total of four Cu atoms. Ascorbate oxidase (AO) is essentially a dimer of laccase-like subunits while ceruloplasmin (CEP) is more complex.6 The AO3 and CEP6 crystal structures confirm spectroscopic results7,8 showing that the T2 and T3 Cu sites are in close proximity (within 4 Å) and together form a trinuclear Cu cluster. Similarly, although the crystal structure of fungal laccase is of a T2 depleted form of the enzyme, it indicates that an analogous trinuclear Cu cluster exists in the native enzyme.9 The T1 Cu site in fungal laccase accepts electrons from the substrate, typically diphenols, aryl diamines, or amino

1 Stanford University.
2 Novo Nordisk Biotechnology.

10.1021/ja991087v CCC: $18.00 © 1999 American Chemical Society Published on Web 07/21/1999
phenols, and then transfers the electrons over 13 Å to the T2/T3 trinuclear Cu cluster where dioxygen is reduced to H2O.1

A plethora of comparative studies have established that the copper coordination of the T2 and T3 centers is very similar among the multicopper oxidases; however, there are differences in the T1 Cu ligation. The classic T1 Cu coordination environment, such as that found in plastocyanin (Figure 1A), involves a short Cu–S from Cys (≈2.1 Å), a long Cu–S from Met (≈2.8 Å), and two fairly typical Cu–N from His (≈2.0 Å).10 This ligation pattern is also observed for the T1 Cu in ascorbate oxidase, two of the three T1 copper of human ceruloplasmin, and the tree laccases. In contrast, sequence alignments suggest that laccases isolated from fungi possess a leucine (Leu) or phenylalanine (Phe) rather than a methionine at the axial position.1,3,9

These amino acids do not contain functional groups that can ligate to the Cu and are too bulky to allow water to bind. The recent crystal structure of Coprinus cinereus laccase confirms that the T1 Cu in this fungal laccase forms a trinuclear planar site with two NH-his and one SCys ligands and, most importantly, no axial ligand (Figure 1B).9

The nature of the blue copper site has been the subject of extensive studies that relate the unique electronic structure of this site with its function as an electron-transfer center.12–14 A combination of experimental and theoretical studies have elucidated a number of important properties of the blue Cu site. There is very little geometry change upon reduction from Cu2+ to Cu+, indicating that the site has a low reorganization energy associated with electron transfer.15,16 The Cu–SCys bond is highly covalent,17–19 providing an efficient hole superexchange pathway for electron transfer through this residue.20 The strength of the Cu–SCys bond appears to be inversely related to the strength of the axial Cu–SNax interaction (the weaker the Met, the stronger the Cys).21 The reduction potential of the oxidized T1 Cu site tends to be higher than that of aqueous Cu2+.22

Further insight into the origin of these properties may be gained by looking at systematic differences in the experimental data and electronic structures of a series of blue copper proteins with axial interactions of varying strength. The electronic structures of the classic T1 site in plastocyanin as well as a number of perturbed T1 sites (nitrite reductase, cucumber basic blue, and stellacyanin) have now been examined.19,21,23,24 The perturbed sites fall into two classes: those exhibiting a tetragonal distortion with increasing axial interaction (pseudooazurin, cucumber basic protein, and nitrite reductase) and those displaying a tetrahedral distortion (stellacyanin).21 In both cases the change in electronic structure in the perturbed centers was linked to the axial ligand interaction. The blue copper site in fungal laccase is of particular interest in relation to other blue copper proteins because it lacks an axial ligand.

In this study, we examine the T1 Cu in three different fungal laccases where the axial ligand is absent, Myceliophthora thermophila (M.t.), Rhizoctonia solani (R.s.), and Polyporus pinisetus (P.p.).23,25 From sequence comparisons and EPR data, these enzymes appear to have identical T1 Cu ligation, and yet they exhibit a range (500–800 mV) of redox potentials (Table 1). In this study, we use absorption, circular dichroism (CD), magnetic circular dichroism (MCD), EPR, and resonance Raman spectroscopies to elucidate the spectral features of these enzymes. Parallel studies of fluoride derivatives are used to differentiate the spectral features of the T1 from those of the trinuclear Cu cluster. The spectroscopic results are combined with density functional calculations to gain insight into the electronic structure of this unique, nonaxially ligated, trinuclear planar T1 Cu site. In addition, we examine a mutant in which the axial Phe of the high potential Polyporus pinisetus laccase was mutated to a Met to gain further insight into the contribution of the axial Met ligand to the electronic structure. Finally, the electronic structure of these enzymes is compared to that of the classic blue copper site in plastocyanin and the origins of their spectral and redox differences are discussed.

### Experimental Section

**Enzyme Characterization.** Myceliophthora thermophila laccase, Rhizoctonia solani laccase, Polyporus pinisetus laccase, and the axial F463M mutant of Polyporus pinisetus laccase were cloned and expressed.

<table>
<thead>
<tr>
<th>Table 1. Comparison of Redox Potentials of the T1 Cu</th>
<th>( E^0 )</th>
<th>( \text{Units of mV vs NHE} )</th>
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<tbody>
<tr>
<td>Myceliophthora thermophila laccase</td>
<td>450–480</td>
<td>( ^6 )</td>
</tr>
<tr>
<td>Rhizoctonia solani laccase</td>
<td>630–680</td>
<td>( ^6 )</td>
</tr>
<tr>
<td>Polyporus pinisetus Laccase</td>
<td>750–790</td>
<td>( ^6 )</td>
</tr>
<tr>
<td>Polyporus pinisetus laccase F463M</td>
<td>680</td>
<td>( ^6 )</td>
</tr>
<tr>
<td>spinach plastocyanin</td>
<td>370</td>
<td>( ^6 )</td>
</tr>
</tbody>
</table>

(24) A perturbed we mean an increase in absorption at 450 nm and a rhombic EPR spectrum.
(25) This enzyme has a high degree of homology with Polyporus versicolor laccase. It should be noted that Polyporus pinisetus is also referred to as Trametes villosa, and Polyporus versicolor is often referred to as Trametes versicolor.
as described previously.\textsuperscript{27-30} Enzyme concentrations were determined using the absorption band at 280 nm ($e_{280} = 146$ mM$^{-1}$ cm$^{-1}$ for M.\textit{C.}, 66 mM$^{-1}$ cm$^{-1}$ for R.S.L., and 80 mM$^{-1}$ cm$^{-1}$ for P.P.L and P.P.L F463M). Copper concentrations were determined spectrophotometrically using 2.2'-biquinoline\textsuperscript{31} or by atomic absorption spectroscopy. The concentration of paramagnetic copper was determined from spin quantitation of EPR spectra (vide infra). Spectra were normalized to copper concentration. All experiments were performed in 100 mM potassium phosphate buffer, pH = 6 (pD = 5.6 for MCD experiments). Chemicals used as buffers were reagent grade and were used without further purification. Water was purified to a resistivity of 15–18 M\textOmega using a Barnstead Nanopure deionizing system.

**Isolation of the T1 Cu.** Fluoride adduct samples were prepared by incubating equimolar amounts of NaF and protein for 24 h at 4°C. Samples were then characterized by absorption, CD, and MCD. To selectively reduce the T1 Cu (vide infra), F$^-$ adduct samples were treated anaerobically with 1–5 electron equiv of sodium ascorbate. Samples were then transferred anaerobically to quartz EPR tubes, MCD cells, or optical quartz cuvettes for further spectroscopic characterization. Glassed samples for MCD were prepared in deuterated buffer and 50% (v/v) D$_2$O/glycerol-$d_1$. Addition of glycerol had no effect on the CD spectrum of the proteins.

**Spectroscopic Studies.** Room-temperature UV/visible absorption spectra were obtained using a Hewlett-Packard HP8452A diode array spectrophotometer in 1.0, 0.2, or 0.1 cm quartz cuvettes. Room-temperature CD and low-temperature (5 K) MCD spectra in the 300–800 nm region were collected with a Jasco J-500-C spectropolarimeter operating with an S-20 photomultiplier tube and an Oxford SM4-7T magnet. CD and MCD spectra in the 700–2000 nm region were recorded with a Jasco J-200-D spectropolarimeter, a liquid nitrogen cooled InSb detector, and an Oxford SM4000-77 magnet. CD samples were run in a 1.0 cm quartz cuvette. MCD samples were run in MCD cells fitted with quartz disks and a 0.3 cm rubber gasket spacer. Depolarization of light by the MCD samples was monitored by the effect it had on the CD spectrum of nickel (+)-tartrate placed before and after the sample. Depolarization was less than 5%. Simultaneous Gaussian fitting of the absorption, CD, and MCD spectra was performed using the PeakFit program (Jandel). EPR spectra were obtained using a Bruker ER 220D-SRC spectrometer. All samples were run at 77 K in a liquid nitrogen finger Dewar. Spin quantitation of the EPR spectra was accomplished using a Cu standard, Cu(ClO$_4$)$_2$, of known copper concentration. All experiments were performed in 100 mM potassium phosphate buffer, pH 6.5 for MCD experiments. Calculations were performed on a series of models with varying Cu–S$_{Cys}$ bond lengths. Reasonable agreement with spectroscopy was given by a model with a Cu–S$_{Cys}$ bond length of 2.067 Å, the distance in the oxidized poplar plastocyanin (PLC) structure\textsuperscript{39} (PDB code: 1PLC). The precision of this bond length in plastocyanin is ±0.04 Å.\textsuperscript{30} The Cu–S–C$^\equiv$ angle was placed at 110.4°, the value in the 1PLC structure. The imidazole nitrogens were placed such that the S–Cu–N angles were identical to the laccase crystal structure.\textsuperscript{29} A coordinate system was chosen to give a Cu d$_z^2$–$d_x^2$–$d_y^2$ ground-state wavefunction;\textsuperscript{35,36} the Cu–S$_{Cys}$ bond is 45° from the x and y axes, and the N$_S$S plane defines the xy plane. This axis system diagonalizes the g tensor for plastocyanin.\textsuperscript{36} The Cartesian coordinates employed for calculations performed for this study are provided as Supporting Information.

**B. Self-Consistent Field Xα Scattered Wave (SFC-Xα-SW) Calculations.** Following the approach described in ref 23, the electronic structure of the T1 center in fungal laccase was probed using the 1982 QCPE release of the SFC-Xα-SW package.\textsuperscript{57-60} The atomic sphere radii were adjusted to reproduce the experimentally observed g values in plastocyanin.\textsuperscript{19} The parameters used for the SFC-Xα-SW calculations are listed as Supporting Information.

**C. ICAO Density Functional Calculations.** Spin-restricted calculations were performed as described in ref 23 using version 2.0.1 of the Amsterdam Density Functional (ADF) program suite.\textsuperscript{41} Basis functions, core expansions, core coefficients, and fit functions for all atoms were used as provided from database IV, which includes Slater-type orbital triple-$\zeta$ basis sets for all atoms and a single-$\zeta$ polarization function for all atoms except Cu. Results and Analysis

**Identification of the Spectral Features of the T1 Cu.** The absorption, CD, and MCD spectra of multicooper oxidases, including fungal laccase, are complicated by contributions from all three Cu sites. The absorption and CD spectra contain transitions involving the T1 and T3 coppers, while the MCD spectrum is a combination of transitions arising from the paramagnetic T1 and T2 coppers. To gain insight into the electronic structure of the T1 Cu, one must isolate its features from those of the T2 and T3 Cu sites. This was accomplished using the differential anion binding properties of the Cu sites. Exogenous ligands (F$^-$, CNO$^-$, N$_3^-$, O$_2^{2-}$) have been shown to bind to the T2 Cu site and in some cases (N$_3^-$ and O$_2^{2-}$) to bridge the T2 and T3 Cu centers.\textsuperscript{32-46} However, the T1 Cu is


in C1 symmetry, in which methyl thiolate is substituted for cysteine and imidazoles replace the histidine residues in the T1 copper coordination sphere. The crystallographic coordinates of \textit{Coprinus cinereus} laccase\textsuperscript{9} guided the construction of the model for the fungal laccase T1 site used in the calculations. The 1.9 Å resolution crystal structure shows a long Cu–S$_{Cys}$ distance of 2.2 Å (vide infra). Calculations were performed on a series of models with varying Cu–S$_{Cys}$ bond lengths. Reasonable agreement with spectroscopy was given by a model with a Cu–S$_{Cys}$ bond length of 2.067 Å, the distance in the oxidized poplar plastocyanin (PLC) structure\textsuperscript{39} (PDB code: 1PLC). The precision of this bond length in plastocyanin is ±0.04 Å.\textsuperscript{30} The Cu–S–C$^\equiv$ angle was placed at 110.4°, the value in the 1PLC structure. The imidazole nitrogens were placed such that the S–Cu–N angles were identical to the laccase crystal structure.\textsuperscript{29} A coordinate system was chosen to give a Cu d$_z^2$–$d_x^2$–$d_y^2$ ground-state wavefunction;\textsuperscript{35,36} the Cu–S$_{Cys}$ bond is 45° from the x and y axes, and the N$_S$S plane defines the xy plane. This axis system diagonalizes the g tensor for plastocyanin.\textsuperscript{36} The Cartesian coordinates employed for calculations performed for this study are provided as Supporting Information.


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unaffected by exogenous ligands. Normally, the T1 Cu accepts electrons from the substrate and transfers them to the T2/T3 centers in a fast (\(>10^3\) s\(^{-1}\)) reaction.\(^1\) However, binding an anion at the T2 Cu can alter the thermodynamic driving force for electron transfer from the T1 to the T2/T3 center and therefore allows selective reduction of the T1. This selective reduction of the T1 Cu allows its features to be distinguished from those of the T2 and T3 coppers. F\(^-\) binds to the T2 with high affinity,\(^2\) and this binding can be monitored by the appearance of superhyperfine coupling between the Cu\(^{2+}\) (\(S = \frac{1}{2}\)) and the F\(^-\) (\(I = \frac{1}{2}\)). Previous studies of *Polyporus versicolor* laccase indicated that F\(^-\) binding to the T2 Cu lowers the redox potential of the T3 Cu center from 780 to 580 mV.\(^2\) This 200 mV change in \(E^\circ\) is sufficient to localize the first electron, supplied by an external reductant, on the T1 Cu.

In the present study, P.p. laccase was treated with 1 equiv of fluoride (F\(^-\)) for 24 h. The EPR spectrum (Figure 2) of the F\(^-\)-treated sample indicated that the T2 Cu features were altered, with the appearance of \(^{19}\)F superhyperfine coupling (\(A_{\text{IP}}^F = 53 \times 10^{-4} \text{ cm}^{-1}\), \(A_{\perp}^F = 125 \times 10^{-4} \text{ cm}^{-1}\))\(^9\) indicating anion binding. There was no detectable change in the spin Hamiltonian parameters of the T1 Cu. Further, addition of F\(^-\) did not alter the absorption, CD, or MCD spectra. These results suggest that the T1 Cu was not significantly perturbed by F\(^-\) binding to the T2 Cu. Addition of a stoichiometric amount of ascorbate to the F\(^-\)-bound sample resulted in complete loss of absorption and CD features in the visible region (see Figure S1 of Supporting Information) and disappearance of the T1 Cu EPR signal, while the F\(^-\)-altered T2 Cu EPR signal remained present (Figure 2). These changes are consistent with reduction of the T1 Cu in the presence of an oxidized F\(^-\)-bound T2 Cu center. Simulations show that the EPR parameters (\(g\) values, hyperfine coupling constants, and \(^{19}\)F superhyperfine coupling constants) of the T2 Cu in the samples containing F\(^-\), with and without ascorbate, are the same, indicating that reduction of the T1 Cu does not affect the T2 Cu. These manipulations enable differentiation of the spectral contributions from the T1 and T2 Cu centers.

Low-temperature MCD spectroscopy directly probes excited states of metal centers with paramagnetic ground states. Therefore, the MCD spectrum of resting P.p. laccase contains contributions from both the T1 and T2 Cu. Selective reduction of the T1 Cu allows identification of the bands due to each Cu site. Figure 3 shows the MCD spectrum of P.p. laccase + F\(^-\) (T1\(_{\text{ox}}\)/T2\(^-\)F\(^-\)\(_{\text{ox}}\)) and P.p. laccase + F\(^-\) + ascorbate (T1\(_{\text{ox}}\)/T2\(^-\)F\(^-\)\(_{\text{ox}}\)). The arrows highlight the changes that occur upon addition of ascorbate. The complete loss of the negative band at 6900 cm\(^{-1}\) and the positive feature \(\sim 17\) 500 cm\(^{-1}\) upon T1 reduction indicates these bands arise from the T1 Cu. In addition, the negative bands \(\sim 13\) 500 and \(\sim 15\) 500 cm\(^{-1}\) lose most of their intensity, suggesting that the T1 Cu makes a significant contribution in this region. The spectrum of P.p. laccase + F\(^-\) + ascorbate has a positive MCD feature at \(\sim\) 11 000 cm\(^{-1}\) and a negative feature at \(\sim 15\) 000 cm\(^{-1}\). These MCD transitions are consistent with previous reports of the T2 Cu MCD spectrum in *Rhus vernicifera* (tree) laccase where the T2 features were selectively observed by replacing the T1 Cu with spectroscopically innocent Hg\(^{2+}\).\(^4\)

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(49) Although the splittings are clearly observed in the spectrum, superhyperfine coupling constants were obtained from EPR simulations.
The MCD spectral features of the T1 Cu in P. p. laccase were obtained by subtracting the spectrum of P. p. laccase + F− + ascorbate (T1 red/T2 F− ox) from the spectrum of P. p. laccase + F− (T1 ox/T2 F− ox). The room-temperature absorption, CD, and low-temperature (5 K) MCD spectra of the T1 Cu in P. p. laccase obtained in this manner are shown in Figure 4A. Simultaneous fitting of these three spectra revealed that six bands were required to fit the region from 5000 to 25 000 cm−1. These bands are depicted by the dashed lines in the absorption spectrum. To be consistent in our comparison with other blue copper proteins, we have used the numbering scheme from plastocyanin.19 The experimental oscillator strengths (fexp) were calculated according to the approximation fexp ≈ 4.61 × 10−9εmaxν1/2, where the absorption maximum (εmax) is expressed in M−1 cm−1 and the full width at half-maximum (ν1/2) is expressed in cm−1. The energies and experimental oscillator strengths of the bands are given in Table 2. The absorption, CD, and MCD spectra of M. t. laccase and R. s. laccase are very similar to those of P. p. laccase and are presented in Supporting Information (Figures S2 and S3). The transition energies and experimental oscillator strengths of all three enzymes are included in Table 2.

In addition to studying the wild-type fungal laccases, we examined a mutant in which the nonligating axial Phe in P. p. laccase was changed to a Met.30 Figure 4B displays the absorption, CD, and MCD of the P. p. laccase F463M mutant. (50) The energy region above 25 000 cm−1 is obscured by contributions from a small heme impurity.
Copper Site in Fungal Laccase

Again, the individual Gaussian resolved transitions obtained from simultaneous fitting of the absorption, CD, and MCD spectra are given by the dashed lines in the absorption spectrum. The energies and experimental oscillator strength of the bands are included in Table 2.

Ligand field and charge-transfer transitions can be differentiated by comparing their relative intensities in absorption and MCD. In particular, the MCD intensity depends on the magnitude of spin–orbit coupling for the center involved in the transition.\(^{51-53}\) Because the spin–orbit coupling parameter for Cu is greater than that for S or N (\(\xi_{dS}(Cu) \approx 828 \text{ cm}^{-1}, \xi_{dS}(N) \approx 70 \text{ cm}^{-1}\)), transitions centered on the Cu (i.e., \(d \rightarrow d\)) have a higher C-term MCD intensity than transitions involving significant S or N character (i.e., charge transfer). Alternatively, absorption intensity is expected to be much higher for charge transfer (CT) transitions because the ligand–ligand overlap (which determines absorption intensity) between the two states involved in the charge-transfer transition should be much larger.

In both the wild-type P.p. laccase and the F463M mutant, band 4 at \(\sim 16,500 \text{ cm}^{-1} (\sim 600 \text{ nm})\) has a very high absorption \(\epsilon_{\text{max}}\) and a relatively low MCD intensity and is the intense feature responsible for the blue color of fungal laccase. In analogy with other well-characterized blue copper proteins\(^{19,21}\) (Figure 4, Table 2) this band involves a \(\pi\)-type Cu–SCys bonding interaction and is thus assigned as a \(\pi \rightarrow Cu\) \(d_{x^2-y^2}\) CT transition. Note that EPR and density functional calculations identify \(d_{x^2-y^2}\) as the highest occupied molecular orbital (HOMO) (vide infra). Band 3 can also be assigned a \(S \rightarrow Cu\) \(d_{x^2-y^2}\) CT transition but as shown previously,\(^{19,54}\) it is characterized by a pseudo-\(\sigma\) bonding interaction. There has been some disagreement as to whether a band is in fact present in the 1800 cm\(^{-1}\) energy region,\(^{55,56}\) however, our data, particularly the CD spectrum, show that it is indeed required to be present on the basis of experiment. Although these two SCys \(\rightarrow Cu\) \(d_{x^2-y^2}\) CT transitions are at similar energies in the fungal laccases, in the P.p. laccase F463M mutant, and in plastocyanin, the oscillator strength of the charge-transfer transitions varies significantly. The total oscillator strength at room temperature in the fungal laccases ranges from 0.1046 to 0.1096, which is about 30\% higher than in plastocyanin (0.0824). The oscillator strength observed in P.p. laccase F463M (0.1032) is about 5\% lower than in the wild type but higher than for plastocyanin. The oscillator strength of a ligand-to-metal charge-transfer transition can be correlated with the donor strength of the ligand.\(^{57}\) A higher oscillator strength in fungal laccase thus indicates that the Cu–SCys bond is stronger and more covalent in the fungal laccases. The decrease in oscillator strength in the F463M mutant demonstrates that the covalency of the Cu–SCys bond is lowered relative to the wild type. The larger covalency of the fungal laccase T1 center observed here is further supported by Q band electron nuclear double resonance (ENDOR) measurements\(^{58}\) of the \(\beta\)-methylenyl \(^3\)H hydrogen couplings (HFCs) for the Cys residue ligated to the Cu center. In this comparative study of T1 sites, different blue Cu centers gave similar \(\rho_S\) values with the exception of a fungal laccase where the spin density (and therefore covalency) was \(-33\\%\) larger.\(^{59}\) Sulfur K-edge (1S \(\rightarrow S\) 3p) X-ray absorption spectroscopy can provide a direct, quantitative measure of the S covalency of the HOMO, and these studies are currently being pursued.

The four lower energy transitions observed in the optical spectra of wild type and F463M P.p. laccase (bands 5–8) are assigned to ligand field transitions. On the basis of MCD sign and intensity, band 5 is attributed to the \(d_{x^2-y^2}\)–\(d_{x^2-y^2}\) transition; this transition is typically the most intense negative feature in the blue copper MCD spectrum.\(^{19,21}\) Band 6 is not resolved in the P.p. laccase MCD spectrum; however, its presence is required by the simultaneous fit of the absorption, CD, and MCD spectra. Presumably, band 6 is not resolved in the MCD spectrum because it overlaps the negative bands resulting from the \(d_{x^2-y^2}\)–\(d_{x^2-y^2}\) and \(d_{x^2-y^2}\)–\(d_{x^2-y^2}\) transitions, since these bands are very close in energy in fungal laccase. However, band 6 is clearly resolved in the P.p. laccase F463M MCD spectrum, and because of its positive sign, it is attributed to the \(d_{x^2-y^2}\)–\(d_{x^2-y^2}\) transition. On the basis of analysis of the MCD sign and comparison to other blue copper proteins (vide infra), band 7 is assigned as \(d_{x^2-y^2}\)–\(d_{x^2-y^2}\) and band 8 is attributed to \(d_{x^2-y^2}\)–\(d_{x^2-y^2}\).

Overall, there are several important spectral differences among the fungal laccases, the F463M mutant, and plastocyanin, which serves as a reference point for describing blue copper centers (Figure 4, Table 2). In the fungal laccases, the \(d \rightarrow d\) bands shift to a higher transition energy than in plastocyanin. This is most clearly manifested in the absorption spectrum in which the \(d \rightarrow d\) bands appear as a shoulder at 13700 cm\(^{-1}\). In plastocyanin these bands exhibit a well-resolved envelope at \(\sim 12500 \text{ cm}^{-1}\). The electronic spectra of the F463M mutant, where the nonligating Phe was replaced with a potentially ligating Met, reveal that the \(d \rightarrow d\) bands are lower in energy than in the wild type but higher than in plastocyanin. Further, the \(d_{x^2-y^2}\), \(d_{x^2-y^2}\), and \(d_{x^2-y^2}\) transitions are grouped closer in energy in the fungal laccases; thus, the \(d_{x^2-y^2}\)–\(d_{x^2-y^2}\) transition is not clearly resolved in the MCD spectrum. In the F463M mutant, these bands are more spread out in energy (Figure 4B), allowing the positive MCD band 6, the \(d_{x^2-y^2}\)–\(d_{x^2-y^2}\) transition, to be observed. In plastocyanin, these bands are still further separated in energy.

In the wild-type fungal laccases, the lowest energy transition \(d_{x^2-y^2}\)–\(d_{x^2-y^2}\) is negative, whereas in the F463M mutant and other axially ligated blue copper proteins it is positive.\(^{19,21}\) The origin of this sign change can be understood by examining the different mechanisms of the MCD C-term intensity. As mentioned earlier, for a transition to have C-term intensity it must be polarized in two perpendicular directions. In the low-symmetry blue copper site this is accomplished by spin–orbit coupling between states that mix their orthogonal polarizations.

(53) MCD intensity of Cu\(^{57}\) at low temperature arises from C-terms. C-term intensity requires polarization in two perpendicular directions. However, the low symmetry of the blue copper site removes all orbital degeneracy; therefore, transitions are polarized in only one direction. For low-symmetry sites, the only mechanism for C-term intensity involves spin–orbit coupling between states that mix their orthogonal polarizations.
(59) We note that by using the appropriate McConnell relation (i.e., \(A \approx \rho_S(\text{Cu}) \times c^0 \times \cos \theta\) with the Cu–S–C\(^{–}\)–H dihedral angles derived from the crystal structure,\(^{10}\) and a value of \(\rho_S(\text{Cu}) (83 \text{ MHz})\) appropriate for a S-centered radical,\(^{7,9}\) and the orientation of the orbital containing the unpaired spin known from single-crystal EPR,\(^{35}\) the spin density at the thiolate S can be estimated for plastocyanin to give \(\rho_S = 0.36\) (in good agreement with the Xe-SW and S K-edge XAS value of \(-38\%\)).
coupling, which mixes transitions of orthogonal polarization. Both excited state and ground-state spin–orbit \( (s \rightarrow o) \) coupling can contribute to the overall C-term intensity. The former leads to the MCD sum rule that the C-term intensity summed over all excited states must equal zero, while the latter leads to deviations from the sum rule. The contributions of both these mechanisms are summarized in the following equation:

\[
C_o = \frac{-1}{12} \sum_{\text{doubly}} \sum_{\text{spin}} \sum_{\text{orbit}} \epsilon_{\text{groups}}^2 \delta_{\text{o}} \{ A_{ij}^{-1} \text{Im}(\psi_j) \sum_{\text{orbit}} \xi(\nabla) \tilde{T}_{i,m} \psi_i \psi_i \psi_i \psi_i + \Delta_{ij}^{-1} \text{Im}(\psi_j) \sum_{\text{orbit}} \xi(\nabla) \tilde{T}_{i,m} \psi_i \psi_i \psi_i \psi_i \} \]

where the first term in braces represents the ground-state \( s \rightarrow o \) coupling and the second term denotes the excited-state \( s \rightarrow o \) coupling. In this equation, \( o \) denotes the acceptor orbital (i.e., the ground state), \( i \) denotes the donor orbital (i.e., the excited state), and \( j \) denotes an intermediate orbital (i.e., an excited state) \( 51 \).

Using this equation and the procedure described in ref 60, we can analyze the individual contributions to the C-term intensity of the \( d_{z^2} \rightarrow d_{x^2-y^2} \) transition for fungal laccase and for plastocyanin. In this case, the donor orbital (excited state) is \( d_{z^2} \), the acceptor orbital (ground state) is \( d_{x^2-y^2} \), and the intermediate orbital is either \( d_{xy} \), \( d_{yz} \), or \( d_{xz} \) pseudo-\( s \). For plastocyanin, the \( d_{z^2} \rightarrow d_{x^2-y^2} \) transition is calculated to be positive. The main contribution to this positive intensity is excited-state spin–orbit coupling with \( d_{xy} \), which involves the \( L_o \) operator coupling \( m \), and \( m \) polarized transitions. In fungal laccase, \( m \) decreases significantly to the point where it is negligible. This derives from the lack of an axial ligand that drops the Cu atom into the \( N_2 S \) plane, eliminating the transition density in the \( z \) direction, which is perpendicular to the plane.

**EPR Spectra and Parameters.** Figure 5 shows the EPR spectra and simulations for the three fungal laccases and the P.p. laccase F463M mutant. Table 3 presents the spin Hamiltonian parameters obtained from EPR spectral simulations. The observed \( g \) values (\( g_{\|} > g_\perp > 2.0023 \)) are consistent with a \( d_{xy} \) half-occupied highest molecular orbital (HOMO). In all three fungal laccases, \( g_{\|} \) is significantly smaller than in plastocyanin (2.208 – 2.194 vs 2.226). There are no significant differences in the spin Hamiltonian parameters of the three fungal laccases. In the F463M mutant, the \( g_{\|} \) value (2.213) is between that of the wild-type enzyme and plastocyanin. Similarly, the \( g_\perp \) value of the three fungal laccases and the P.p. laccase F463M mutant is smaller than that observed in plastocyanin. The \( g \) value expressions obtained from ligand field theory are directly proportional to the amount of metal character in the half-occupied HOMO (i.e., covalency) and inversely proportional to the ligand field transition energies. \( 63 \) Analyses of \( d \rightarrow d \) bands in the absorption, CD, and MCD spectra reveal that the ligand field transitions have shifted higher in energy in the fungal laccases relative to plastocyanin. Assuming a similar covalency for plastocyanin and the \( T1 \) site in fungal laccase, the following expressions can be used to estimate the \( g \) value dependence on the ligand field transition energies.

\[
g_{\|} = 2.3 + 0.06 \Delta_{T1} \quad \text{and} \quad g_{\perp} = 2.3 - 0.06 \Delta_{T1}
\]

\( \Delta_{T1} \) is the transition energy of the intermediate excited state minus the transition energy of the excited state.

**Table 3. Spin Hamiltonian Parameters Obtained from EPR Simulations**

<table>
<thead>
<tr>
<th></th>
<th>( g_{|} )</th>
<th>( g_\perp )</th>
<th>( A_{|} )</th>
<th>( A_\perp )</th>
</tr>
</thead>
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<tr>
<td>M.t.</td>
<td>2.201</td>
<td>2.045</td>
<td>7</td>
<td>2.247</td>
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<tr>
<td>R.s.</td>
<td>2.208</td>
<td>2.043</td>
<td>10</td>
<td>2.265</td>
</tr>
<tr>
<td>P.p.</td>
<td>2.194</td>
<td>2.046</td>
<td>8</td>
<td>2.248</td>
</tr>
<tr>
<td>P.p. F463M</td>
<td>2.213</td>
<td>2.047</td>
<td>9</td>
<td>2.248</td>
</tr>
<tr>
<td>Plastocyanin*</td>
<td>2.226</td>
<td>2.053</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

*Published previously in ref 35.

\( \Delta_{T1} \) McGarvey, B. R. In *Transition Metal Chemistry*; Carlin, B. L., Ed.; 1966: Chapter 3, pp 89–201.
\[ \Delta g_{J}(\text{laccase}) = \frac{E_{J}(\text{PLC})}{E_{J}(\text{laccase})} \Delta g_{J}(\text{PLC}) \]  
\[ \Delta g_{\perp}(\text{laccase}) = \frac{E_{\perp}(\text{PLC})}{E_{\perp}(\text{laccase})} \Delta g_{\perp}(\text{PLC}) \]

In these equations, \( \Delta g_{J} \) is the deviation of that \( g \) value from the spin-only value (2.0023) and \( E \) is the experimental transition energy in cm\(^{-1}\). Using these expressions, the experimental energies of the \( d_{x^2-y^2} \rightarrow d_{x^2-y^2} \) transitions \( (E_{J}) \), and \( g_\perp \) of plastocyanin (see Table 2 for values), \( g_\perp \) for the T1 site is predicted to be 2.186 for M.t., 2.187 for R.s., 2.184 for P.p., and 2.197 for the P.p. F463M mutant. These calculated values are in close agreement with, but are all lower than, the experimental values. However, it should also be noted that these calculated values will increase slightly when the experimentally derived covalency (vide supra) is taken into consideration. When \( E_{\perp} \) is used, \( g_\perp \) of the laccases is predicted to be 2.046 for M.t., 2.045 for R.s., 2.045 for P.p., and 2.047 for the P.p. F463M mutant. The calculated values for the T1 Cu in all three fungal laccases and the F463M mutant are quite similar to the experimental ones, indicating that the increased ligand field transition energies account for the smaller \( g \) values in these enzymes.

Relative to plastocyanin, the \( A_\parallel \) value is higher in the fungal laccases \((63) \times 10^{-4} \text{ cm}^{-1}\) for plastocyanin vs \((182-90) \times 10^{-4} \text{ cm}^{-1}\), while that of the P.p. laccase F463M axial mutant \((78) \times 10^{-4} \text{ cm}^{-1}\) is between that of the wild-type P.p. laccase and that of plastocyanin. The \( A_\perp \) values are too small to be obtained reliably. The hyperfine coupling constant depends on several competing factors, including (a) Fermi contact, which results from unpaired spin density at the Cu nucleus, (b) direct spin dipolar coupling, which arises from the interaction of the electron spin \( (S) \) with the nuclear spin \( (I) \) of the metal, and (c) indirect (orbital) dipolar coupling, which occurs as a result of coupling between the electron orbital magnetic moment \( (\mu) \) and the nuclear spin \( (I) \) of the metal. The following ligand field expressions are used to describe these contributions to the hyperfine coupling:\(^{63}\)

\[ A_\parallel = P_\alpha \left( -\kappa - \frac{4}{7} \alpha^2 + \frac{3}{7} \Delta g_\perp + \Delta g_\parallel \right) \]
\[ A_\perp = P_\alpha \left( -\kappa + \frac{2}{7} \alpha^2 + \frac{11}{14} \Delta g_\perp \right) \]

where \( P_\alpha \) is 396 \times 10^{-4} \text{ cm}^{-1}, \( \kappa \) is the Fermi contact term, and \( \alpha^2 \) is the percent metal character in the \( d_{x^2-y^2} \) orbital. If we make the reasonable assumptions that \( \kappa \) and \( \alpha^2 \) are the same in the fungal laccases and plastocyanin and that the hyperfine coupling is negative, experimental \( g \) values can be used to calculate the hyperfine coupling. The lower values of \( \Delta g_\parallel \) and \( \Delta g_\perp \) in the fungal laccases relative to plastocyanin will decrease the indirect orbital dipolar contribution to the hyperfine. Because the indirect dipolar contribution is positive, a decrease in this term will result in the hyperfine becoming more negative (i.e., larger in magnitude). When the experimental \( g \) values are used, the value of \( A_\parallel \) changes from \(-63 \times 10^{-4} \text{ cm}^{-1}\) for plastocyanin to \(-74 \times 10^{-4} \text{ cm}^{-1}\) for M.t. laccase, \(-71 \times 10^{-4} \text{ cm}^{-1}\) for R.s. laccase, \(-76 \times 10^{-4} \text{ cm}^{-1}\) for P.p. laccase, and \(-69 \times 10^{-4} \text{ cm}^{-1}\) for P.p. F463M. Thus, the decrease in \( g \) values accounts for about 50% of the difference in hyperfine coupling between plastocyanin and fungal laccase. Another possible contribution to the difference in hyperfine couplings could be a change in 4s mixing into the HOMO. The contribution of 4s to the Fermi contact term is large and positive. Therefore, even a small decrease in 4s mixing would cause the hyperfine coupling to become more negative. Density functional calculations (vide infra) on fungal laccase indicate that the amount of 4s mixing does indeed decrease from 1% in plastocyanin\(^{23}\) to 0% in fungal laccase because of the planar geometry of fungal laccase. This decrease would result in the hyperfine coupling becoming more negative by about 16.8 \times 10^{-4} \text{ cm}^{-1}\) in the fungal laccases. When combined with the change in ligand field, these contributions predict a parallel hyperfine coupling of \(-91 \times 10^{-4} \text{ cm}^{-1}\) for M.t.L, \(-87 \times 10^{-4} \text{ cm}^{-1}\) for R.s.L, and \(-93 \times 10^{-4} \text{ cm}^{-1}\) for P.p.L, in reasonable agreement with the experimentally observed values. The value of \(-69 \times 10^{-4} \text{ cm}^{-1}\) predicted for F463M P.p. laccase follows the anticipated trend based on ligand field transition energies; however, it is lower than the experimentally observed value of \(78\) \times 10^{-4} \text{ cm}^{-1}\). The predicted \( A_\parallel \) value cannot be corrected for the change in 4s mixing because no structure is available for electronic structure calculations.

**Resonance Raman.** Figure 6 presents the resonance Raman spectra of the three fungal laccases and the PpL F463M mutant.
obtained with excitation at 647.1 nm into the Scys → Cu charge-transfer band. As for other blue copper proteins,33,65 the spectra are characterized by a complex envelope of rr bands centered around 400 cm⁻¹, all of which are associated with Cu–S vibrations. In all of the fungal laccases, the envelope of bands occurs at higher frequency than plastocyanin.33 The most intense peak in the resonance Raman spectrum varies as follows: 428 cm⁻¹ (M.t. laccase), 435 cm⁻¹ (R.s. laccase), 428 cm⁻¹ (P.p. laccase), and 426 cm⁻¹ (F463M mutant) compared to 425 cm⁻¹ for plastocyanin33 and 408 cm⁻¹ for P. aeruginosa azurin.66 The intensity-weighted average, (ψCu,S), of the peaks in the 400 cm⁻¹ envelope has been shown to be an indicator of the Cu–Scys bond strength as described in ref 33. For the fungal laccases the HOMO of the T1 center in fungal laccase is highly covalent, with the increased oscillator strength observed in the fungal laccases being characterized by a complex envelope of rr bands centered at 403 cm⁻¹ for P. p. laccase,1 ) 33 or 407.6 cm⁻¹ for plastocyanin (vide infra). Finally, the calculation reveals that the increase derives from the loss of the axial Met ligand into the HOMO in fungal laccase (1% vs 0%), which contributes character to the larger S Cys spin density implied in sphere overlap.

Electronic Structure Calculations. Xα-SW electronic structure calculations were performed to gain further insight into the origin of the differences between the electronic structure of the T1 center in fungal laccase and plastocyanin. The ground-state energies and one-electron wave functions are summarized in Table 4. Plots of the contours of the HOMO both perpendicular and parallel to the N₂S plane are displayed in Figure 7.

The electronic structural properties of the HOMO are important, since this orbital is associated with the electron transfer reactivity of this site. Table 4 and Figure 7 suggest that the HOMO of the T1 center in fungal laccase is highly covalent, containing only 54% Cu character.67 The majority of the remaining orbital character comes from Scys (37%). The S character in the HOMO is characterized by a π-type Scys–Cu interaction between the Cu dₓ²−₂z² orbital and the S πt orbital, a situation very similar to that observed for plastocyanin.19,23,55,68 The calculated Scys character in the HOMO of the T1 in fungal laccase (37%) corresponds to a 2% increase from the amount of S character in the HOMO of plastocyanin (35%).23,69 The small increase in Scys character in the HOMO of fungal laccase, relative to plastocyanin, is consistent with the higher oscillator strength (vide supra), the increased Scys spin density implied from the β-methylene 1H proton hyperfine couplings observed with ENDOR (vide supra),58 and the increased (ψCu,S) obtained from resonance Raman data. While the increase in thiolate character predicted by the calculations is smaller than the experimentally observed increase, the calculations clearly show that the increase derives from the loss of the axial Met ligand in fungal laccase (vide infra). Finally, the calculation reveals that relative to plastocyanin, there is a decrease in 4s mixing into the HOMO in fungal laccase (1% vs 0%), which contributes to the larger A Hannah value observed for fungal laccase (vide supra).

(69) The value of 38% for the S character in plastocyanin originated from the calculations of ref 19, which used a C₆-idealized Cu site with a Cu–Scys distance of 2.13 Å. These calculations were experimentally calibrated to the EPR g-values by varying the sphere radii. For accurate comparison with the C₁ fungal laccase site, we use the plastocyanin calculations of Larsson et al.68 These calculations employ the same sphere radii as Gewirth and Solomon (and the present study), a Cu–Scys distance of 2.067 Å, and a C₁ representation of the plastocyanin active site. More recent plastocyanin Xα-SW calculations give a value of 35% for the S character in the HOMO of plastocyanin. The quantitative difference (3%) in S character between these two plastocyanin calculations is due to differences in charge partitioning in the intersphere regions and differences in sphere overlap.
The four remaining fully occupied d orbitals are next in energy (Figure 8); immediately below the HOMO in energy is the d_{xy} orbital, followed by the d_{z^2} orbital, and finally the d_{x^2-y^2} pair. Overall, in fungal laccase the splitting of the d manifold appears to increase relative to plastocyanin. In addition, the separation between d_{xy}, d_{xz}, and d_{yz} decreases, causing these transitions to be grouped close in energy. These observations are consistent with the experimentally observed trends in the transition energies of fungal laccase relative to plastocyanin.

The next four orbitals are formally charge transfer in nature, involving S_{Cys} π and pseudo-σ interactions, as well as π_{1} type orbitals on each of the imidazole (Im) rings. Although they are in the experimentally observable energy region, the Im π_{1} orbitals are not expected to have significant CT intensity, since they have little overlap with the HOMO; the electron density in the π_{1} orbitals is predominantly on nonligating atoms of the Im rings, which do not contribute to the HOMO. Below the Im π_{1} orbitals in energy are the Im π_{2} CT orbitals, which do have a significant contribution from the ligating N(His). These are followed by the S_{Cys} σ interaction that mainly involves the S^′ and C^0 of the thiolate.

In passing, we note that calculations using the 2.19 Å Cu–S_{Cys} from the crystal structure give a covalent HOMO with only 26% thiolate character. This HOMO is similar in general appearance to that in Figure 7. However, the decreased covalency, relative to plastocyanin, for the calculation using the crystallographic 2.19 Å Cu–S distance is inconsistent with the experimentally observed increase in covalency and the strength of the Cu–S bond indicated by the resonance Raman spectra. The 2.067 Å bond length used in the calculations (Figure 8 and Figure 9) is within the precision of the fungal laccase crystal structure at 1.9 Å resolution.

Discussion

The major differences between the T1 Cu centers in the crystal structures of plastocyanin and fungal laccase are the presence of the axial ligand and the position of the Cu atom with respect to the N_{His}–N_{His}–S_{Cys} plane. This study focuses on the effect that such geometric changes will have on the electronic structure of the T1 Cu site. Absorption, CD, MCD, EPR, and resonance Raman data demonstrate that the high-potential T1 Cu site in fungal laccase exhibits a number of significant electronic structure differences compared to the classic blue copper site in plastocyanin. The d → d bands move to higher energy, reflecting an increase in the strength of the ligand field. This shift in the ligand field causes the g values to decrease. The decreased g values in fungal laccase account for about 50% of the observed increase in the hyperfine coupling, with the remaining change coming from a decrease in 4s mixing. The increased oscillator strength and the previously published ENDOR results suggest a 30% increase in S covalency in the fungal laccases. Similarly, resonance Raman data provide direct evidence that the Cu–S bond is stronger in the fungal laccases. These experimental results are supported by Xα-SW calculations, which predict an increase in the ligand field and an increase in covalency of the Cu–S_{Cys} bond upon going from the plastocyanin site to the fungal laccase T1 site.

Origin of the Changes in Electronic Structure.

The origin of the experimentally observed changes in the electronic structure between the T1 site in plastocyanin and fungal laccase can be determined by using density functional calculations to evaluate hypothetical intermediate geometries. The main geometrical differences between plastocyanin and the T1 Cu site in fungal laccase involve elimination of the axial SMet ligand at 2.82 Å, a 0.36 Å shift of the Cu atom into the N_{S}S plane, and small differences in the S–Cu–N angles. Figure 8 summarizes the electronic structure changes associated with a series of geometric perturbations that transform the plastocyanin structure into the fungal laccase structure. For all structures considered, the HOMO was similar in appearance to that in plastocyanin: highly covalent with a strong π-type antibonding interaction between the Cu d_{2s}–2 orbital and the S_{Cys} π orbital. In intermediate structure A, the axial Met of plastocyanin is removed while maintaining the remaining structure. This perturbation causes the covalency to increase from 35% in plastocyanin to 37%, as observed in the fungal laccase calculation. A series of ADF calculations in which the Cu–S_{Met} distance is progressively lengthened and then removed suggest that this increase in S_{Cys} donor character results from charge compensation for the lost axial S_{Met} ligand residue. Figure 8 (top) includes the results of these calculations on Δq(Cys) (the change in charge donation from the Cys). For intermediate structure A, the fully occupied levels of the d manifold shift to deeper energy relative to plastocyanin by an amount that is about half the shift observed in the fungal laccase calculation. Finally, intermediate structure B is created by shifting the Cu atom into the N_{2}S plane while maintaining ligand distances identical to those of plastocyanin and intermediate structure A. Here, the percent covalency (from Xα) and the amount of Cys charge donation estimated by ADF calculations are roughly the same as in A. However, moving the Cu atom into the N_{2}S plane shifts the d manifold to even deeper energy relative to the d_{2s–2} orbital.
consistent with the experimentally observed increase in LF transition energies in fungal laccase. This geometric perturbation also reproduces the experimentally observed grouping of the \( d_{xy} \), \( d_{yz} \), and \( d_{xz} \) transitions.

Finally, making small adjustments to the angular positions of the Cu ligating atoms (as in the fungal laccase structure) has little effect on either the covalency of the site or the relative energies of the \( d \) orbitals.\(^{(71)} \) This series of calculations reveals that the key feature in the formation of the fungal laccase site is the removal of the axial ligand and concomitant increase in the \( S_{Cys} \) donor strength. Note that these distortions are coupled, since the absence of an axial ligand will result in a shift of the Cu atom into the \((N_{His})_{2}SCys\) plane.

**Origin of the High Redox Potential.** Numerous proposals have suggested that the high reduction potential typically observed in the fungal laccases results from the lack of the axial methionine ligand.\(^{(16,72,73)} \) Such a geometry would stabilize the reduced site at the expense of the oxidized site because of the loss of a ligand donor interaction, albeit a weak one. This would cause the reduction potential to increase. No significant differences in the electronic structure of the T1 site in M.t. laccase, R.s. laccase, and P.p. laccase could be discerned even though the reduction potential of the T1 Cu in these enzymes differs by 300 mV (Table 1). This large range in potential suggests that, as is generally recognized, other factors such as solvent accessibility, the orientation of dipoles, and hydrogen bonding play an important role in tuning the \( E^o \) of this site.\(^{(74)} \) However, this does not preclude the importance of the ligand environment in significantly contributing to the reduction potential. The \( E^o \) values of the T1 site in all three fungal laccases studied are substantially higher than typically observed for blue copper proteins. To evaluate the contribution of the ligand environment, it is instructive to systematically examine the impact of each ligand by site-directed mutagenesis within a fixed protein environment.

The F463M mutant of the high-potential P.p. laccase enables the role of the axial methionine to be evaluated while keeping the remaining protein environment constant. The absorption, CD, MCD, EPR, and resonance Raman data for the mutant all reveal a systematic perturbation to the T1 Cu electronic structure, consistent with the mutant site becoming more like the site in plastocyanin. From the electronic spectra and EPR parameters, the ligand field of the F463M mutant is between that of the wild-type enzyme and plastocyanin. The increase in the \((\nu_{Cu-S})\) value obtained from resonance Raman spectroscopy indicates that the strength of the Cu–S-Cys bond decreases in F463M. Further, the covalency of the Cu–S-Cys bond decreases, as reflected by the oscillator strength of the absorption spectrum. These results suggest that the methionine contribution to the electronic structure of the T1 site is beyond a simple change in the local dielectric caused by the Phe → Met mutation and is consistent with a weak bonding interaction. Importantly, upon changing the axial Phe to Met, the \( E^o \) decreases 100 mV, from 780 to 680 mV.\(^{(30)} \) The extent of the change is consistent with reciprocal studies on *Pseudomonas aeruginosa* azurin in which the naturally occurring axial Met was mutated to a variety of other amino acids including those with noncoordinating groups such as Leu \( E^o \) increased 102 mV) and Ile \( E^o \) increased 128 mV).\(^{(75)} \)

**Ligand Field Trends with Axial Interaction.** Given the experimental assignment of the ligand field transitions (see Appendix), correlations can be made between the energy of the \( \zeta^2 \) transition and the strength of the interaction in the \( z \) direction (i.e., approximately along the Cu–axial ligand bond). Examination of *Alcaligenes denitrificans* azurin (A.d. Az) and its M121Q mutant reveals that the \( \zeta^2 \) transition (band 8) decreases in energy when the stronger O-(Gln) ligand (\( r_{Cu-O} = 2.26 \) Å) replaces the weak S(Met) (\( r_{Cu-S} = 3.11 \) Å) in the axial position.\(^{(76)} \) Thus, the energy of the \( \zeta^2 \) transition decreases as the strength of the axial ligand increases, as would be expected considering \( d_{z^2} \) is antibonding with respect to the axial ligand. In P.p. laccase the energy of the \( \zeta^2 \) (band 8) transition is remarkably high at ~6900 cm\(^{-1}\), consistent with the complete absence of an axial ligand. In the P.p. laccase F463M mutant, the energy of band 8 decreases to 6500 cm\(^{-1}\), indicating a stronger axial interaction than in laccase.

We also note that the splitting between the \( x^2-y^2 \) and \( xy \) orbitals is related to the presence of a Jahn–Teller force that could occur in the oxidized blue copper site; the smaller the splitting, the more the site will be subject to distortion along the tetragonal coordinate described in ref 23 and also in refs 55 and 56. The geometry of the blue copper site in fungal laccase splits these orbitals sufficiently in energy such that structural reorganization upon oxidation is expected to be minimized, therefore allowing efficient electron transfer. It should also be recognized that the high covalency of the Cu–S-Cys bond provides an efficient superexchange pathway for electron transfer from the T1 Cu to the T2/T3 Cu cluster.

**Summary**

We have provided a detailed description of the electronic structure of the nonaxially ligated T1 Cu site in fungal laccase. The primary differences between this site and that of the classic blue copper site in plastocyanin are the increased covalency of the Cu–S-Cys bond and the increased strength of the ligand field. Both of these changes derive from removal of the axial ligand. Experimental studies on the F463M mutant of fungal laccase indicate that replacing the nonligating axial Phe with a weakly ligating axial Met significantly perturbs the structure of the T1 Cu, decreasing the covalency of the Cu–S-Cys bond, the ligand field strength, and the reduction potential (by 100 mV). In addition to the direct ligand environment, the protein matrix is also found to play a critical role in determining the reduction potential of the T1 Cu site. Our experimental results are supported by electronic structure calculations that show that removal of the axial ligand causes the Cu–S-Cys to compensate for the reduced donor interaction. This study clearly establishes that the presence of the axial ligand impacts the electronic structure of the blue copper site.

**Acknowledgment.** We gratefully acknowledge Anders Pedersen and Gideon Davies for providing us with the crystal structure coordinates for *Coprinus cinereus* laccase and Dr. Frank Neese for extremely valuable discussions regarding MCD theory. This research was supported by NIH Grant DK31450.

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\(^{(71)} \) Relative to intermediate structure B, the small shift in the energy of the \( xy \) orbital for the final fungal laccase model likely results from the small increase in the N–Cu–N angle, which places the Cu–N bonds slightly closer to the \( xy \) orbital, thereby increasing the antibonding interaction.


Appendix: Assignment of the Ligand Field Transitions

There continues to be a discrepancy between the ordering of two ligand field transitions predicted by electronic structure calculations, and the experimental spectral assignment of these transitions. These differences may be due to poor descriptions for the Cu and S wave functions in such highly covalent centers. The two highest energy ligand field bands are shown by both experiment and calculations to be associated with the $\chi_z$ and $\chi_y$ transitions, which typically exhibit a pseudo-A-term MCD feature. However, calculations of blue copper sites predict that the lowest energy band (8) should be the $xy \rightarrow x^2 - y^2$ transition and the next highest energy band (7) should be the $z^2 \rightarrow x^2 - y^2$ transition, whereas experimental results indicate the opposite assignment. The original assignment of the experimental ligand field bands in ref 19 attributed the low-energy transition (band 8) to $z^2 \rightarrow x^2 - y^2$ and the next transition (band 7) to $xy \rightarrow x^2 - y^2$ on the basis of analysis of MCD signs. Similarly, the method of calculating C-term MCD signs outlined in ref 60, which includes spin-orbit coupling with both the ground and excited state as well as the transition polarizations, reinforces this early assignment; for plastocyanin, the $z^2 \rightarrow x^2 - y^2$ transition is calculated to be positive and is thus assigned to band 8 while the $xy \rightarrow x^2 - y^2$ is calculated to be negative and is assigned to band 7. Further insight into this assignment can be obtained by examining the experimental transition energies of bands 7 and 8 over a series of blue Cu proteins (Table 5) along with the $g_i$ value, which provides an independent estimate of the $xy$ energy ($\Delta g_i \approx \frac{1}{E_{x^2-y^2}}$, see eq 2). This feature of the ligand field assignment is important, since the $z^2 \rightarrow x^2 - y^2$ transition energy can provide an experimental probe of the strength of the axial ligand field.

The blue Cu site in Achromobacter cycloclastes nitrite reductase (Ac NiR) has a $g_i$ value of 2.19, the classic blue Cu site in plastocyanin (PLC) has a $g_i$ value of 2.226, and Rhus vernicifera stellacyanin (Rv Stc) has a $g_i$ value of 2.287. Relative to PLC (5000 cm$^{-1}$), band 8 is at higher energy in both Ac NiR and PLC (10 800 cm$^{-1}$) and Rv Stc (5500 cm$^{-1}$), but the differences are small. The large difference in the $g_i$ values of Rv Stc and Ac NiR is therefore difficult to rationalize if band 8 is attributed to $d_{xy}$. Alternatively, band 7 in PLC (10 800 cm$^{-1}$) is at an energy between that of Rv Stc (8750 cm$^{-1}$) and Ac NiR (11 900 cm$^{-1}$), and therefore, when band 7 is assigned as the $d_{xy} \rightarrow d_{x^2-y^2}$ transition, the trend in $g_i$ is a straightforward reflection of the different ligand fields associated with the different sites. Similarly, in cucumber basic protein (CBP, $g_i = 2.207$), band 8 is slightly higher in energy (5800 cm$^{-1}$) than in Ac NiR (5600 cm$^{-1}$), yet the $g_i$ value of the former is larger. Conversely, band 7 in CBP (10 800 cm$^{-1}$) is at lower energy than in Ac NiR (11 900 cm$^{-1}$), and therefore, the change in $g_i$ is again consistent with assigning this transition to $d_{xy}$. To minimize the influence of differences in protein structure on the transition energies and $g$ values, the wild type Alcaligenes denitrificans azurin (Az) can be compared with a point mutant, M121Q, where the axial Met ligand has been replaced with a Glm. Both bands 7 and 8 decrease in energy (Table 5) upon mutation; the larger magnitude ($\sim 2000$ cm$^{-1}$) of the decrease in band 7 compared to band 8 ($\sim 300$ cm$^{-1}$) is again consistent with the radical change in $g_i$ from 2.255 in Az to 2.29 in M121Q. On the basis of these considerations, it seems clear that band 7 can be experimentally assigned to the $d_{xy} \rightarrow d_{z^2} + d_{x^2-y^2}$ transition, while band 8 can be assigned to the $d_{xy} \rightarrow d_{x^2-y^2}$ transition.

Supporting Information Available: Absorption and CD spectra of the F$^-$ derivative of P.p. laccase before and after the addition of ascorbate, Abs, CD, and MCD spectra of M.t. and R.s. laccase, tables of the Cartesian coordinates used in SCF-Xa calculations (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA991087V

### Table 5. Spectral Summary d $\rightarrow$ d Bands of Blue Copper Proteins

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<th>Rv Stc</th>
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<th>CBP</th>
<th>Ad Az</th>
<th>Ad Az</th>
<th>M121Q</th>
<th>P.p. laccase</th>
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<td>2.207</td>
<td>2.255</td>
<td>2.287</td>
<td>2.194</td>
</tr>
</tbody>
</table>

* Band positions are given in cm$^{-1}$. From ref 23. From ref 21. NiR (5600 cm$^{-1}$) and Rv Stc (5500 cm$^{-1}$), but the differences are small. The large difference in the $g_i$ values of Rv Stc and Ac NiR is therefore difficult to rationalize if band 8 is attributed to $d_{xy}$. Alternatively, band 7 in PLC (10 800 cm$^{-1}$) is at an energy between that of Rv Stc (8750 cm$^{-1}$) and Ac NiR (11 900 cm$^{-1}$), and therefore, when band 7 is assigned as the $d_{xy} \rightarrow d_{x^2-y^2}$ transition, the trend in $g_i$ is a straightforward reflection of the different ligand fields associated with the different sites. Similarly, in cucumber basic protein (CBP, $g_i = 2.207$), band 8 is slightly higher in energy (5800 cm$^{-1}$) than in Ac NiR (5600 cm$^{-1}$), yet the $g_i$ value of the former is larger. Conversely, band 7 in CBP (10 800 cm$^{-1}$) is at lower energy than in Ac NiR (11 900 cm$^{-1}$), and therefore, the change in $g_i$ is again consistent with assigning this transition to $d_{xy}$. To minimize the influence of differences in protein structure on the transition energies and $g$ values, the wild type Alcaligenes denitrificans azurin (Az) can be compared with a point mutant, M121Q, where the axial Met ligand has been replaced with a Glm. Both bands 7 and 8 decrease in energy (Table 5) upon mutation; the larger magnitude ($\sim 2000$ cm$^{-1}$) of the decrease in band 7 compared to band 8 ($\sim 300$ cm$^{-1}$) is again consistent with the radical change in $g_i$ from 2.255 in Az to 2.29 in M121Q. On the basis of these considerations, it seems clear that band 7 can be experimentally assigned to the $d_{xy} \rightarrow d_{z^2} + d_{x^2-y^2}$ transition, while band 8 can be assigned to the $d_{xy} \rightarrow d_{x^2-y^2}$ transition.