Spectroscopic Studies of *Rhus vernicifera* and *Polyporus versicolor* Laccase. Electronic Structures of the Copper Sites

David M. Dooley,[1a] Jill Rawlings,[1b] John H. Dawson,[1c] Philip J. Stephens,[1b] Lars-Erik Andréasson,[1d] B. G. Malmström,[1d] and Harry B. Gray*[1e]

Contribution No. 5826 from the Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, California 91125.
Received June 28, 1978

Abstract: Low-temperature absorption and room temperature circular dichroism (CD) and magnetic circular dichroism (MCD) spectroscopic measurements have been made on the multicopper oxidases *Rhus vernicifera* laccase and *Polyporus versicolor* laccase. Near-infrared bands at 6000, 9500, and 11 700 cm^{-1} for *Rhus* (tree) laccase and 7250 and 11 000 cm^{-1} for *Polyporus* (fungal) laccase are assigned to d-d transitions of the type 1 (blue) Cu(II). The low energies of these bands, when considered with the EPR g values, are consistent only with a tetragonally (D_{2d}) distorted tetrahedral geometry for type 1 Cu(II). Ligand field stabilization energy contributions to the redox potential of the type 1 copper are 269 (tree) and 203 mV (fungal). Analysis of all the spectroscopic results suggests that the characteristic bands near 13 000, 16 000, and 23 000 cm^{-1} in the native tree and fungal enzymes be assigned to the charge-transfer transitions πS(thiolate) $\rightarrow$ $d_{x^2-y^2}$, σS(thiolate) $\rightarrow$ $d_{x^2-y^2}$, and πN(imidazole) $\rightarrow$ $d_{x^2-y^2}$ in a $^2B_2(d_{x^2-y^2})$ ground-state blue center of probable ligand composition CuN_2SX (N = His, S = Cys, X = Met or substitute). Binding of fluoride at the type 2 site in both tree and fungal enzymes perturbs the type 1 circular dichroism spectrum. Bands attributable to the copper types 2 and 3 are observed in visible region absorption and CD spectra of NO-treated tree laccase and a high pH (>8.7) form of fungal laccase. Both NO-treated tree and high pH fungal proteins display intense absorption at 30 000 cm^{-1} and a weak shoulder near 16 700 cm^{-1}. Prominent activity in the CD spectra is centered at 18 000 and 24 000 cm^{-1} in NO-treated tree laccase, and 15 000, 19 500, and 22 500 cm^{-1} in high pH fungal laccase. The 16 700 cm^{-1} absorption shoulder and 24 000 cm^{-1} CD activity are absent in type 2 depleted, NO-treated tree laccase. The energies of the observed bands rule out near tetrahedral geometry for types 2 and 3 copper, but are consistent with structures based on tetragonal six, five, or square-planar four coordination.

The laccases are multicopper oxidases that contain a blue or type 1 copper site.[2] The two best characterized enzymes are from a fungus (*Polyporus versicolor*) and the Japanese lacquer tree (*Rhus vernicifera*). These oxidases couple the oxidation of a variety of substrates to the four-electron reduction of oxygen to water. The type 1 reduction potential (415 mV)[2] in *Rhus vernicifera* laccase is slightly more positive than those found for the single blue copper proteins (184-370 mV),[3] but it is less positive than that of the fungal enzyme (767 mV).[2] X-ray crystal-structure analyses have shown that the ligands bound to each of the blue copper centers in poplar plastocyanin[4] and *Pseudomonas aeruginosa* azurin[5] are two histidines, a cysteine, and a methionine; the coordination geometry is less well determined from the X-ray experiments, but extensive electronic spectroscopic studies have shown[6] that a flattened tetrahedral structure is most probable for the blue copper sites in plastocyanin, azurin, and stellacyanin.

In addition to the type 1 copper, the laccases contain three other copper atoms that are distributed in type 2 (1 Cu atom/molecule) and type 3 (2 Cu atoms/molecule) sites. The type 2 copper displays an EPR spectrum that is typical of an axial Cu(II) complex.[2] No visible absorption or CD bands have previously been associated with this center. It is known,[7] however, that type 2 copper binds anions, and several experiments have indicated[8] that this copper plays a key catalytic role, possibly functioning as a mediator of electron transfer into the type 3 coppers.

The type 3 coppers absorb at 330 nm[2] and are diamagnetic,[9] but their structure remains to be elucidated. We have recently obtained spectroscopic evidence that a catalytically active oxygen intermediate is associated with the type 3 site of *Rhus* laccase during turnover conditions.[10] At 3 °C and pH 6.0, intense absorption at 340 nm and a weaker system at 475 nm attributable to an oxygen-laccase intermediate were observed. The intermediate also exhibits a positive CD band at 362 nm. These spectral features are quite similar to those found in the binuclear copper proteins oxyhemocyanin[11] and oxytyrosinase.[12]

We have now completed an extensive series of room temperature CD, MCD, and low-temperature absorption spectroscopic experiments on both laccases. Bands in the near-infrared region attributable to type 1 d-d transitions have been observed and analyzed in terms of a flattened tetrahedral blue site geometry. Analysis of the type 1 EPR spectra of both laccases has also been performed. In addition, visible absorption and CD spectral features attributable to the copper types 2 and 3 in both laccases have been observed for the first time. Finally, we have carefully analyzed all the available spectroscopic data on various blue proteins; this analysis has allowed us to pinpoint spectroscopic features that may signal structural variations in the different blue copper centers.

Experimental Section

Rhus vernicifera acetone powder was obtained from Saito and Co., Ltd., Tokyo. Laccase was extracted and purified to a ratio $A_{280}/A_{614} \leq 15.4$ by the method of Reinhammar.[13] This material ran as a single band of the correct molecular weight during NaDodSO$_4$-polyacrylamide gel electrophoresis. We have observed that the A_{280}/A_{614} ratio may be lowered to 11-12 by either extensive dialysis vs. pure water or by chromatography on Sephadex G-150. Evidently a low molecular weight UV absorbing material is removed by the above procedures. Laccase from *Polyporus versicolor* was cultured and purified at Caltech by standard methods[14] from strains provided by Dr. Rölf Brandén and one of us (L.-E.A.). Samples of the enzyme were also prepared in Göteborg from fungal strain PRL-572; a slightly modified Fåhraeus-Reinhammar procedure[14a] was used. No significant differences in the properties of the two preparations were found. Fluoride ion was removed from the purified proteins by dialysis against 50 mM acetate buffer (pH 5.5) containing 20 mM ascorbate for approximately 10 h. All fungal laccase samples had A_{280}/A_{610} ratios of 16.5-16.8 and $A_{280}/A_{260} > 2.0$.[14]

Enzyme concentrations for visible CD and MCD measurements were between 5×10^{-5} and 1×10^{-4} M, tree laccase samples were in potassium phosphate buffer (μ = 0.1 M, pH 6.0), and fungal laccase samples were in sodium phosphate buffer (μ = 0.1 M, pH 6.0). Instrument base lines were determined with the same buffers. Tree laccase solutions for use in the near-infrared region were prepared in D_2O; deuterated 0.1 M sodium acetate buffer (pD 5.7) was used to

0002-7863/79/1501-5038$01.00/0 © 1979 American Chemical Society

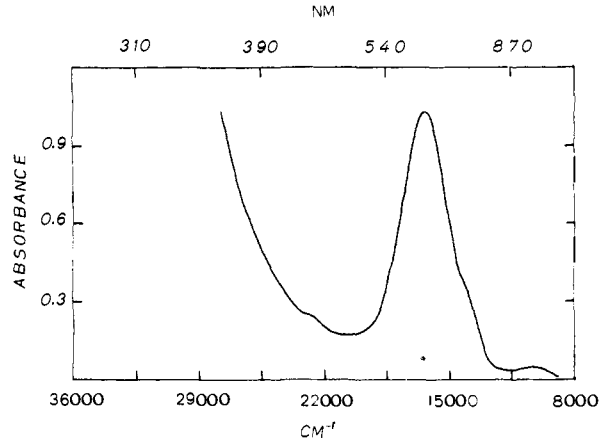

Figure 1. Absorption spectrum of fungal laccase on a Plexiglas disk at 20 K.

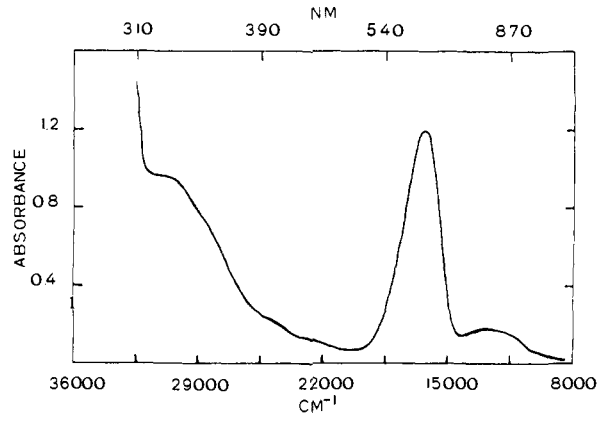

Figure 2. Absorption spectrum of tree laccase on a quartz disk at 20 K.

prepare fungal laccase samples. Equilibration was carried out by concentrating the protein in an Amicon ultrafiltration cell and then adding approximately a tenfold excess of D_2O or deuterated buffer. This procedure was repeated 4–5 times before the solution was concentrated to its final value.

Deuterated acetate buffer was prepared by dissolving well-dried anhydrous sodium acetate in D_2O and then adjusting the pD to 5.7 at 6 °C by addition of DCl. At this concentration of buffer and pD, the total chloride ion concentration is ~1 mM, an order of magnitude less than the concentration where inhibition of fungal laccase by this anion occurs.[7,15] However, to check that this concentration of chloride had no effect on the near-infrared measurements, spectra of fungal laccase in 0.05 M sodium acetate buffer (pH 5.5) in H_2O were also obtained. Buffered solutions of fungal laccase were used to prevent reduction of the type 1 site that occurs at pH >6.5.[16] Spectra were recorded for samples having concentrations of 1–2 mM in small volume quartz cells with path lengths of 1 or 2 mm to minimize solvent absorption. Reliable data could be obtained out to ~2.1 μm where D_2O overtones diminished the light level to the limit of detector response. D_2O or deuterated buffer was used to obtain instrument base lines.

Specific reduction of the type 1 copper in fungal laccase was accomplished by titration with NaOH (or NaOD for use in the near-infrared) or by extensive dialysis against phosphate-borate buffer (pH 8.7 at 4 °C). Specific reduction of the type 1 center in tree laccase was achieved via reaction with nitric oxide.[17] Solutions of tree laccase, ~0.5 mM for visible region experiments and ~2 mM for near-infrared work, were placed in cuvettes or EPR tubes that could be directly attached to a vacuum line. Oxygen was purged via three–four cycles of gentle evacuation and flushing with argon. Approximately 1 atm of NO was then admitted after passage through a dry ice-acetone bath; the protein solution was again evacuated and this procedure repeated at least three times to ensure complete equilibration. An atmosphere of NO was left over the samples, and the cuvette or EPR tube was removed from the line. After we warmed the solution to about 45 °C for 5 min in a water bath, the blue color was observed to bleach and the laccase solutions became light green. Type 2 depleted tree laccase was prepared as previously described.[18] The degree of reduction or removal of copper was checked by EPR measurements. Spectra were recorded on a Varian E-line Century Series spectrometer at 5 K utilizing an Air Products Heli-Trans system. Frequencies were determined with a PRD Electronics, Inc. frequency meter.

Protein films for low-temperature absorption measurements were prepared by evaporating ~1 mM solutions of the proteins on plexiglass or quartz disks in a desiccator filled with Drierite at 4 °C. Films were not allowed to dry past the stage where they were still slightly elastic. A lightly greased rubber gasket was then placed around the film and a second disk added as a cover. The film was then masked with aluminum foil to prevent light leaks. The assembly was mounted in the sample space of a Cryogenic Technology Model 20 cryocooler utilizing a mixture of copper filings in silicone grease to ensure good thermal contact. Samples were cooled slowly to minimize cracking of the film. Several hours were required for the plexiglass disks to achieve thermal equilibrium with the cold stage of the cryocooler at low temperature (<70 K).

Absorption spectra were recorded on Cary 17 and 14 spectrometers. Visible CD spectra were taken with Cary 61 and JASCO J-40 instruments. CD spectra in the near-infrared region were recorded on a laboratory constructed instrument described elsewhere.[19] Standard sensitivity settings were 0.05° and between 5×10^{-4} and 5×10^{-3} ΔA for the visible and near-infrared regions, respectively. MCD spectra were obtained (with the Cary 61) using a Varian Associates superconducting magnet at fields of 40 kG. MCD spectra were corrected for natural CD by computer subtraction of the field-off spectrum from the field-on spectrum. Camphor-10-sulfonic acid was used to calibrate the visible CD. The sign of the CD in the near-infrared region was determined with a Ni(II) tartrate solution and calibrated with a quarter wave plate. CD and MCD spectra are reported in terms of the differential molar extinction coefficient $\Delta\epsilon$. In the case of MCD, $\Delta\epsilon$ is normalized to a field of +10 kG.

Reagent grade chemicals were used without further purification. D_2O used was 99.8% D (Stohler Isotope Chemicals). Protein concentrations are based on the extinction coefficients at 614 nm (5700 L mol^{-1} cm^{-1}) for tree laccase and 610 nm (4900 L mol^{-1} cm^{-1}) for fungal laccase.[2] A Brinkmann Model 101 was used for all pH determinations. pD was obtained by adding 0.4 to the pH meter reading.

Results

The 20 K absorption spectrum of fungal laccase is shown in Figure 1. The resolution obtained at low temperature is rather disappointing, being only marginally better than that obtained at room temperature. A weak shoulder is resolved at 23 000 cm^{-1}, the shoulder at 13 000 cm^{-1} is more pronounced, and a weak absorption in the near-infrared region, not previously observed, is evident near 10 000 cm^{-1}. Much better spectral results were obtained for tree laccase at 20 K (Figure 2). The intense band at ~16 000 cm^{-1} is narrowed considerably at low temperature. A broad, asymmetric peak, probably composed of two overlapping transitions, is clearly resolved to lower energy of the intense "blue" band. Significantly, a less intense band is observed to even lower energies, appearing as a shoulder near 10 000 cm^{-1}. A transition at this energy has not previously been detected in tree laccase. Weak shoulders are observed at energies near 23 000 cm^{-1} and the near-UV band associated with the type 3 coppers is clearly resolved from the large protein absorbance. The low-temperature spectrum of tree laccase is similar in many respects to the spectra obtained[6] for plastocyanin, azurin, and stellacyanin.

The visible CD spectra of tree and fungal laccase are presented in Figures 3 and 4, respectively, and are in good agreement with those reported earlier.[20,21] Examination of the visible CD spectrum of NO-treated tree laccase (vide infra) establishes that the bands shown in Figure 3 are due to the type 1 site. Such an assignment is reasonable for tree laccase, as its visible CD spectrum shows considerable similarity to those of

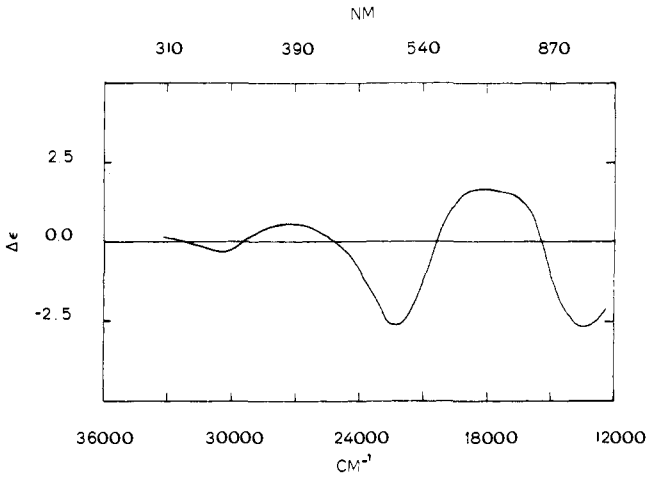

Figure 3. CD spectrum of tree laccase in $\mu = 0.1$ M potassium phosphate buffer (pH 6.0) at room temperature.

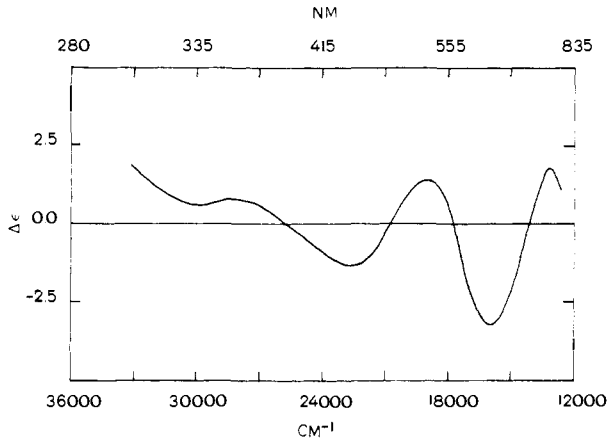

Figure 4. CD spectrum of fungal laccase in 0.1 M sodium phosphate buffer (pH 6.0) at room temperature.

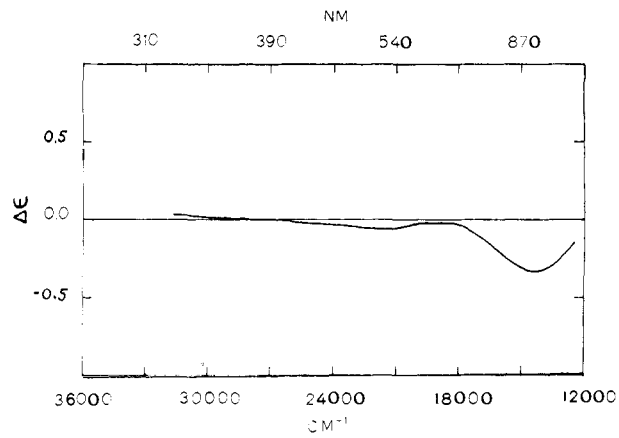

Figure 5. MCD spectrum of tree laccase in $\mu = 0.1$ M potassium phosphate buffer (pH 6.0) at room temperature.

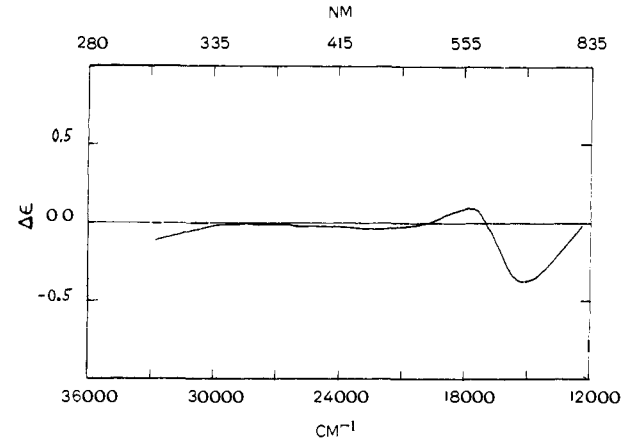

Figure 6. MCD spectrum of fungal laccase in 0.1 M sodium phosphate buffer (pH 6.0) at room temperature.

single blue copper proteins. In this context it should be noted that azurin, plastocyanin, and stellacyanin have positive CD activity at energies greater than 27 000 cm^{-1}. It is surprising that the relatively intense (ϵ(ox-red) = 2800 M^{-1} cm^{-1}) absorption at 30 000 cm^{-1} due to the type 3 coppers does not have any CD activity associated with it.

All of the observed bands in the fungal laccase spectrum may be assigned to the type 1 chromophore, as these bands disappear upon specifically reducing this copper by either raising the pH to 8.7 or by adding 1 equiv of ascorbate to the fluoride-inhibited enzyme.[22] Considering the remarkable similarity of the visible CD spectra of proteins containing the blue or type 1 copper center,[6,21,23] it is important to note that the spectrum of fungal laccase is decidedly different. In particular, although the features at 28 500 and 22 650 cm^{-1} have the same sign and position as observed for other blue proteins, fungal laccase displays two oppositely signed bands between 21 000 and 14 000 cm^{-1}, and a positive maximum at ~13 000 cm^{-1}; all other blue proteins show a broad positive maximum (often asymmetric) between 21 000 and 14 000 cm^{-1} and a negative peak near 13 000–14 000 cm^{-1}.

The visible MCD curves of tree and fungal laccase are presented in Figures 5 and 6, respectively. The spectrum of tree laccase shows a striking resemblance to the MCD spectra of plastocyanin,[6] azurin,[6] and ceruloplasmin;[23] a negative maximum is observed at 14 250 cm^{-1} in tree laccase, 14 400 cm^{-1} in azurin, 14 100 cm^{-1} in plastocyanin, and 14 600 cm^{-1} in ceruloplasmin. In contrast, fungal laccase displays an MCD curve unique among the blue copper proteins examined to data. A negative maximum is observed at 15 150 cm^{-1} and a smaller positive peak at 17 850 cm^{-1}, the curve crossing the base line at 16 950 cm^{-1}. It should be noted that the MCD spectrum of stellacyanin is also unlike that of any other blue copper protein. MCD spectral data for all blue copper proteins examined to date are summarized in Table I.

Table I. Bands in the Visible MCD Spectra of Blue Copper Proteins

blue protein	$\bar{\nu}$, cm^{-1}	$\Delta\epsilon$
plastocyanin[a]	14 100	−0.40
azurin[a]	14 400	−0.25
tree laccase	14 250	−0.35
ceruloplasmin[b]	14 600	−0.88
stellacyanin[a]	13 500	−0.30
	16 500	−0.40
	22 000	−0.20
fungal laccase	15 150	−0.40
	17 850	+0.10

[a] From ref 6. [b] From ref 23.

Figures 7 and 8 show the near-infrared CD spectra of tree and fungal laccase, respectively. Shoulders are observed at about 11 700 and 9500 cm^{-1} on the tail of the negative activity from the lowest energy visible band in the tree laccase spectrum. A peak is clearly resolved at 6000 cm^{-1}. Bands are evident at 11 000 and 7250 cm^{-1} in the fungal laccase spectrum. Positive CD activity is still present at the lowest energies ob-

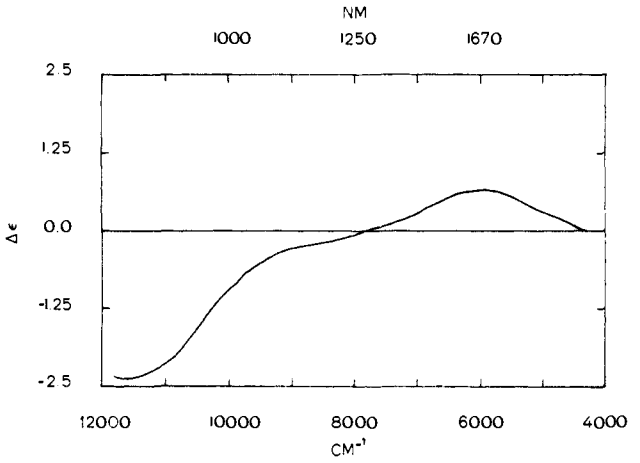

Figure 7. Near-infrared CD spectrum of tree laccase in D_2O at room temperature.

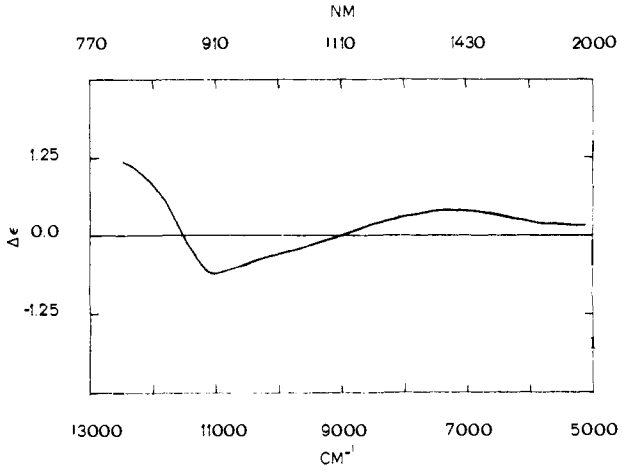

Figure 8. Near-infrared CD spectrum of fungal laccase in deuterated 0.1 M sodium acetate (pD 5.7) at room temperature.

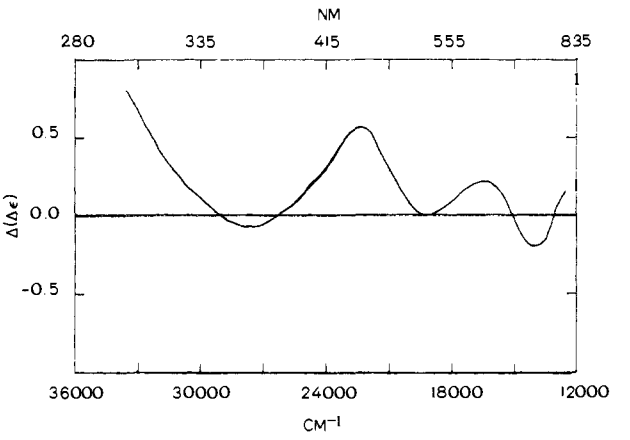

Figure 9. Spectrum obtained by subtracting the tree laccase CD spectrum from that of the fluoride-treated protein. Incubation time was sufficient for fluoride binding to be complete. Both spectra were taken in $\mu = 0.1$ M potassium phosphate buffer (pH 6.0) at room temperature.

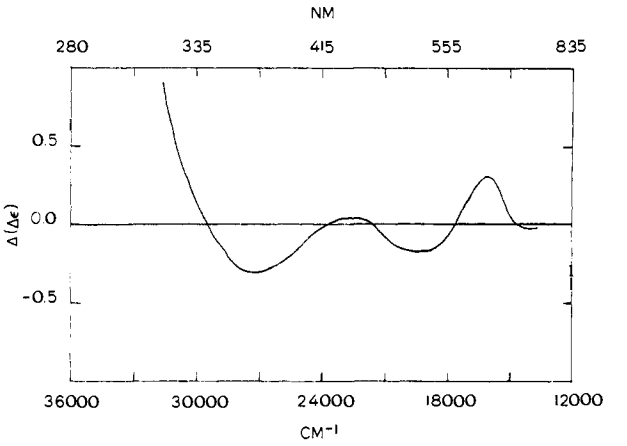

Figure 10. Spectrum obtained by subtracting the fungal laccase CD spectrum from that of the fluoride-treated protein. Incubation time was sufficient for fluoride binding to be complete. Both spectra were taken in 0.1 M sodium phosphate buffer (pH 6.0) at room temperature.

tainable under our experimental conditions. Assignment of these low-energy features to type 1 copper was confirmed in a direct way for tree and fungal laccase by measuring the near-infrared CD spectra of the NO-treated and high pH forms of these proteins. For both laccases the intensities of the near-infrared bands decrease in direct proportion to intensity losses in the visible absorption and CD bands of the blue chromophore. No activity was found after the proteins were reduced by the addition of dithionite. At the concentrations used to record the CD spectra, no MCD activity was observed except for an increasing negative signal at energies greater than 10 000 cm^{-1} in the tree laccase spectrum.

As the CD spectrum should be sensitive to small conformational changes affecting the structure of the type 1 copper, we examined the effect of F^- on the visible CD of tree and fungal laccase (Figures 9 and 10). Only slight changes are observed for tree laccase at energies less than approximately 21 000 cm^{-1}. However, the intensity at 22 400 cm^{-1} is noticeably decreased and an increase in $|\Delta\epsilon|$ is seen above 31 000 cm^{-1}, where the largest and most reproducible change is found[15] in the absorption spectrum. The entire CD spectrum of fungal laccase is slightly altered upon addition of F^-, although the various bands are apparently affected to different extents. The largest change occurs above 30 000 cm^{-1}, consistent with changes observed[15] in the absorption spectrum.

Incubation of tree laccase in the presence of NO at 45 °C results in reduction of the type 1 copper.[17] The EPR spectrum (Figure 11) indicates nearly complete reduction, as the signals

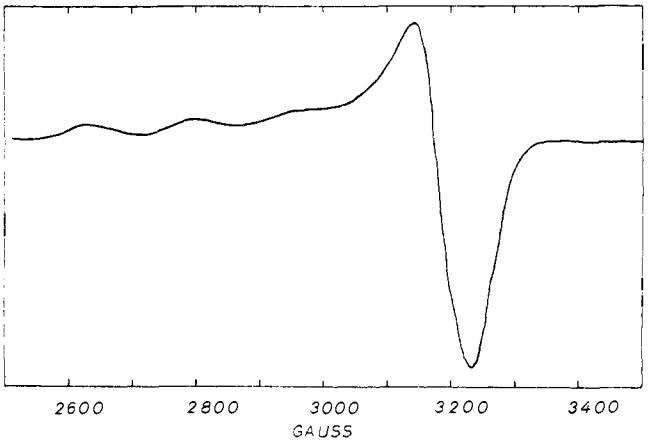

Figure 11. EPR spectrum (9.1760 GHz) of tree laccase plus nitric oxide at 75 K. Microwave power was 20 mW and the modulation amplitude was 1.6 G.

attributable to type 1 copper are not evident. All the observed signals are due to a modified[24,25] type 2 copper ion. Visible and near-ultraviolet absorption spectra of NO-treated tree laccase are shown in Figure 12. The presence of the intense near-ultraviolet absorption establishes that the type 3 site is oxidized under these conditions. A shoulder is present near 600 nm

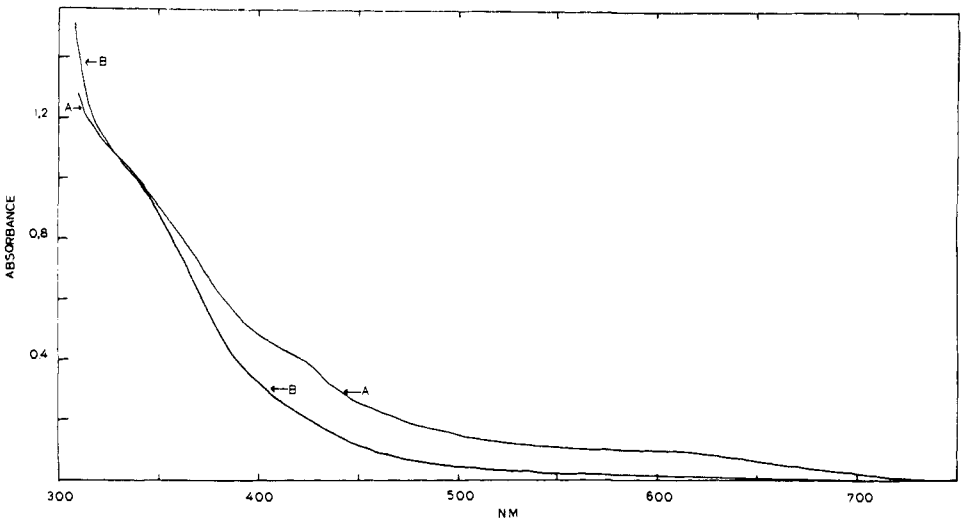

Figure 12. Absorption spectra of native (A) and type 2 depleted (B) tree laccase measured at room temperature after reaction with nitric oxide in 0.06 M potassium phosphate buffer (pH 7.6).

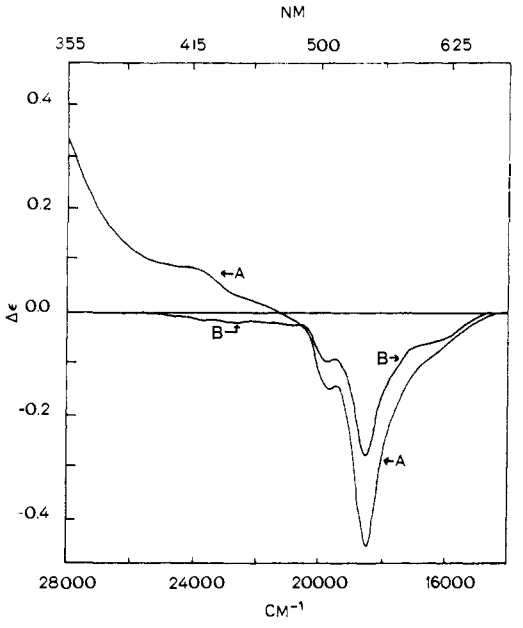

Figure 13. CD spectra of native (A) and type 2 depleted (B) tree laccase measured at room temperature after reaction with nitric oxide in 0.06 M potassium phosphate buffer (pH 7.6).

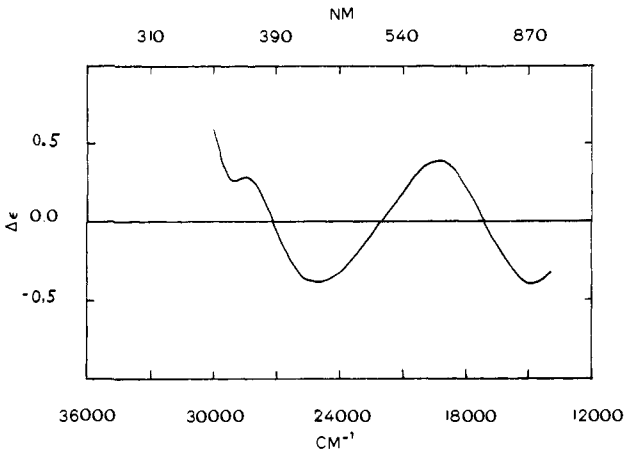

Figure 14. CD spectrum of fungal laccase measured at room temperature in deuterated 0.1 M sodium acetate (pD 5.7) after titration with $NaOD/D_2O$.

(16 700 cm^{-1}), which may be assigned to the type 2 copper, based on the results obtained for type 2 depleted laccase plus nitric oxide (also shown in Figure 12). In the CD spectrum of NO-treated native laccase, a band is clearly observed at 18 500 cm^{-1}, with shoulders near 16 000 and 20 000 cm^{-1} (Figure 13). These features are also present (but are less intense) in the NO-treated type 2 depleted laccase spectrum (Figure 13). Additionally, the NO-treated native protein displays strong positive activity above 21 000 cm^{-1}; no such signals are seen for the type 2 depleted protein, indicating that this activity is a property of the type 2 site. Observed values of $\Delta\epsilon$ are in the ranges established for d–d transitions of cupric peptide and amino acid complexes.[26]

At pH >6.5 the type 1 copper in fungal laccase is reduced; the type 2 and type 3 coppers remain oxidized.[16] The absorption spectrum (not shown) is nearly identical with that of NO-treated tree laccase (Figure 12), as evidenced by a shoulder near 600 nm and rising absorption at higher energies due to the band at 330 nm (30 000 cm^{-1}) in the near-UV region. However, three peaks are resolved in the CD spectrum of a fungal laccase sample that was titrated with $NaOD/D_2O$ solution until the blue color disappeared (Figure 14). Negative peaks are observed at 25 500 and near 15 000 cm^{-1}, and a positive feature is found at 19 500 cm^{-1}. The intensities are of the same magnitude as those seen in the spectrum of NO-treated tree laccase. Essentially the same CD spectrum was observed after dialyzing fungal laccase against phosphate-borate buffer (pH 8.7) at 4 °C, except that some residual intensity was apparent in regions where bands due to type 1 copper occur, indicating that the type 1 center was not completely reduced. Examination of the EPR spectrum confirmed that some type 1 was still oxidized under these conditions. No MCD activity was observed for the type 1 reduced fungal protein at fields of 40 kG. Dialyzing the sample against pH 6.0 phosphate buffer restored approximately 70% of the original absorbance at 610 nm.

Discussion

The low-energy bands associated with the blue copper centers in the laccases are attributable to d–d transitions. As in the single blue copper proteins,[6] the observed energies are clearly inconsistent with a blue copper structure based either on a six-coordinate octahedral or a four-coordinate square planar geometry (d–d transition energies associated with such geometries are much higher[27]). Possible geometries derived from trigonal (C_{3v}) distortions of five-and four-coordinate

complexes may be eliminated by consideration of the EPR parameters, particularly the variation of the g values (both magnitude and ordering) and their dependence on the energies of the ligand field states.[6] For example, $g_\parallel \simeq 2.0 < g_\perp$ for axially compressed trigonal-bipyramidal Cu(II) complexes, the predicted ordering for a d_{z^2} ground state.[28] A compressed trigonal four-coordinate geometry will also have a d_{z^2} ground state for Cu(II), and thus $g_\parallel \simeq 2.0$ and $g_\perp = 2 - 6k\lambda/\Delta W(^2E - {}^2A_1)$.[29] This g-value ordering is observed for copper-doped ZnO[30] and BeO[31] and is opposite to that found in blue copper proteins. Furthermore, at least one of the values of $|A_\parallel|$ or $|A_\perp|$[30-32] is much larger than that observed for blue copper sites.

The observed spectroscopic properties of laccase type 1 copper may be interpreted by assuming a tetragonally distorted (D_{2d}) tetrahedral site structure.[6] The electronic energies (W) of the d-electron states of Cu(II) in such a D_{2d} ligand field are as follows:

$$W(^2A_1) = W(a_1 = d_{z^2}) = -12(3\cos^2\beta - 1)Ds - 3\delta Dt$$

$$W(^2B_1) = W(b_1 = d_{xy}) = 12(3\cos^2\beta - 1)Ds + \frac{1}{2}(35\sin^4\beta - \delta)Dt$$

$$W(^2E) = W(e = d_{xz}, d_{yz}) = -6(3\cos^2\beta - 1)Ds + 2\delta Dt$$

$$W(^2B_2) = W(b_2 = d_{x^2-y^2}) = 12(3\cos^2\beta - 1)Ds - \frac{1}{2}(35\sin^4\beta + \delta)Dt$$

where $\delta = 35\cos^4\beta - 30\cos^2\beta + 3$, $Ds = Ze\langle r^2\rangle/21a^3$, $Dt = Ze\langle r^4\rangle 21a^5$; the ground state is $^2B_2$; and the coordinate system and β are as defined previously (the limits are $\beta = 54.74°$ (T_d) and 90° (D_{4h})).[6] Considerable confidence may be placed in this model, as it accords closely with the known β dependence of the energies of the ligand field states in $CuCl_4^{2-}$ ions possessing β values between 65 and 90°.[6,33,34]

The ligand field analysis of single blue copper proteins has shown[6] that β values near 60° fit the low energy d-d transitions and at the same time give reasonable energies at the square-planar and tetrahedral limits. Our calculations on tree laccase illustrate this point: Assigning $\Delta W(^2E_2 - {}^2B_2) = 6000\ cm^{-1}$ and $\Delta W(^2B_1 - B_2) = 9500\ cm^{-1}$, $\Delta W(^2A_1 - {}^2B_2)$ is calculated to be 12 150 cm^{-1} for $\beta = 61°$, $Ds = 698\ cm^{-1}$, and $Dt = 464\ cm^{-1}$. ($\Delta W(^2A_{1g} - {}^2B_{1g})$, the D_{4h} limit, is calculated to be 21 390 cm^{-1}.) In contrast, β values of 60 and 64° yield $Ds = 897\ cm^{-1}$, $Dt = 483\ cm^{-1}$, $\Delta W(^2A_1 - {}^2B_2) = 12\,928\ cm^{-1}$, and $Ds = 365\ cm^{-1}$, $Dt = 416\ cm^{-1}$, $\Delta W(^2A_1 - {}^2B_2) = 9993\ cm^{-1}$, respectively. Falk and Reinhammar have reported[21] a negative shoulder at 11 700 cm^{-1} in the CD spectrum of tree laccase, and we have observed both absorption and a CD shoulder at this energy; the transition is logically $\Delta W(^2A_1 - {}^2B_2)$, indicating that $\beta = 61°$ is to be preferred. The 11 700-cm^{-1} absorption band is several times more intense than the 9500-cm^{-1} band, consistent with D_{2d} selection rules; $^2B_2 \rightarrow {}^2A_1$ is electric dipole allowed, but $^2B_2 \rightarrow {}^2B_1$ is forbidden.

The EPR spectrum of fungal laccase is decidedly rhombic;[2] therefore, the D_{2d} ^2E state must be split into two components in this case. One component of $^2B_2 \rightarrow {}^2E$ may fall at 7250 cm^{-1}; alternatively, both components of the ^2E state may be lower in energy than 5000 cm^{-1}, with $^2B_2 \rightarrow {}^2B_1$ at 7250 cm^{-1} and the 11 000-cm^{-1} feature being $^2B_2 \rightarrow {}^2A_1$. The former assignment is adopted for the reasons given below.

The expressions for the g values in a tetragonally distorted tetrahedral field are

$$g_\parallel = 2 - 8k_\parallel\lambda^0/\Delta W(^2B_1 - {}^2B_2)$$

$$g_\perp = 2 - 2k_\perp\lambda^0/\Delta W(^2E - {}^2B_2)$$

where k and λ^0 ($= -828\ cm^{-1}$) are orbital reduction and free ion spin-orbit coupling parameters, respectively. Note that the g values, particularly $g_\parallel$, are very sensitive to the energies of the $^2B_2 \rightarrow {}^2E$ and $^2B_2 \rightarrow {}^2B_1$ electronic transitions. Values of $k_\parallel = 0.38$ and $k_\perp = 0.16$ have been computed from a fit of the theory to the EPR parameters measured for stellacyanin, azurin, and plastocyanin.[6] Very similar values of k adequately fit the observed EPR spectrum of tree laccase (vide infra). The experimental g values are $g_z = 2.287$, $g_y = 2.077$, $g_x = 2.025$ for stellacyanin,[25] $g_\parallel = 2.260$, $g_\perp = 2.052$ for azurin,[35] $g_\parallel = 2.226$, $g_\perp = 2.053$ for plastocyanin,[36] $g_\parallel = 2.298$, $g_\perp = 2.047$ for tree laccase,[25] and $g_z = 2.190$, $g_x = 2.052$, $g_y = 2.033$ for fungal laccase.[37] If the values of $k_\parallel$ and $k_\perp$ for the type 1 copper in fungal laccase are assumed to be approximately the same as for the other blue copper proteins, then $\Delta W(^2B_1 - {}^2B_2)$ must be greater than 10 000 cm^{-1} and $\Delta W(^2E - {}^2B_2)$ greater than 5500 cm^{-1}. Taking $\Delta W(^2B_1 - {}^2B_2) = 11\,000\ cm^{-1}$, $k_\parallel$ is computed to be 0.32. If the higher energy component of the split ^2E is assigned a ΔW of 7250 cm^{-1}, then we can estimate $k_\perp$ to be 0.14. This places the unobserved lower energy component of ^2E at 4460 cm^{-1} (mean ^2E energy = 5855 cm^{-1}). Positive activity to the low-energy side of the 7250-cm^{-1} maximum (Figure 8) is consistent with another transition of the same sign lying just below the observed energy region. Assuming $\beta = 60°$, $\Delta W(^2E - {}^2B_2) = 5855\ cm^{-1}$, and $\Delta W(^2B_1 - {}^2B_2) = 11\,000\ cm^{-1}$, we obtain $Ds = 796\ cm^{-1}$ and $Dt = 559\ cm^{-1}$, with the D_{4h} $\Delta W(^2A_{1g} - {}^2B_{1g})$ limit being 24 690 cm^{-1}. The highest energy d-d transition ($^2B_2 \rightarrow {}^2A_1$) is predicted at 13 500 cm^{-1}. A band attributable to $^2B_2 \rightarrow {}^2A_1$ is not observed, presumably because it falls in a region of the spectrum that is dominated by intense charge-transfer features.

In contrast to fungal laccase, the EPR spectrum of tree laccase is very nearly axial, even at 35 GHz.[25] Values of $g_\parallel = 2.265$ and $g_\perp = 2.044$ are calculated using the k values derived from analysis of the single blue copper proteins and the observed energy level splittings for tree laccase. Increasing $k_\parallel$ to 0.43 allows the calculated value of $g_\parallel$ to match the experimental value. The orbital reduction factors for all of the blue copper centers examined so far are considerably less than those calculated for square-planar copper peptide complexes, where $0.55 \leqslant k_\parallel \leqslant 0.64$ and $0.45 \leqslant k_\perp \leqslant 0.71$,[38] indicating that electron delocalization and covalent bonding are particularly pronounced in the type 1 copper site.

The unusually low value of $|A_\parallel|$ exhibited by the blue copper site is also accounted for by a tetragonally distorted tetrahedral geometry. Previous calculations have shown that by mixing 4p character into the primarily 3d levels, which is allowed in near tetrahedral symmetries but forbidden in most other cases, it is possible to understand small $|A_\parallel|$ values.[39,40] Copper occupies tetragonally distorted tetrahedral sites in NH_4F[40] and $Zn[C(NH_2)_3]_2(SO_4)_2$[41] with $|A_\parallel| < 4 \times 10^{-4}\ cm^{-1}$ and $< 5 \times 10^{-3}\ cm^{-1}$, respectively, and $g_\parallel > g_\perp \simeq 2.1$. This ordering of g values was also observed in the EPR spectrum of the near tetrahedral complex Cu(α,α'-dibromodipyrromethene)$_2$, but $|A_\parallel|$ was not resolved.[39] Cs_2CuCl_4 has approximately D_{2d} symmetry with $\beta \simeq 65°$; the ligand field bands for this complex occur at approximately 5000, 8000, and 9000 cm^{-1}.[6] EPR spectra of the pure crystal and of copper-doped Cs_2ZnCl show $g_\parallel \simeq 2.38$, $g_\perp \simeq 2.09$, and $|A_\parallel| \simeq 2.5 \times 10^{-3}\ cm^{-1}$.[42] Bis(4-phenylamino-2-phenyliminopent-3-enato-*N,N'*)copper(II) also displays a ligand field transition near 6000 cm^{-1}; its EPR spectrum is characterized by $g_\parallel \simeq 2.218$, $g_\perp = 2.070$, and $|A_\parallel| = 10.7 \times 10^{-3}\ cm^{-1}$.[43]

Calculated ligand field parameters for all the blue copper centers examined to date are given in Table II. Values of Ds and Dt are those resulting directly from the fit. Calculations were performed using the observed energies of the $^2B_2 \rightarrow {}^2B_1$ and $^2B_2 \rightarrow {}^2E$ transitions and requiring reasonable values for $^2A_1 \rightarrow {}^2B_1$ and the D_{4h} limit ($^2A_{1g} \rightarrow {}^2B_{1g}$). Owing to the

Table II. Blue Copper Ligand Field[a] Parameters and Reduction Potentials

blue protein	$^2B_2 \rightarrow {}^2E$	$^2B_1$	$^2A_1$	β, deg (Ds, Dt, D_{4h} limit in cm^{-1})	LFSE,[b] mV	E^0, mV
plastocyanin[c]	5000	9150	11200	60 (691, 465, 21200)	345	347[d]
azurin[c]	5800	10200	(13000)[e]	61 (700, 480, 21600)	250	330[d]
tree laccase	6000	9500	11700	61 (698, 464, 21400)	269	415[f]
ceruloplasmin[g]	6100	10000	11500	61 (717, 464, 21800)	260	535[h]
stellacyanin[c]	5250[i]	8800[j]	11110[j]	60 (765, 444, 22800)	340	184[d]
fungal laccase	7250[i]	11000	(13500)[e]	60 (796, 559, 24700)	203	767[f]

[a] Band positions in cm^{-1} taken from near-infrared CD spectra except where noted. [b] Ligand field destabilization relative to the Cu(II) aquo ion. [c] From ref 6. [d] From ref 3. [e] Calculated value. [f] From ref 2. [g] From ref 23; $^2B_2 \rightarrow {}^2A_1$ energy estimated from low-temperature absorption data. [h] Average of the potentials (ref 2) for the two blue copper ions. [i] Higher energy component of a split ^2E. [j] Low-temperature absorption data; the CD band positions are 8100 and 10500 cm^{-1} (ref 6); two fits with $\beta = 61°$ are also acceptable (taking $^2B_1$ at 8800 cm^{-1}, $\beta = 61°$ (588, 430, 18416), with $^2B_2 \rightarrow {}^2A_1$ at 10843 cm^{-1}; $^2B_1$ at 8100 cm^{-1}, $\beta = 61°$ (620, 395, 18849), with $^2B_2 \rightarrow {}^2A_1$ at 10526 cm^{-1}).

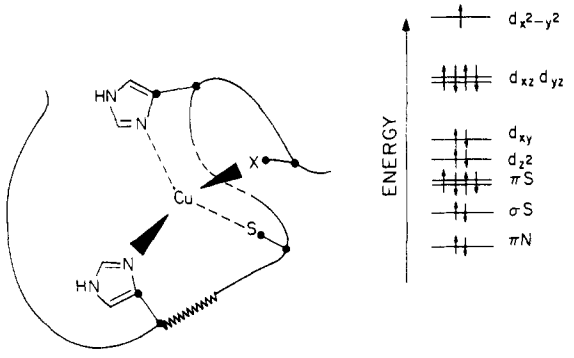

Figure 15. Structural representation of the blue copper site in proteins (X = Met-S or a substitute). Included is a diagram of the electronic energy levels of a flattened tetrahedral (effectively D_{2d}) blue copper center. Only the highest occupied πN orbital is shown, and X electronic levels are not included in the energy level diagram. The ground state for Cu(II) is $^2B_2(d_{x^2-y^2})$.

previously mentioned ambiguity in the interpretation of the spectrum of fungal laccase, a wider range of fits was attempted, primarily by relaxing constraints on the D_{4h} limit. All such attempts gave less satisfactory results than those calculated for $\beta = 60°$, and the values shown in Table II are to be preferred.

The ligand field stabilization energies (LFSE) are calculated to be −6731 and −7244 cm^{-1} for the tree and fungal blue coppers, respectively. As the value for $Cu(H_2O)_6^{2+}$ is −8900 cm^{-1},[27a] the laccase blue coppers are destabilized by 269 mV (tree) and 203 mV (fungal) relative to $Cu(H_2O)_6^{2+}$. Under the unlikely assumption that all other factors contributing to the reduction potentials are equal, then the LFSE treatment predicts potentials of 422 (tree) and 356 mV (fungal) for the laccases (based on 153 mV for aquo Cu(II)). The predicted and experimental values of the potential for tree laccase are in remarkably good agreement, which is probably accidental. Solvation differences and other factors must be more important than the LFSE in determining the potential of fungal laccase. However, it may be significant that for every blue copper center analyzed to date the LFSE makes a positive contribution to the reduction potential (Table II). Thus the LFSE contribution allows us to understand the generally high positive potentials of blue copper proteins, if not some of the trends within the series.

Examination of the spectroscopic parameters and reduction potentials set out in Table II suggests that the blue site in tree laccase very likely is structured in the same manner as that in plastocyanin or azurin (Figure 15 with X = Met). The blue site in fungal laccase, however, may vary in ligand composition, as it possesses an unusually high potential as well as a relatively strong (and slightly rhombic) ligand field. If there is a ligand variation in fungal laccase, it need not necessarily be in the X position of Figure 15. Variation could also occur at one of the histidine positions.[44] It is virtually certain, however, in view of the chemical[45] and spectroscopic evidence, that at least one cysteine ligand is bound to the blue copper in the fungal enzyme.

Visible CD spectral data also suggest that there are some differences in the blue sites in the tree and fungal enzymes. The visible CD curve of tree laccase (Figure 3) resembles those of the small blue copper proteins.[6] In contrast, the visible CD spectrum of fungal laccase (Figure 4) differs substantially below energies of 21000 cm^{-1}. Careful inspection of the spectra reveals that the two lowest energy transitions in fungal laccase are of opposite sign than those observed for all other blue copper sites.[6] It should be noted that stellacyanin, azurin, and plastocyanin display[6] positively signed shoulders at 18000–19000 cm^{-1}; the corresponding transitions in the laccases are more pronounced and equally as intense as the peak expected near 16000 cm^{-1}. Consequently, a broad (20500 to 15500 cm^{-1}) positive band results in tree laccase. In fungal laccase these two transitions are opposite in sign and well resolved.

Evaluation of the Kuhn anisotropy factor, $\gamma = |\Delta\epsilon|/\epsilon$, is useful in assigning the various CD and absorption bands, as those with magnetic dipole character will display values of γ that are considerably larger than transitions that are only electric dipole allowed.[6] In a site of approximate D_{2d} symmetry, π charge-transfer transitions are both electric and magnetic dipole allowed, whereas σ charge transfer transitions are only electric dipole allowed. Values of γ for the various spectral bands in the tree and fungal enzymes are set out in Table III. Assignment of the prominent band at ~16000 cm^{-1} to σS(thiolate) $\rightarrow d_{x^2-y^2}$ is consistent with a large body of data.[6,23,46] Reasonable assignments for the bands at about 23000 and 13000 cm^{-1} are πN(imidazole) $\rightarrow d_{x^2-y^2}$ and πS(thiolate) $\rightarrow d_{x^2-y^2}$, respectively (see Figure 15). The highest energy ligand field transition, $^2B_2 \rightarrow {}^2A_1$, may also contribute intensity to the 13000-cm^{-1} band in the case of fungal laccase. The positive CD feature in the 18000–19000-cm^{-1} region is tentatively attributed to a $\sigma S^* \rightarrow d_{x^2-y^2}$ transition, where S* is a thioether-type sulfur atom. This assignment follows that given[6] for the single blue copper proteins (S* is a methionine sulfur in plastocyanin and azurin; it is proposed[6,46h] to be one of the sulfurs in a disulfide unit in stellacyanin). If this interpretation of the CD band is correct, then X must be a sulfur atom in both the tree and fungal enzymes.

Types 2 and 3. Specific reduction of the blue chromophore in both tree and fungal laccase has allowed us to identify absorption bands and CD activity attributable to the other copper sites (Table IV). Observed intensities of the visible absorption and CD spectra indicate the transitions are most likely d–d in nature, and the energies are consistent with six-, five-, or square-planar four-coordinate Cu(II).[27] Tetrahedral geometry

Table III. Transition Assignments and Kuhn Anisotropy Factors

blue protein	ν, cm^{-1} [a]	ϵ [b]	$\lvert\Delta\epsilon\rvert$	γ	assignment
tree laccase	6000	(100) [c]	0.70	(0.0070) [c]	$^2B_2 \rightarrow {}^2E$
	9500	200	0.25	0.0012	$^2B_2 \rightarrow {}^2B_1$
	11700	600	2.5	0.0042	$^2B_2 \rightarrow {}^2A_1$
	13000	600	2.7	0.0045	$\pi S \rightarrow d_{x^2-y^2}$
	16125	5700	1.5	0.0003	$\sigma S \rightarrow d_{x^2-y^2}$
	23000	530	2.6	0.0049	$\pi N \rightarrow d_{x^2-y^2}$
fungal laccase	7250	(100) [c]	0.45	(0.0045) [c]	$^2B_2 \rightarrow {}^2E$
	11000	170	0.6	0.0035	$^2B_2 \rightarrow {}^2B_1$
	13000	830	2.0	0.0024	$\pi S \rightarrow d_{x^2-y^2}$ and $^2B_2 \rightarrow {}^2A_1$
	16000	4900	3.2	0.0006	$\sigma S \rightarrow d_{x^2-y^2}$
	19000	950	1.4	0.0015	d
	22650	800	1.3	0.0016	$\pi N \rightarrow d_{x^2-y^2}$

[a] Band positions from near-infrared and visible CD spectra. [b] Values taken from a gaussian resolution of the 20 K absorption spectrum. [c] Estimated value. [d] This band and the corresponding CD feature in tree laccase (not listed) may possibly represent $\sigma S^* \rightarrow d_{x^2-y^2}$ transitions; see text.

may be ruled out for copper types 2 and 3 in tree and fungal laccase by the absence of d-d transitions at energies near or below 10000 cm^{-1}. As both types 2 and 3 copper remain oxidized in high pH fungal laccase, the transitions cannot be assigned to one type specifically. For NO-treated tree laccase, however, it is apparent that the 16700 cm^{-1} absorption is associated primarily with the type 2 site. Both types 2 and 3 copper contribute to the 18500-cm^{-1} CD minimum (Figure 13), as the intensity in the NO-treated tree laccase spectrum decreases $\sim$1/3 upon removal of the type 2 copper. Finally, we should note that the weak feature present in the NO-treated tree laccase spectrum (Figure 12) near 425 nm ($\sim$23500 cm^{-1}) was not reproducible.

Our findings suggest that the type 2 and type 3 copper ions possess coordination geometries typical of many small monomeric Cu(II) complexes or bridged binuclear units. Several other metalloproteins are known to contain binuclear or "type 2" copper sites. Oxyhemocyanin[11] and tryosinase[12] have both absorption and CD bands attributable to d-d transitions of Cu(II) in the region where transitions appear in the spectra of the type 1 reduced laccases. Bovine erythrocyte superoxide dismutase, which contains a Cu(II)N$_4$ chromophore, displays a λ_{max} of 680 nm (14700 cm^{-1}) in its visible absorption spectrum. Extensive dialysis of ceruloplasmin against ascorbate results in loss of the type 1 chromophore.[23,48] Upon reoxidation, the ascorbate-modified ceruloplasmin shows absorption and CD spectra remarkably similar to those of high pH fungal laccase.[23]

Addition of F$^-$ perturbs the entire visible and near-ultraviolet absorption[15] and CD spectra of tree and fungal laccase. Examination of the effect of F$^-$ under conditions where the blue copper of fungal laccase is reduced indicates[49] that the perturbation of the CD spectrum above 30000 cm^{-1} is associated with changes in the type 3 chromophore or is due to a new feature associated with the type 2 copper-fluoride interaction, as suggested previously.[15] In any case, the data rule out autoreduction of the type 1 copper as an explanation for the effect of F$^-$ on the visible absorption or CD spectrum and indicate that anion binding at the type 2 site perturbs the type 1 site.

The evidence now available suggests[8] that intraenzyme electron-transfer steps are part of the catalytic mechanism of the laccases. However, magnetic susceptibility measurements have established[9] that the copper types 1 and 2 must be separated by several angstroms and are probably not bridged by a common ligand. Consequently, perturbations of the type 1 chromophore by anion binding at the type 2 site may have mechanistic significance, as such alterations could reflect conformational changes of the enzyme designed to facilitate intramolecular electron transfer. The difference CD spectra (Figures 9 and 10) are consistent with this idea, particularly considering the fact that the bands associated with the type 1 site are not affected equally, suggesting that certain portions of the polypeptide chain are more sensitive to changes at the type 2 site than other type 1 ligand-containing sections.

Table IV. Types 2 and 3 Copper Absorption and CD Bands

blue protein	absorption, cm^{-1}	circular dichroism, cm^{-1}
tree laccase	16700 (sh); 30000	16000(sh); 18500; 20000(sh); 24000
fungal laccase	16700 (sh); 30000	15000; 19500; 22500; 28500(sh)

Acknowledgment. We thank Drs. E. Bunnenberg and C. Djerassi for allowing us access to their spectroscopic and computer facilities at Stanford University. This research was supported by the National Science Foundation (CHE77-11389). D.M.D. acknowledges a National Institutes of Health Predoctoral Traineeship (1974-1978). J.H.D. acknowledges a National Institutes of Health Postdoctoral Fellowship (IF32CA05748-01) (1976-1978).

References and Notes

(1) (a) Amherst College; (b) University of Southern California; (c) University of South Carolina; (d) University of Göteborg; (e) address correspondence to this author at the California Institute of Technology.
(2) (a) J. A. Fee, *Struct. Bonding (Berlin)*, **23**, 1 (1975); (b) R. Malkin and B. G. Malmström, *Adv. Enzymol.*, **33**, 177 (1970).
(3) N. Sailasuta, F.C. Anson, and H. B. Gray, *J. Am. Chem. Soc.*, **101**, 455 (1979).
(4) P. M. Colman, H. C. Freeman, J. M. Guss, M. Murata, V. A. Norris, J. A. M. Ramshaw, and M. P. Venkatoppa, *Nature (London)*, **272**, 319 (1978).
(5) E. T. Adman, R. E. Stenkamp, L. C. Sieker, and L. H. Jensen, *J. Mol. Biol.*, **123**, 35 (1978).
(6) (a) E. I. Solomon, J. W. Hare, and H. B. Gray, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 1389 (1976); (b) E. I. Solomon, J. W. Hare, D. M. Dooley, J. H. Dawson, P. J. Stephens, and H. B. Gray, *J. Am. Chem. Soc.*, submitted for publication.
(7) R. Malkin, B. G. Malmström, and T. Vänngård, *FEBS Lett.*, **1**, 50 (1968).
(8) (a) R. A. Holwerda and H. B. Gray, *J. Am. Chem. Soc.*, **96**, 6008 (1974); (b) R. Brändén and B. Reinhammar, *Biochim. Biophys. Acta*, **405**, 236 (1975); (c) L.-E. Andréasson, R. Brändén, and B. Reinhammar, *ibid.*, **438**, 370 (1976); (d) R. Brändén and B. Reinhammar, *ibid.*, **405**, 236 (1975); (e) L.-E. Andréasson and B. Reinhammar, *ibid.*, **445**, 579 (1976); (f) R. Brändén and J. Deinum, *FEBS Lett.*, **73**, 144 (1977).
(9) D. M. Dooley, R. A. Scott, J. Ellinghaus, E. I. Solomon, and H. B. Gray, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 3019 (1978).
(10) D. A. Baldwin, D. M. Dooley, and H. B. Gray, *J. Am. Chem. Soc.*, manuscript in preparation.
(11) K. W. Nickerson and K. E. Van Holde, *Comp. Biochem. Physiol. B*, **39**, 855 (1971).
(12) R. L. Jolley, L. H. Evans, N. Makino, and H. S. Mason, *J. Biol. Chem.*, **249**, 355 (1974).
(13) B. Reinhammar, *Biochim. Biophys. Acta*, **205**, 35 (1970).
(14) (a) G. Fåhraeus and B. Reinhammar, *Acta Chem. Scand.*, **21**, 5 (1967); (b) R. Mosbach, *Biochim. Biophys. Acta*, **73**, 204 (1962).
(15) R. Brändén, B. G. Malmström, and T. Vänngård, *Eur. J. Biochem.*, **36**, 195

(1973).
(16) J. A. Fee, B. G. Malmström, and T. Vänngård, *Biochim. Biophys. Acta*, **197**, 136 (1970).
(17) G. Rotilio, L. Morpurgo, M. T. Graziani, and M. Brunori, *FEBS Lett.*, **54**, 163 (1975).
(18) M. T. Graziani, L. Morpurgo, G. Rotilio, and B. Mondovi, *FEBS Lett.*, **70**, 87 (1976).
(19) (a) G. A. Osborne, J. C. Cheng, and P. J. Stephens, *Rev. Sci. Instrum.*, **44**, 10 (1973); (b) L. A. Nafie, T. A. Keiderling, and P. J. Stephens, *J. Am. Chem. Soc.*, **98**, 2715 (1976).
(20) S.-P. Tang, J. E. Coleman, and Y. P. Myer, *J. Biol. Chem.*, **243**, 4286 (1968).
(21) K.-E. Falk and B. Reinhammar, *Biochim. Biophys. Acta*, **285**, 84 (1972).
(22) R. Malkin, B. G. Malmström, and T. Vänngård, *Eur. J. Biochem.*, **10**, 324 (1969).
(23) J. H. Dawson, D. M. Dooley, R. Clark, P. J. Stephens, and H. B. Gray, *J. Am. Chem. Soc.*, following paper in this issue.
(24) Our EPR spectrum of NO-treated tree laccase is similar, but not identical, to the type 2 spectrum observed during anaerobic reduction of the enzyme by hydroquinone.[25] It is likely that either hydroquinone or NO (or both) perturbs the type 2 site. Both EPR spectra are consistent with a tetragonal type 2 copper site.
(25) B. G. Malmström, B. Reinhammar, and T. Vänngård, *Biochim. Biophys. Acta*, **205**, 48 (1970).
(26) (a) R. B. Martin, in "Metal Ions in Biological Systems", Vol. I, H. Siegel, Ed., Marcel Dekker, New York, 1974, Chapter 4; (b) A. S. Brill, "Transition Metals in Biochemistry", Springer-Verlag, Berlin, 1977.
(27) (a) J. W. Hare, Ph.D. Thesis, California Institute of Technology, 1976; (b) B. J. Hathaway and A. A. G. Tomlinson, *Coord. Chem. Rev.*, **5**, 1 (1970); (c) B. S. Hathaway and D. E. Billing, *Ibid.*, **5**, 143 (1970).
(28) (a) W. E. Hatfield and E. R. Jones, *Inorg. Chem.*, **6**, 1502 (1970); (b) J. S. Wood, *J. Chem. Soc. A*, 1582 (1969).
(29) C. J. Ballhausen, "Introduction to Ligand Field Theory", McGraw-Hill, New York, 1962.
(30) R. E. Dietz, H. Kamimura, M. D. Sturge, and A. Yariv, *Phys. Rev.*, **132**, 1559 (1963).
(31) M. DeWit and A. R. Reinberg, *Phys. Rev.*, **163**, 261 (1967).
(32) C. A. Bates and P. E. Chandler, *J. Phys. C*, **4**, 2713 (1971).
(33) R. D. Willett, J. A. Haugen, J. Lebsack, and J. Morrey, *Inorg. Chem.*, **13**, 2510 (1974).
(34) R. L. Harlow, W. J. Wells, G. W. Watt, and S. H. Simonsen, *Inorg. Chem.*, **14**, 1768 (1975).
(35) A. S. Brill, G. F. Bryce, and J. H. Maria, *Biochim. Biophys. Acta*, **154**, 342 (1968).
(36) W. E. Blumberg and J. Peisach, *Biochim. Biophys. Acta*, **126**, 269 (1966).
(37) B. G. Malmström, B. Reinhammar, and T. Vänngard, *Biochim. Biophys. Acta*, **156**, 67 (1968).
(38) C. F. Bryce, *J. Phys. Chem.*, **70**, 3549 (1966).
(39) C. A. Bates, W. S. More, K. J. Standley, and K. W. H. Stevens, *Proc. Phys. Soc.*, **79**, 73 (1962).
(40) I. H. Parker, *J. Phys. C*, **4**, 2967 (1971).
(41) V. G. Krishnan, *J. Chem. Phys.*, **68**, 660 (1978).
(42) M. Sharnoff, *J. Chem. Phys.*, **42**, 3383 (1965).
(43) D. Attanasio, A. A. G. Tomlinson, and L. Alagna, *J. Chem. Soc., Chem. Commun.*, 618 (1977).
(44) We note that pulsed EPR studies suggest that imidazole (histidine) is a type 1 copper ligand in tree laccase (B. Mondovi, M. T. Graziani, W. B. Mims, R. Oltzik, and J. Peisach, *Biochemistry*, **16**, 4198 (1977)); there is also EPR evidence[8b] for nitrogen ligation to type 1 copper in the fungal enzyme, but the exact number and nature of these nitrogen donors are not known.
(45) (a) C. Briving and J. Deinum, *FEBS Lett.*, **51**, 43 (1975); (b) H. Beinert, *Coord. Chem. Rev.*, **23**, 119 (1977).
(46) (a) D. R. McMillin, R. A. Holwerda, and H. B. Gray, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 1389 (1974); (b) D. R. McMillin, R. C. Rosenberg, and H. B. Gray, *ibid.*, **71**, 4760 (1974); (c) V. M. Miskowski, J. A. Thich, R. Solomon, and H. J. Schugar, *J. Am. Chem. Soc.*, **98**, 8344 (1976); (d) J. S. Thompson, T. J. Marks, and J. A. Ibers, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 3114 (1977); (e) A. R. Amundsen, J. Whelan, and B. Bosnich, *J. Am. Chem. Soc.*, **99**, 6730 (1977); (f) J. L. Hughey IV, T. G. Fawcett, S. M. Rudich, R. A. Lalancette, J. A. Potenza, and H. J. Schugar, *ibid.*, **101**, 2617 (1979); (g) O. Siiman, N. M. Young, and P. R. Carey, **96**, 5583 (1974); (h) N. S. Ferris, W. H. Woodruff, D. B. Rorabacher, T. E. Jones, and L. A. Ochrynowycz, *ibid.*, **100**, 5939 (1978).
(47) S. J. Lippard, A. L. Burger, K. Ugurbil, J. S. Valentine, and M. W. Pantoliano, *Adv. Chem. Ser.*, **No. 162** (1977).
(48) C. B. Kasper and H. F. Deutsch, *J. Biol. Chem.*, **238**, 2325 (1963).
(49) D. M. Dooley, Ph.D. Thesis, California Institute of Technology, 1979.

Spectroscopic Studies of Ceruloplasmin. Electronic Structures of the Copper Sites

John H. Dawson,[1a] David M. Dooley,[1b] R. Clark,[1c] Philip J. Stephens,[1c] and Harry B. Gray*[1d]

Contribution No. 5889 from the Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, California 91125.
Received September 21, 1978

Abstract: Low temperature absorption and room temperature circular dichroism and magnetic circular dichroism spectra have been measured for the blue copper oxidase ceruloplasmin. Azide- and thiocyanate-bound ceruloplasmins have been examined by the two latter techniques. Low energy ligand field transitions have been located in the native (6100, 10 000, and 11 500 cm^{-1}) and azide-bound (8940 and 12 000 cm^{-1}) proteins that are of similar energy to those previously attributed to a flattened tetrahedral type 1 (blue) copper in other blue copper proteins. The MCD spectrum, featuring a single negative band at 14 600 cm^{-1}, is quite similar to those of the other blue copper proteins. The combined spectroscopic evidence suggests that both blue copper sites in ceruloplasmin are structured in the same manner as the CuN_2SS^* (N = His, S = Cys, S* = Met) unit in azurin and plastocyanin. Examination of the ligand field spectra shows conclusively that structural changes in both type 1 coppers occur on anion binding. Analysis of the d–d spectrum of azide-bound ceruloplasmin requires a larger distortion away from a tetrahedral structure than is indicated for native type 1 copper. An ascorbate-modified (type 1 depleted) derivative of ceruloplasmin has been characterized; its absorption (16 500 cm^{-1}), circular dichroism (13 700, 17 000, 19 800, 22 200, 25 300, and 32 000 cm^{-1}), and electron paramagnetic resonance ($g_\perp = 2.06$, $g_\parallel = 2.26$; $A_\parallel = 170$, $A_N = 14$ G) spectra have been measured. The band positions observed in absorption and CD rule out tetrahedral or near-tetrahedral geometries for the type 2 and type 3 coppers. A tetragonal structure for type 2 copper with four nitrogen-donor ligands is suggested by the nine-line superhyperfine splitting pattern seen in the $g_\perp$ region of ascorbate-modified ceruloplasmin.

Ceruloplasmin,[2] the major copper-containing blood plasma protein, is the only mammalian member of a small group of enzymes called the blue copper oxidases.[3-9] Since its original discovery by Holmberg,[10] ceruloplasmin has been intensely investigated, especially after establishment of its clinical importance as the affected protein in the hereditary copper metabolic disorder known as Wilson's disease. Although capable of oxidizing a variety of organic molecules in vitro, its physiological substrate is thought to be ferrous ion.[2] Ceruloplasmin may also function as a copper transport protein,[2] as it will reversibly bind up to ten cupric ions in addition to the intrinsic, catalytically important coppers.[11]

The blue copper oxidases (e.g., ceruloplasmin, ascorbate oxidase, and the laccases) contain at least four copper ions in three spectroscopically distinguishable sites.[3-9] Intensely blue in color, the type 1 (blue) copper has an abnormally small

0002-7863/79/1501-5046$01.00/0 © 1979 American Chemical Society