1260, 1220, 1119, 1079, 983. UV[42] (CH_3CN, nm (absorbance)) 673 (0.15), 602 (0.10), 471 (0.12), 452 (1.08), 358 (0.51), 318 (1.07), 291 (0.71), 218 (0.90).

Hexamethoxytriphenylene–Tris(dicyanomethylene)cyclopropane 1:1 Complex, HMT·8. All operations were carried out in the glovebox. Hexamethoxytriphenylene[44] (10 mg, 0.024 mmol) was dissolved in 4 mL of dry CH_2Cl_2. Dissolution required stirring for several minutes. Freshly prepared HCTMC (5 mg, 0.022 mmol) was added as a solid. The reaction mixture immediately turned violet. It was allowed to stir for 45 min, and the solid was collected on a medium-porosity fritted funnel. After drying under vacuum the violet solid weighed 10 mg.

Anal. Found (Calcd for $C_{36}H_{24}N_6O_6$): C, 59.48 (67.92); H, 3.75 (3.80); N, 11.35 (13.20). Ratio (calcd/found) for C = 87.6, N = 86.0%. IR (KBr) 2211 (n), 2195 (sh), 1616, 1502, 1467, 1439, 1417, 1352, 1262, 1211, 1157, 1042, 967, 846, 779, 708, 669, 646, 626.

Preparation and ESR Spectroscopy of Dications 18 and 19. A suspension of **17**·PF_6^- (15 mmol, 0.016 mg) in 0.40 mL of CH_3CN under argon was cooled in a −35 °C bath. To this suspension was added with stirring 0.15 mL of a 0.175 M solution of $NOSbF_6$ in CH_3CN (1.6 equiv) via a Teflon needle. The resulting blue-green solution of **19** was evacuated briefly and then transfered rapidly via cannula to a −35 °C ESR tube under argon. A $\Delta m = 2$ line was observed as well as z and xy lines and a broad, intense singlet at $g \sim 2$. A D value of 0.0219 was found ($E = 0$), which agrees well with the value found for **3**, $D = 0.022$. An identical procedure was followed for the preparation of **18** for which $D = 0.022$.

Acknowledgment. We are indebted to Dr. Frank DiSalvo, Joe Waszczak, and Dr. Lynn Schneemeyer at AT&T Bell Laboratories for the magnetic susceptibility determinations on the complexes and to Dr. Jerry Torrance for magnetic susceptibility measurements on the radical cations. We also thank Dr. Bruce Johnson at General Electric for the differential scanning calorimetry measurements. We thank J. Miller, J. Torrance, and F. Wudl for advising us of their results in advance of publication. We acknowledge the National Science Foundation for support and for a predoctoral fellowship to T.J.L.

Chemical and Spectroscopic Studies of the Coupled Binuclear Copper Site in Type 2 Depleted *Rhus* Laccase: Comparison to the Hemocyanins and Tyrosinase

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Abstract: The active site of native laccase contains four copper ions, one of which can be removed generating the type 2 depleted (T2D) form which contains a blue (type 1, T1) and a coupled binuclear (type 3, T3) copper center. Deoxy (T3: [Cu(I)Cu(I)]), half met (T3: [Cu(II)Cu(I)]), met and uncoupled met-N_3^- (T3: [Cu(II)Cu(II)]) T2D laccase active site derivatives have been prepared, and their chemical and spectroscopic study provides insight toward a spectroscopically effective model for the coupled binuclear copper site in T2D laccase. CO, Cl^-, Br^-, I^-, N_3^-, and SCN^- are found to bind to the type 3 site in deoxy T2D, and from competition studies, all appear to coordinate to one exchangeable position at the deoxy active site; thus, while dioxygen is found not to react with the type 3 site in T2D, this site is still accessible to small molecule binding. Peroxide oxidizes deoxy to met T2D, and spectral studies of this derivative indicate that like met hemocyanin and tyrosinase, met T2D contains two tetragonal, EPR nondetectable Cu(II) ions which are thus antiferromagnetically coupled. Addition of N_3^- to met T2D at pH <6 produces a triplet EPR signal characteristic of two uncoupled dipole-interacting Cu(II)'s indicating that a protonatable endogenous bridge is present at the type 3 site in laccase. One-electron reduction of met T2D by ferrocyanide generates half met T2D. For all anions investigated, one exogenous ligand binds to the cupric ion in half met and is readily removed by dialysis ($K \sim 10^2$ M^{-1}). This behavior is in marked contrast to anion binding in half met hemocyanins and tyrosinase ($K > 10^4$ M^{-1}, where exogenous ligands bridge the binuclear copper site) and indicates a nonbridged binding mode for exogenous ligands to the type 3 site in laccase. The $N_3^- \rightarrow$ Cu(II) charge-transfer spectrum of the met-N_3^- derivative, where the absorption and CD transitions are maximum at the same energy, also supports this nonbridging coordination geometry of exogenous ligands, which is reasonably attributed to there being only one exhangeable coordination position at the type 3 site. Thus, this comparison of the coupled binuclear copper site in T2D laccase to that in the hemocyanins and tyrosinase indicates a strong geometric and electronic structural similarity in the endogenous bridge but an important difference in the exogenous ligand coordination mode; this difference in active site structure provides insight into the differences in dioxygen reactivity of these coupled binuclear copper active sites.

A number of important biological systems contain a coupled binuclear copper active site[1] that interacts with dioxygen. The simplest of these metalloproteins are the hemocyanins and tyrosinase which reversibly bind oxygen. Tyrosinase[2] in addition functions to hydroxylate phenols to *o*-diphenols (monooxygenase activity) and to oxidize the resulting diphenol to the *o*-quinone (diphenolase activity). The multicopper oxidases[3] *Rhus* and *Polyporus* laccase, ceruloplasmin and ascorbic acid oxidase, contain a number of copper centers that include one or two coupled binuclear copper sites (designated as "type 3" copper) and are the more complicated of the binuclear copper containing proteins. These enzymes couple one-electron oxidations of substrate to the four-electron reduction of dioxygen to water.

In the hemocyanins and tyrosinase, a series of systematically varied coupled binuclear copper active site derivatives[1] have been

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prepared. Of importance with respect to this study are the half met and met protein forms. The half met form contains an EPR detectable [Cu(II)Cu(I)] site and the met form, like oxyhemocyanin, contains a [Cu(II)Cu(II)] active site that exhibits no EPR signal. Detailed chemical and spectroscopic study of these derivatives demonstrates the active sites of all hemocyanins and tyrosinase to be extremely similar,[4] as described by the spectroscopically effective model for the oxyhemocyanin coupled binuclear copper site in ref 1. In this description, oxygen reacts with deoxy to generate oxy, oxidizing the site to two Cu(II)'s and binding as peroxide.[5] The two tetragonal copper(II)'s are bridged by a protonatable[6] endogenous ligand RO^- (Ph–O^-, HO^-, or RO^-). This endogenous bridge provides a superexchange pathway that mediates antiferromagnetic coupling resulting in a diamagnetic ground state and no EPR signal. X-ray crystallographic results[7] on deoxy hemocyanin indicate no evidence for phenoxide or alkoxide at the active site, suggesting the endogenous ligand may be hydroxide; these studies further indicate that three histidine residues coordinate each of the deoxy copper centers. Consistent with the crystallographic results on deoxy, resonance Raman,[8] spin–echo,[9] and extended X-ray absorption fine structure (EXAFS)[10] spectroscopic studies indicate that histidine residues are coordinated upon oxidation of the coppers. Exogenous ligands also bridge the exchange coupled Cu(II)'s, and in the case of oxy, optical[11] and resonance Raman[5] studies indicate the peroxide is bound in a μ-1,2 bridging geometry.

Laccase, the simplest of the multicopper oxidases, contains single blue (or type 1, T1), normal (or type 2, T2), and coupled binuclear (T3) copper centers for a total of four copper ions at its active site. Optical and EPR contributions are observed for native *Rhus vernicifera* laccase characteristic of each individual type of copper center.[12] The T1 Cu(II) is observed strongly in both the electronic absorption (ϵ_{614} = 5700 M^{-1} cm^{-1}) and EPR ($A_\parallel$ = 43 × 10^{-4} cm^{-1}, $g_\parallel$ = 2.30, $g_\perp$ = 2.06, 2.03) spectra of laccase. While the optical features due to the T2 copper are weak and dominated by the intense overlapping T1 charge-transfer (CT) bands, the T2 Cu(II) EPR contribution is clear ($A_\parallel$ = 206 × 10^{-4} cm^{-1}, $g_\parallel$ = 2.24, $g_\perp$ = 2.05). Like the coupled binuclear copper site in oxy and met hemocyanin, the T3 site is EPR nondetectable, and if this is due to exchange coupling between two Cu(II)'s, magnetic susceptibility studies[13] estimate $-2J > 550$ cm^{-1}. The binuclear T3 copper site has been associated with an absorption band at 330 nm (ϵ = 2800 M^{-1} cm^{-1}) which reduces as a two-electron acceptor.[14]

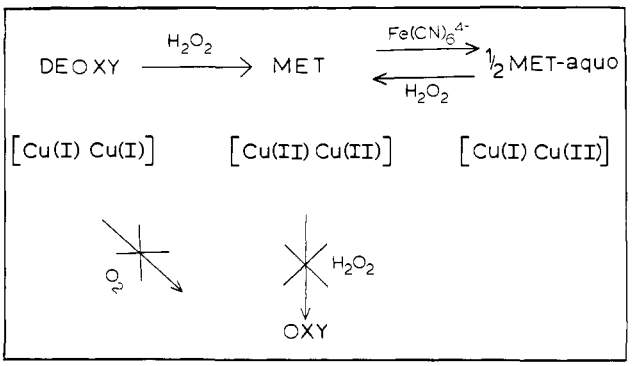

Figure 1. Copper ion oxidation state composition in T2D laccase derivatives.

An important simplification for study of the coupled binuclear or T3 site in laccase is the reversible preparation of a type 2 copper depleted (T2D) protein derivative, first reported in 1976 by Graziani et al.[15] In the EPR spectrum, the features due to the T2 Cu(II) are removed leaving only the Cu(II) signal associated with the oxidized T1 center. In the optical spectrum, the T2D form exhibits no 330-nm absorption band,[16] this feature in the native enzyme having been associated with the T3 site. Further, this T2D derivative was reported[16] not to exhibit N_3^- binding to T3 copper, also in contrast to native laccase for which intense N_3^- → Cu(II) CT absorption intensity is observed[17,18] between 400 and 500 nm upon addition of the inhibitor N_3^-. In 1981, we reported[19] that addition of H_2O_2 leads to the reappearance of the 330-nm band ($\Delta\epsilon \sim 2000$ M^{-1} cm^{-1}). New absorption intensity is also observed in the ligand field region, and azide addition to T2D laccase after reaction with H_2O_2 now does generate an N_3^- → Cu(II) CT feature. These results suggested that T2D laccase contains a reduced T3 site that could be reoxidized by H_2O_2; X-ray absorption spectroscopy (XAS) confirmed this reduction,[19] where T2D laccase samples before and after addition of peroxide exhibited edge structures qualitatively similar to those of Cu(I) and Cu(II) imidazole complexes, respectively. These XAS edge studies have been extended,[20] and as shown in the following paper,[21] through XAS difference edge analysis we are now capable of rigorously quantitating the amount of Cu(I) present in a sample of unknown Cu(I) and Cu(II) composition. These studies demonstrate that the T2D sample studied here contains 98 ± 22% Cu(I) before and 0 ± 8% Cu(I) after oxidation by H_2O_2. Visible–UV/XAS peroxide titration studies of T2D laccase further determine that the increase in 330-nm absorption intensity correlates linearly with the percent of oxidation of the binuclear copper site, indicating that the peroxide oxidizes, but does not bind, to the T3 site in T2D laccase.

In this paper, chemical and spectral studies of the coupled binuclear T3 copper site in T2D laccase derivatives will be presented and compared to the coupled binuclear copper site in the hemocyanins and tyrosinase. In preliminary communications,[22]

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the preparation of a stable, mixed valence or half met T3 copper derivative of T2D laccase was reported. Here, the chemistry and spectroscopy of T2D is further developed. Through this research, a series of systematically varied active site derivatives (Figure 1) have been investigated. As T2D contains a reduced T3 site, it is referred to as the deoxy form of T2D (T1: [Cu(II)]; T3: [Cu(I)Cu(I)]). T2D laccase is oxidized by peroxide (or aqueous nitrite at pH <7.0)[22b] and is referred to as met T2D (T1: [Cu(II)]; T3: [Cu(II)Cu(II)]). A form of T2D laccase parallel to oxyhemocyanin is not accessible,[23] as deoxy T2D does not react with O_2 and peroxide does not bind to met T2D (see the following paper). One-electron reduction of met with ferrocyanide (or nitrite)[22b] produces the half met (T1: [Cu(II)]; T3: [Cu(II)Cu(I)]) protein form. The half met derivatives (like deoxy) undergo oxidation by peroxide to yield met T2D laccase. These T2D derivatives, while prepared by different chemical paths, directly parallel the well-characterized[1] hemocyanin and tyrosinase active site derivatives and hence allow a comparison of the multicopper oxidase T3 site to the hemocyanin and tyrosinase coupled binuclear copper sites.

Experimental Section

Laccase was purified[24,25] from the acetone powder (Saito and Co., Osaka) of the Japanase lacquer tree *Rhus vernicifera* to a ratio of A_{280}:A_{614} = 15.0–16.5. Two modifications to the published procedure were the centrifugation of the stirred acetone powder suspension in 0.01 M potassium phosphate, pH 6.0, and its filtering over Celite prior to Buchner filtration for its batch extraction by CM-Sephadex C-50. The T2 copper was selectively removed through the anaerobic ferrocyanide, dimethylglyoxime, and EDTA dialysis procedure reported in ref 15. In recent preparations, T2D laccase was additionally eluted through a short column of DEAE-A50 in order to remove residual $Fe(CN)_6^{3-}$, resulting in T2D protein for which A_{330}:A_{614} ~ 0.35–0.45. A number of spectroscopic techniques have been used to quantitate the amount of iron which remains in the final T2D laccase: (1) atomic absorption (AA) analyses (vide infra) indicate 0.10 ± 0.05 Fe per mol of T2D protein; (2) room temperature optical absorption studies of concentrated T2D in the 400-nm region indicate 0.05 ± 0.03 ferricyanide (ϵ = 1000 M^{-1} cm^{-1}) per mol of T2D protein; and (3) magnetic circular dichroism (MCD) spectra of deoxy T2D laccase at 4.2 K and 10 000 G display transitions at 402 nm (+) and 305 nm (−) which are similar to the charge-transfer bands observed in the MCD spectrum of ferricyanide at 300 K.[26] After field and temperature correction, the intensity of the 402-nm feature indicates 0.02 $Fe(CN)_6^{3-}$ per mol of T2D.[27] This ~5% ferric impurity contributes to the EPR spectra of T2D (e.g., the lowest field peak in Figure 2, see Results), but as it is present at low concentration and as its EPR signal is not perturbed on ligand binding, all of the spectral changes presented are reasonably attributed to the T2D active site copper. Met T2D was prepared by reaction of 0.1–0.2 mM deoxy T2D laccase with 3–6 mM H_2O_2, followed by dialysis. Anaerobic reduction of met T2D with 0.7–1.0 protein equivalent ferrocyanide followed by aerobic dialysis in phosphate buffer generated the half met derivative[22a] which from double integrated EPR intensity contains 1.5 ± 0.1 spin/mol.

Water is purified to a resistivity of 15–18 mΩ cm through a Sybron Barnstead Nanopure deionizing system. All studies are in 0.1 M potassium phosphate, pH 6.0, unless otherwise stated and utilize reagent grade chemicals without further purification. Typically, exogenous ligand reactivity studies were performed by direct addition of the anion (in a 5- or 10-μL aliquot of appropriate concentration) to the protein followed by 24–40 h of incubation at 4 °C. Equilibrium binding constants were calculated as described by Byers.[28] Reagent grade peroxide (30% aqueous solution) was standardized by permanganate.[29]

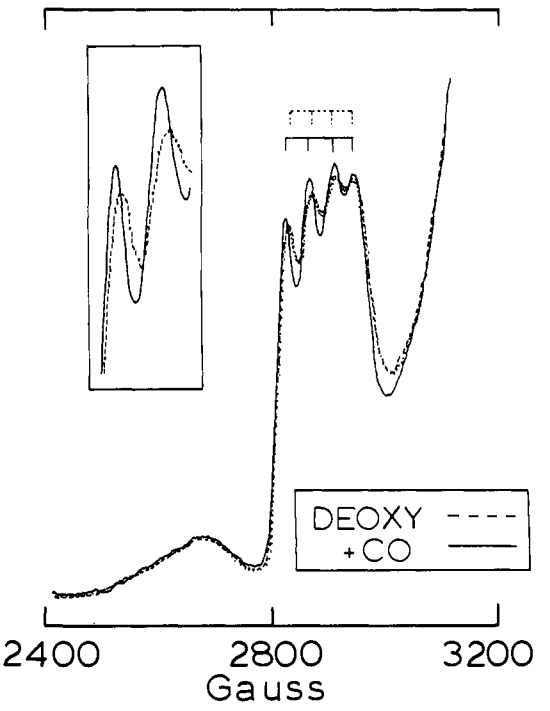

Figure 2. Superposition of *g* parallel regions of EPR spectra at 77 K: (---) original deoxy T2D laccase; (—) after 1-h reaction with 1 atm of CO; (···) after removal of CO. The vertical hatchmarks indicate the increase in hyperfine splitting after reaction with CO. Inset: Expanded scale illustration of changes in the first two T1 parallel hyperfine peaks.

Experiments under 1 atm of CO (99.5%, Matheson) were performed in 2-mL serum-stoppered disposable test tubes by evacuation of the T2D laccase solutions followed by re-equilibration with CO for 45–60 min. For higher pressures of CO (~2 atm), protein solutions were placed in a 20-mL high-pressure glass reaction vessel (Labglass, NJ). Solutions were made anaerobic by repeated vacuum pump/N_2 purge cycling on an N_2 Schlenk line equipped with a chromous[30] perchlorate bubbler.

Total copper ion concentrations in T2D derivatives were routinely determined by atomic absorption spectroscopy with a Perkin-Elmer 2380 AA spectrophotometer equipped with an HGA-400 graphite furnace. The maximum intensity for Cu was monitored at 324.7 nm with a slit width of 0.7 nm. T2D laccase samples were also analyzed for their residual iron content, monitoring intensity at 248.3 nm with a slit width of 0.2 nm. A copper or iron AA standard solution (Alfa) was diluted in the appropriate buffer matrix for each run.

Optical absorption spectra at 298 K were measured in 1-cm quartz cells on a Cary 14 spectrophotometer. Spectra at 77 K were recorded in 50% v/v glycerol/0.1 M potassium phosphate, pH 6.0, between quartz disks with typical path lengths of 1.4 mm. For these spectra, an Air Products LTD-3-110 Heli-Tran liquid helium transfer refrigerator and temperature controller were used in combination with a cryotip sample holder mounted to a Cary 17 spectrophotometer. CD spectra were recorded in 1-cm cells on a Jasco J-500C spectropolarimeter operating with an S1 and S20 photomultiplier tube for the near-IR and visible–UV spectral regions, respectively. CD calibration was done with camphor sulfonic acid and positive $\Delta\epsilon$'s correspond to $\epsilon_{left}-\epsilon_{right}$ circularly polarized light.

A Bruker ER 220 D-SRC EPR spectrometer (operating at 100 KHz modulation, 10 mW incident power, and 12.5 G modulation amplitude, unless otherwise stated) was used for frozen protein solutions at a microwave frequency ~9.27 GHz at 77 K; sample temperatures between 7 and 60 K were obtained at ~9.40 GHz with an Air Products LTD-3-110 Heli-Tran liquid helium transfer refrigerator and a Lake Shore Cryotronics, Inc. cryogenic temperature controller, Model DTC-500. Computer EPR simulation of the triplet signal was performed with the program GNDIMER[31] obtained from Professor John Pilbrow at Monash University, Australia. EPR difference spectra were calculated on a Nicolet Instrument Corp. NIC-1180E Data Processor which was interfaced to the Bruker EPR spectrometer.

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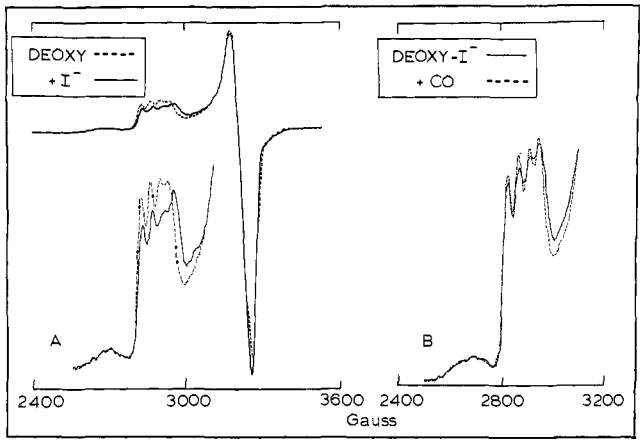

Figure 3. Superposition of EPR spectra at 77 K. (a) (---) 0.31 mM deoxy T2D laccase; (—) after reaction with 0.03 M KI. The $g_{\|}$ region is also shown at 6.3× higher gain. (B) g parallel region of (—) 0.22 mM deoxy-I^- T2D in 0.02 M KI; (---) after 1-h reaction with 1 atm of CO.

Results

A. Deoxy T2D. Deoxy T2D contains a cuprous T3 site in the presence of a cupric T1 center.[20,21] The oxidized T1 site in laccase is spectroscopically similar[32] to that of plastocyanin[33] and azurin,[34] suggesting that exogenous ligands can coordinate only to the T3 coppers at the T2D laccase active site. In deoxy T2D, the d^{10} electron configuration renders the T3 site inaccessible to direct spectroscopic probes of its exogenous ligand binding. However, it has been observed[19] that the spectral features of the T1 Cu(II) are dependent on the oxidation state of the T3 copper, and in parallel we find that ligand binding at the deoxy T3 site can be indirectly studied through changes induced in the EPR spectrum of the T1 Cu(II) center.

Reaction of deoxy T2D with 1 atm of CO(g) perturbs the T1 Cu(II) EPR signal as shown in Figure 2. On binding CO, the total four line parallel hyperfine splitting of the T1 center increases from 116 ± 2 to 131 ± 3 × 10^{-4} cm^{-1} and the relative apparent intensities of the T1 hyperfine peaks change. While the magnitude of this change is limited, it is reproducibly observed, and moreover, upon removal of CO the original EPR spectrum of deoxy T2D is regained; increasing the CO pressure to 2 atm results in no additional EPR spectral perturbation.

The EPR spectral changes associated with I^- binding are shown in Figure 3A. With 100 protein equivalents of I^-, the total T1 parallel hyperfine splitting is increased to 130 × 10^{-4} cm^{-1}. From titration studies (up to 0.04 M I^-) one I^- binds per deoxy site, and the regeneration of the unperturbed deoxy spectrum upon short-term dialysis (data not shown) ascertains that no irreversible effect has occurred. Spectral changes similar to those for I^- are observed when deoxy T2D is reacted with Cl^- or Br^-, but not F^- (vide infra). N_3^- and SCN^- also perturb the T1 Cu(II) EPR signal; from the increase in the T1 hyperfine constant upon reaction with N_3^-, $K_{eq} \sim 100$ M^{-1} at 77 K, and dialysis again restores the original deoxy EPR spectrum.

While N_3^- and I^- are relatively good ligands for Cu(I) and Cu(II), F^- can be a poor ligand for copper and thus helps probe the protein's interaction with anions. Reaction of 0.1 mM deoxy T2D with 0.02 M F^- leads to no perturbation of the T1 EPR spectrum. The changes induced by I^- (Cl^-, Br^-, N_3^-, and SCN^-) therefore do not appear to be associated with nonspecific anion binding. Further, CO is normally a ligand only for the cuprous oxidation state and does perturb the T1 EPR spectrum of deoxy (but not met) T2D. Hence, these ligands are reasonably considered to all be binding at the binuclear cuprous site in deoxy T2D.

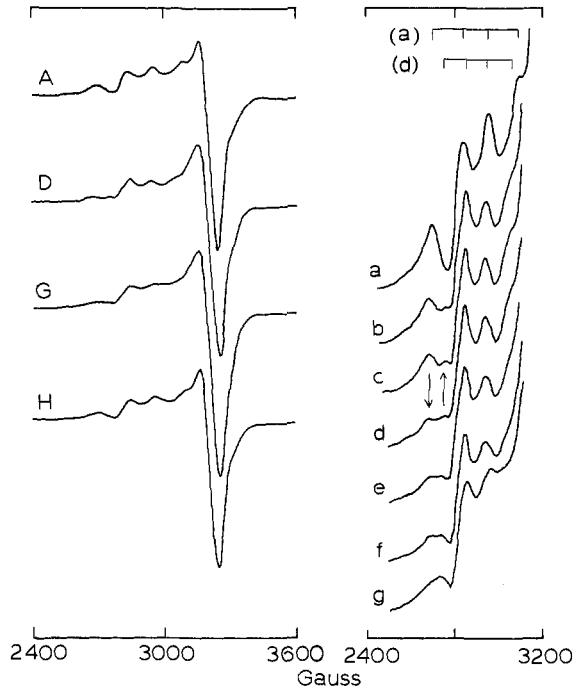

Figure 4. EPR spectra at 77 K: N_3^- titration of half met T2D laccase. (A, a) Original half met aquo T2D; (b) +2.0; (c) +5.0; (D, d) +10; (e) +25; (f) +50; (G, g) +150 protein equivalents NaN_3; (H) after dialysis. The lower case spectra illustrate the $g_{\|}$ region at 6.3× higher gain. The positions of the half met-aquo (upper) and half met-N_3^- (lower) T3 parallel hyperfine peaks are indicated on the right. The arrows at 2700 and 2750 G indicate the change in field for the first T3 Cu(II) hyperfine peak upon azide coordination.

Ligand competition between CO and I^- is shown in Figure 3B for deoxy-I^- T2D (in 100 protein equivalents of I^-) reacted with 1 atm of CO. The changes induced by CO in the relative intensities of the parallel hyperfine peaks are similar to those in Figure 2, indicating that some I^- is being displaced by CO from the deoxy site. The total parallel hyperfine splitting remains increased relative to deoxy T2D and removal of CO regenerates the original deoxy-I^- spectrum; CO also reacts with deoxy-N_3^- T2D. While the observed changes in the deoxy EPR spectra are limited, the CO/I^- and CO/N_3^- competition studies show no new spectral features evident of a ternary complex and there is no evidence for the binding of more than one exogenous ligand, suggesting that N_3^-, I^-, and CO (as well as Cl^-, Br^-, and SCN^-) all coordinate to one exchangeable position at the deoxy active site.

B. Half Met T2D. Half met T2D has an EPR active T3 site ($A_{\|}$ = 138 × 10^{-4} cm^{-1}, $g_{\|}$ = 2.28, and $g_{\perp}$ = 2.07). Quantitative XAS edge and double-integrated EPR studies characterize[20a] this protein form as containing ~50% met T2D which has a T1 Cu(II) contributing to the EPR and ~50% half met T2D which has EPR contributions from both the T3 [Cu(II)Cu(I)] and T1 Cu(II) sites.

Azide titration of half met T2D is followed by 77 K EPR in Figure 4. Upon addition of N_3^-, the T3 EPR signal changes. Related 77 K EPR titration studies of met T2D (vide infra) demonstrate that in ≥25 protein equivalents of N_3^-, the met T1 Cu(II) signal is also perturbed and therefore contributes to the spectral changes in Figure 4e–g. At <25 equivalents of N_3^- (Figure 4b–d), however, the observed EPR changes are associated only with N_3^- binding to the half met T3 site. The intensity of the $\Delta m_s = -^3/_2$ hyperfine peak at lowest field goes down and a new feature grows in at 55 G higher field (indicated by arrows). Additional changes clearly occur in the higher field portion of the $g_{\|}$ region between spectra a and d. While these changes may be complicated by some perturbation of the T1 Cu(II) EPR signal in half met, it is possible to estimate from difference spectra (vide

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(33) Colman, P. J.; Freeman, H. C.; Guss, J. M.; Murata, M.; Norris, V. A.; Ramshaw J. A. M.; Venkatappa, M. P. *Nature (London)* **1978**, *272*, 319–324.
(34) (a) Adman, E. T.; Jensen, L. H. *Isr. J. Chem.* **1981**, *21*, 8–12. (b) Norris, G. E.; Anderson, B. F.; Baker, E. N. *J. Mol. Biol.* **1983**, *155*, 501–521.

Table I. Relative Anion Affinities at Half Met T2D, Half Met Hemocyanin,[36] and Met Apo Hemocyanin[36] Protein Sites

Half Met-L T2D Laccase $N_3^- > SCN^- > CH_3CO_2^- \geq NO_2^- >$ halides $\gg F^-$
Half Met-L Hemocyanin $NO_2^- > N_3^- > I^-, Br^- > SCN^-, Cl^- > CH_3CO_2^- \gg F^-$
Met Apo-L Hemocyanin $N_3^-, SCN^- > NO_2^-, CH_3CO_2^-$ > halides $\gg F^-$

infra) that the changes in the half met T3 signal correspond to a decrease in $A_\parallel$ to 119×10^{-4} cm^{-1} with little change in $g_\parallel$. From the increase in the half met-N_3^- lowest hyperfine line at 2750 G (arrow, Figure 4), one N_3^- binds to the half met site with $K_{eq} \sim$ 6000–8000 M^{-1} at 77 K. In support of the EPR results, related optical N_3^- titration data (vide infra) monitoring both the shift in the half met-aquo d–d bands (CD) and the new $N_3^- \rightarrow$ Cu(II) CT intensity (absorption) also indicate binding of a single N_3^-; from the room temperature optical changes, $K_{eq} \sim 150$ M^{-1}, an order of magnitude lower than at 77 K. Of key importance, short term dialysis (<5 h) leads to reappearance of the original half met aquo T2D EPR signal (Figure 4H is superimposable on Figure 4A), indicating that the N_3^- is no longer bound to the T3 copper site.

In addition to N_3^-, we find that NO_2^-, acetate$^-$, SCN^-, and Br^- all modify the T3 Cu(II) EPR signal, as shown by the EPR difference spectra half met-X minus met-X (Figure 5, Br^- not shown) which estimate the spectral contribution of the T3 Cu(II)-X center. In each of the T3 Cu(II)-X$^-$ EPR signals, $g_\parallel > g_\perp > 2.00$, indicating a $d_{x^2-y^2}$ tetragonal ground state for the half met Cu(II) center. While the perpendicular region is complicated due to changes in the T1 Cu(II),[35] the parallel region shows four discernible hyperfine lines for all anions and no evidence beyond subtraction artifacts for additional copper hyperfine lines which indicates a localized (i.e., class I) mixed valence character for the half met T3 site. EPR titration studies analogous to those presented for N_3^- in Figure 4 indicate that for all these anions only one ligand binds to the half met site and it is readily removed by short-term dialysis. When half met-aquo and half met-N_3^- are reacted with 2 atm of CO, there is no perturbation of the T3 Cu(II)-aquo or -N_3^- EPR signals. From ligand competition studies, the relative binding affinities of the exogenous anions at the half met T2D binuclear copper site have been estimated and are summarized in Table I. For comparison, the relative ligand affinities of half met and met apo hemocyanin[36] are also given.

The near-IR CD spectrum of deoxy T2D is shown in Figure 6A. Several broad features are observed which maximize at 850 nm ($\Delta\epsilon \sim -2.4$ M^{-1} cm^{-1}), 730 nm ($\Delta\epsilon \sim -2.8$ M^{-1} cm^{-1}), 600 nm ($\Delta\epsilon \sim +1.5$ M^{-1} cm^{-1}), and 535 nm ($\Delta\epsilon \sim +1.4$ M^{-1} cm^{-1}) and correspond to the T1 Cu(II) site in deoxy T2D. The near-IR CD transitions of half met-aquo (which contains ~60% met T2D) are also shown in Figure 6A. Correction of the half met T2D spectrum for the met spectral contribution (cf. Figure 9) with appropriate scaling results in the theoretical 100% half met-aquo spectrum shown in Figure 6B. Relative to the T1 Cu(II) spectrum of deoxy T2D, half met-aquo displays features at ~995 nm ($\Delta(\Delta\epsilon) = +1.29$ M^{-1} cm^{-1}) and 625 nm ($\Delta(\Delta\epsilon) = +0.97$ M^{-1} cm^{-1}, not shown in Figure 6B). The effect of N_3^- on 100% half met-aquo T2D, again corrected for the met-N_3^- spectral contribution, is also shown in Figure 6B. Half met-N_3^- appears to contain a negative feature at ~995 nm and a positive feature at ~810 nm relative to the half met-aquo T2D. Two points complicate interpretation of these spectral changes. The optical features of the T1 Cu(II) have been shown[19] to depend on the oxidation state of the T3 site, and in CD spectroscopy it is difficult to differentiate the gain of

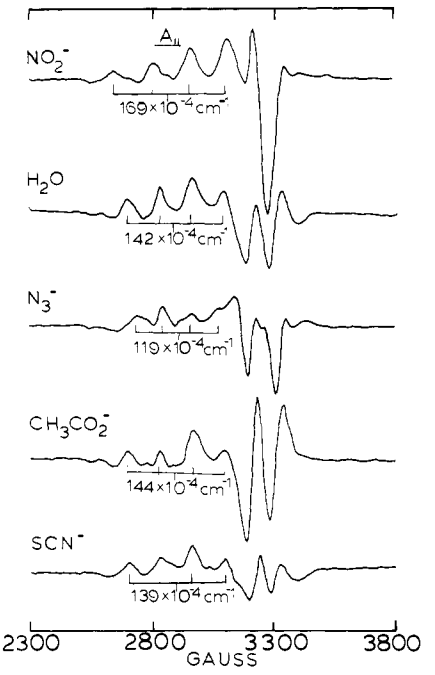


Figure 5. Unsmoothed EPR difference spectra at 77 K: (half met-X) minus (met-X) T2D laccase derivatives. The hyperfine splitting in $g_\parallel$ is indicated for each anion. The $g_\parallel$ values are the following: NO_2^-, 2.31; H_2O, 2.28; N_3^-, 2.27; $CH_3CO_2^-$, 2.28; and SCN^-, 2.28.[35]

a new spectral feature from the loss of a pre-existing feature of opposite sign. However, resonance Raman studies[37] indicate that at room temperature, N_3^- binding at the T3 site in T2D derivatives does not perturb the T1 Cu(II). The 995-nm feature that is present in the 100% half met-aquo but not deoxy T2D and which disappears ($\Delta(\Delta\epsilon) = -1.29$ M^{-1} cm^{-1}) upon coordinating N_3^- is therefore assigned as a Cu(II) ligand field band of the T3 site in half met T2D. The energy of this ligand field transition is consistent with the tetragonal effective Cu(II) symmetry observed from the EPR parameters. The further spectral change at 810 nm ($\Delta(\Delta\epsilon) = +1.36$ M^{-1} cm^{-1} in Figure 6B) is reasonably attributed to this half met T3 Cu(II) ligand field transition with N_3^- bound. This new CD feature at ~810 nm (12 500 cm^{-1}) correlates with a broad increase in the absorption intensity at ~750 nm (13 300 cm^{-1}) upon binding N_3^-.[38] Lastly, there is no new CD (or 77 K absorption) intensity at wavelengths shorter than 1100 nm; thus, no intervalence transition is indicated, consistent with the localized (class I) mixed valence EPR properties of the half met T3 site.

The electronic absorption spectra of half met-aquo titrated with N_3^- ($K_{eq} \sim 150$ M^{-1}) are shown in Figure 7. A reasonably intense band is observed at 400 nm together with a second feature at ~480 nm which is more clearly resolved at 77 K. After correcting the optical spectrum for the $N_3^- \rightarrow$ Cu(II) CT intensity of the residual met-N_3^- T2D which is centered at 450 nm (Figure 10A), significant intensity ($\Delta\epsilon \sim$ 1000–1500 M^{-1} cm^{-1}) remains at ~480 and 400 nm, which must reflect $N_3^- \rightarrow$ Cu(II) CT transitions at the pure half met T3 site. Whereas the ligand field d–d and EPR changes could have been either directly due to T3 Cu(II) coordination or to an indirect consequence of coordination at the Cu(I), the concurrent presence of $N_3^- \rightarrow$ Cu(II) CT transitions requires that N_3^- is binding equatorially at the oxidized Cu(II) center of the half met site. Note that comparison to the met-N_3^- spectral changes in Figure 10 indicates that intensity in the 330-nm region must increase in half me-N_3^-.

(35) The apparent splitting in the g perpendicular region is due to changes in the T1 Cu(II) and is an artifact which arises in spectral subtraction; only the g parallel region is reasonably estimated.

(36) (a) Himmelwright, R. S.; Eickman, N. C.; Solomon, E. I. *J. Am. Chem. Soc.* **1979**, *101*, 1576–1586. (b) Himmelwright, R. S.; Eickman, N. C.; Solomon, E. I. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 237–242.

(37) Porras, A. G.; Spira-Solomon, D. J.; Solomon, E. I., unpublished results.

(38) Due to selection rules for ligand field split d–d transitions of approximately tetragonal Cu(II), the absorption feature is expected to peak at higher energy relative to the CD feature. The former is determined by the $xz,yz \rightarrow x^2 - y^2$ transition while the latter is governed by the large magnetic dipole character of the $xy \rightarrow x^2 - y^2$ transition.

Table II. 330-nm Absorption Intensity in T2D Laccase Derivatives (Data Represent Four Different Preparations of T2D Laccase from a Total of Three Purifications of Native Laccase)

expt	$\Delta\epsilon_{330}$ (met − deoxy)[a]	half met T2D		$\Delta\epsilon_{330}$ (half met − deoxy)/ $\Delta\epsilon_{330}$ (met − deoxy)	$\Delta\epsilon_{330}$ (half met + H_2O_2) − deoxy/ $\Delta\epsilon_{330}$ (met − deoxy)
		T3[Cu(II)Cu(I)][b]	T3[Cu(II)Cu(II)][c]		
1	2240	0.53	0.47	0.39	1.00
2	2410	0.35	0.65	0.57	1.02
3	2350	0.46	0.54	0.55	0.95
4	2120	0.38	0.62	0.40	0.94
av	2280 (130)	0.43 (0.08)	0.57 (0.08)	0.47 (0.10)	0.98 (0.04)

[a] Met prepared by peroxide reaction of deoxy. [b] Half met prepared by ferrocyanide reduction of met, dialyzed. Relative half met T3 sites from double integrated EPR. [c] Based on footnote *b*, assuming 1.00 half met plus met protein sites.

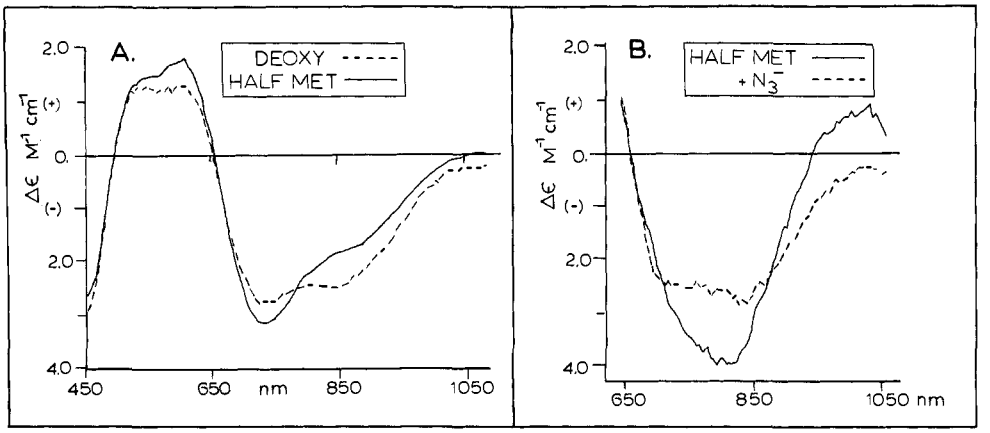


Figure 6. (A) CD spectra at 298 K: deoxy T2D and half met T2D (met T2D anaerobically reduced with one protein of equivalent ferrocyanide, dialyzed). (b) Corrected CD spectra at 298 K: half met T2D laccase (part A) minus 60% met T2D (Figure 9A) scaled to 100% half met protein sites and half met N_3^- T2D laccase (250 × N_3^-) minus 60% met-N_3^- T2D (250 × N_3^-, Figure 9A) scaled to 100% half met-N_3^- protein sites (unsmoothed data, 0.25 mM protein).

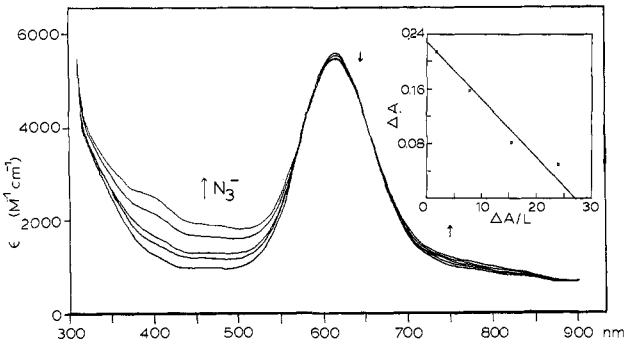


Figure 7. Electronic absorption spectra at 298 K: N_3^- titration of half met T2D laccase. Arrows indicate direction of change with 10, 25, 100, and 250 protein equivalents of NaN_3 (0.19 mM protein). Inset: Binding constant analysis plot[28] of the titration data at 500 nm, corrected for 60% (met-T2D + N_3^-) and scaled to 100% half met protein sites.

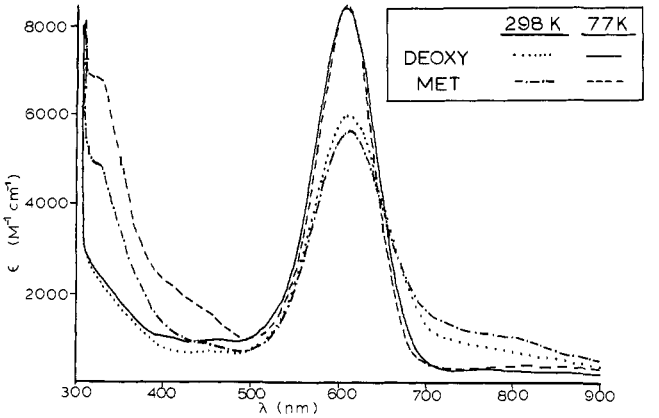


Figure 8. Electronic absorption spectra: met and deoxy T2D derivatives at 298 and 77 K (50% v/v glycerol/0.1 M potassium phosphate, pH 6.0; 1.45 mm path length; 1.1 mM protein).

C. Met T2D. Through comparison of met and deoxy T2D, the spectral features associated with the oxidized T3 site can be determined. Studies aimed at elucidating and assigning transitions of the T3 pair in the visible/near-UV spectral regions will be presented first, followed by an examination of N_3^- binding to the fully oxidized T3 site.

In Figure 8 are shown the 298 K absorption spectra of met and deoxy T2D. The broad $\Delta\epsilon_{750} \sim 150$ M^{-1} cm^{-1} of met T2D can be assigned, by analogy to hemocyanin and tyrosinase,[1] as the d–d transitions in two tetragonal cupric centers. No T3 copper EPR signal is observed in met T2D and this, combined with the Cu(II) XAS edge spectrum,[20,21] requires antiferromagnetic coupling between the two Cu(II) ions and a superexchange mediating endogenous bridge. Figure 8 also shows a near-UV band at ~330 nm which is present in met but not deoxy T2D and is therefore associated with the [Cu(II)Cu(II)] T3 site.

Upon cooling to 77 K (Figure 8) the 600-nm absorption band sharpens in both met and deoxy T2D; only in met T2D, however, is absorption intensity between 500 and 300 nm significantly increased. As this CT intensity is not associated with peroxide binding, it must relate to endogenous ligation at the coupled binuclear cupric site. Further, intensity in the 330-nm region is not dramatically affected upon binding exogenous ligands to met T2D, suggesting the dominant contribution is from endogenous protein ligand → Cu(II) CT transitions. At least one broad new feature is indicated at ~440 nm and the 330-nm band sharpens upon cooling, now maximizing at 320 nm; these spectra demonstrate that a number of transitions must contribute to the broad 330-nm absorption at 298 K.

Comparison of the 330-nm region in deoxy, half met, and met T2D indicates that 330-nm intensity derives primarily from the fully oxidized T3 site. In a series of experiments on four different T2D preparations (Table II), 330-nm absorption and integrated EPR intensity were monitored as (i) deoxy was oxidized to met, (ii) the resultant met was reduced to half met, and (iii) the resultant half met was reoxidized to met. Normalizing the $\Delta\epsilon_{330}$ (2280 ± 130 M^{-1} cm^{-1}) for oxidation of deoxy to met to an

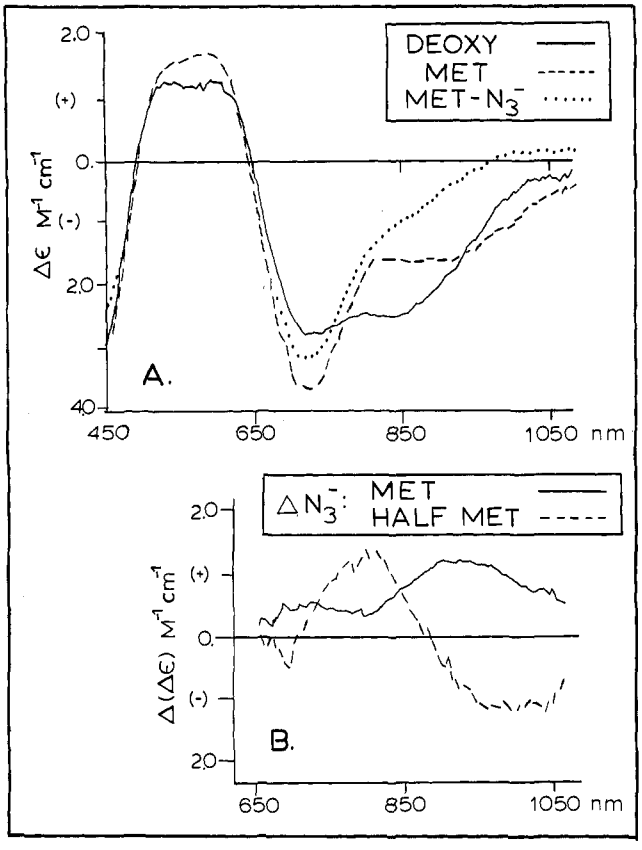


Figure 9. (A) CD spectra at 298 K: deoxy T2D, met T2D, and met T2D + 250 × N_3^-. Digitized spectra have been base line subtracted by computer and are not smoothed (0.27 mM protein). (B) CD difference spectra: (—) met-N_3^- minus met-aquo T2D; (---) met-corrected half met-N_3^- minus half met-aquo T2D.

intensity of 1.00, half met is observed to have a relative intensity at 330 nm of 0.47 ± 0.10 (i.e., an ~50% loss in 330-nm intensity). Within the experimental uncertainty of the integrated EPR intensities, this decrease in 330-nm intensity quantitatively correlates with the one-electron reduction of met to half met T2D, as half met T2D has been determined (Table II) to consist of 0.43 ± 0.08 and 0.57 ± 0.08 half met and met T3 sites, respectively. If 330-nm intensity decreased linearly with reduction of the individual Cu(II) ions, an ~25% loss in 330-nm intensity would be expected. That 53 ± 10% of the 330-nm intensity is lost with one-electron reduction of 43 ± 8% of the T3 sites indicates that the majority of this absorption intensity is associated only with the binuclear cupric form of the T3 site. This could indicate that (a) all of the strongest transitions which comprise the met 330-nm band are associated with the Cu(II) which is reduced in half met, which is unlikely, as this would require an unreasonable distribution of the absorption intensity and therefore ligation between the two copper ions, or (b) much of the 330-nm intensity could require the presence of a coupled binuclear cupric unit.

The ligand binding of the T3 cupric site in met T2D laccase has been studied by probing changes in the d–d and CT features of its two cupric ions. The near-IR CD spectra of met and deoxy T2D are shown in Figure 9A. In comparing these spectra, at least four transitions would appear to be associated with oxidation of the T3 site: $\Delta(\Delta\epsilon)_{990} = -0.47$ M^{-1} cm^{-1}, $\Delta(\Delta\epsilon)_{830} = +1.03$ M^{-1} cm^{-1}, $\Delta(\Delta\epsilon)_{720} = -0.90$ M^{-1} cm^{-1}, and $\Delta(\Delta\epsilon)_{580} = +0.42$ M^{-1} cm^{-1}. While some of these spectral changes are likely associated with the d–d transitions of the binuclear cupric site, potential structural changes in the T1 site concomitant to oxidation of the T3 site, as evidenced by resonance Raman studies,[19] could also contribute to the CD spectral changes. Interpretation of these spectral changes is thus complex and the met CD spectrum is not simply the addition of the d–d transitions of a second Cu(II) ion to the half met CD spectrum in Figure 6. However, as resonance Raman studies[37] indicate that at room temperature the T1 Cu(II) is not

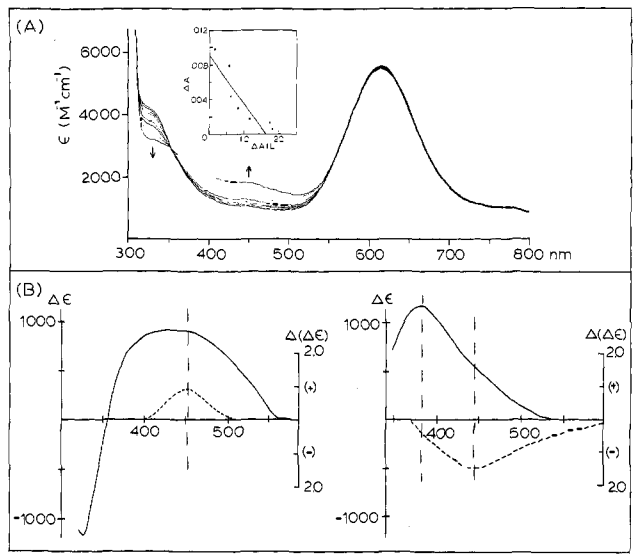


Figure 10. (A) Electronic absorption spectra at 298 K: N_3^- titration of met T2D laccase. Arrows indicate direction of change with 2, 5, 10, 25, 50, 100, and 500 protein equivalents of NaN_3 (0.14 mM protein). Inset: Binding constant analysis plot[28] of the titration data at 450 nm. (B) 298 K (—) electronic absorption and (---) CD difference spectra: Left, met-N_3^- minus met T2D laccase (absorption: 0.14 mM T2D, 0.07 M NaN_3; CD: 0.27 mM T2D, 0.07 M NaN_3). Right, met-N_3^- minus met hemocyanin.[39a] $\Delta\epsilon$ and Δ ($\Delta\epsilon$) values are in units of M^{-1} cm^{-1}.

perturbed on binding N_3^- at the T3 site, it is reasonable to associate the met CD spectral changes generated by N_3^- with structural changes in the binuclear cupric T3 site.

In Figure 9A is also shown the near-IR CD spectrum of met-N_3^- T2D laccase. Upon coordination of N_3^- at the T3 site, $\Delta(\Delta\epsilon)_{940} = +1.22$ M^{-1} cm^{-1} and $\Delta(\Delta\epsilon)_{720} = +0.51$ M^{-1} cm^{-1}. A comparison of the (met-N_3^- minus met-aquo) and (half met-N_3^- minus half met-aquo) difference spectra (Figure 9B) can be used to probe the effects of azide coordination at these T3 sites. In both derivatives, one low-energy feature appears to shift to higher energy (note that in the case of met-N_3^- T2D, the positive difference peak corresponds to the loss of a negative feature in met-aquo T2D). The fact that a single CD band exhibits similar energy changes suggests that N_3^- is binding similarly in half met and met T2D and thus that only one of the met Cu(II)'s is being perturbed.

The visible/near-UV absorption spectra of met-aquo T2D titrated with N_3^- are shown in Figure 10A. A broad new feature is centered at ~450 nm ($\Delta\epsilon$ = 900 M^{-1} cm^{-1}) and through resonance Raman studies[18] it has been assigned as an $N_3^- \rightarrow$ Cu(II) CT transition. Analysis of the titration data at 450 nm (see inset) indicates that a single N_3^- binds with K_{eq} = 170 M^{-1}. The decrease in 330-nm absorbance ($\Delta\epsilon \approx -1000$ M^{-1} cm^{-1}) and the positive CD change at 940 nm (Figure 9) also yield single K_{eq} = 220 and 130 M^{-1}, respectively, and indicate that a single N_3^- is responsible for the three spectral changes. In Figure 10B are shown the 500–300 nm absorption and CD difference spectra of met-N_3^- relative to met-aquo T2D (≥95% of the protein complexed by N_3^-). Gaussian analysis[39b] of the data is consistent with the broad absorption band being comprised of two transitions at 455 nm ($\epsilon \sim 900$ M^{-1} cm^{-1}) and 390 nm ($\epsilon \sim 1000$ M^{-1} cm^{-1}). In the corresponding CD difference spectrum, a positive feature ($\Delta(\Delta\epsilon)$ = +0.72 M^{-1} cm^{-1}) is observed, coincident with the 455-nm N_3^- → Cu(II) CT absorption feature. Also shown in Figure 10B are the analogous met-N_3^- hemocyanin difference absorption and CD spectra for later discussion.

The 77 K EPR changes associated with N_3^- binding to met T2D laccase at pH 6.0 are shown in Figure 11. The integrated EPR intensity is constant throughout the N_3^- titration, demonstrating

(39) (a) Pate, J. E.; Karlin, K. D.; Reed, C. A.; Sorrell, T. N.; Solomon, E. I., manuscript in preparation. (b) Pate, J. E.; Solomon, E. I., unpublished results.

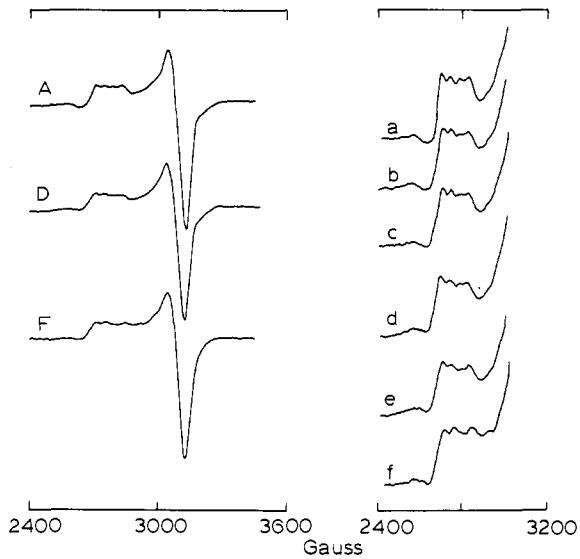


Figure 11. EPR spectra at 77 K: N_3^- titration of met T2D laccase. (A, a) Original met-aquo T2D; (b) +2; (c) +5; (D, d) +25; (e) +50; (F, f) +100 protein equivalents of NaN_3. The lower case spectra illustrate the $g_{\|}$ region at 2.5× higher gain.

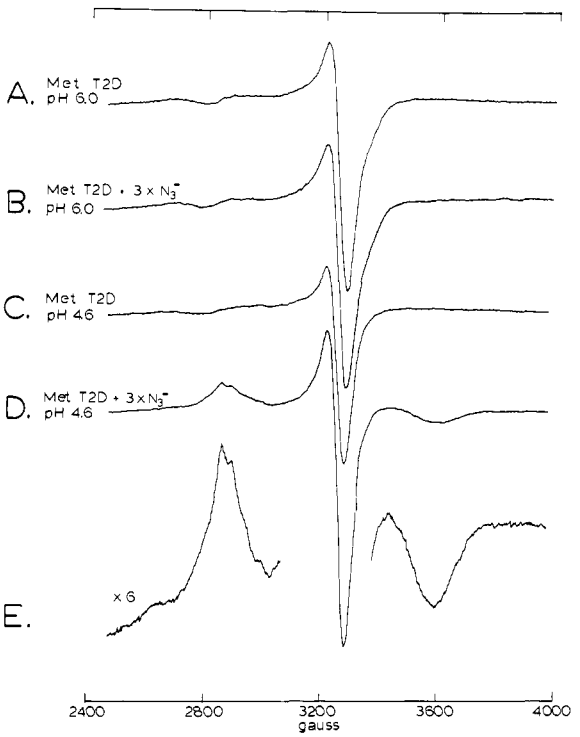


Figure 12. EPR spectra at 10 K: (A) met T2D laccase, 0.1 M potassium phosphate, pH 6.0; (B) sample A plus 3 protein equivalents of NaN_3; (C) met T2D laccase, 0.1 M sodium acetate, pH 4.6; (D) sample C plus 3 protein equivalents of NaN_3; (E) sample D at 6× higher gain (200 mW microwave power, ~0.25 mM protein).

that the new, broadened features for ≥50 protein equivalents of N_3^- (spectra e and f) are associated only with the T1 Cu(II). Hence at low temperature, N_3^- binding at the oxidized T3 center results in a structural change at the T1 copper. Dialysis restores the original met T1 Cu(II) EPR signal, indicating there is no irreversible protein damage by N_3^-. Similar low-temperature effects on the T1 Cu(II) are observed with SCN^-.

While the 77 K EPR spectra of both met-aquo and met-N_3^- T2D laccase show only T1 copper signal intensity, studies of these derivatives at 10 K and low pH provide additional information about the T3 [Cu(II)Cu(II)] site. The EPR spectrum of met-aquo T2D at 10 K over the pH range 7.6–4.0 (Figure 12A,C) shows no signal intensity associated with the T3 site. However, when

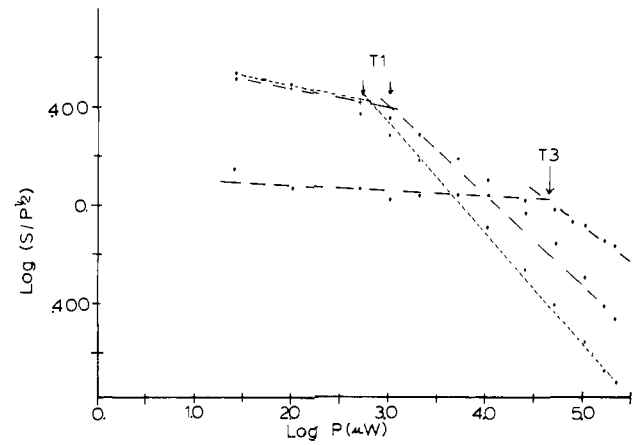


Figure 13. Power (P) saturation curves at 10 K for the EPR signal intensities (S) in (---) met and (– – –) met-N_3^- T2D laccase. Data for the T3 g = 1.86 signal in met-N_3^- T2D and the T1 perpendicular intensity in (+) met-N_3^- and (·) met T2D laccase are indicated.

3 protein equivalents of N_3^- are added to met T2D at pH <6.0 (Figure 12D), an additional broad derivative-shaped signal appears between 2700 and 3900 G; no other transitions are observed between 0 and 10000 G. N_3^- titration of deoxy T2D at 8 K shows no such signal, but if the resultant deoxy-N_3^- is further titrated with peroxide, the broad derivative shaped signal develops; moreover, there is a direct correlation between the 8 K intensity at 3590 G (g = 1.86) and the 298 K increase in 330-nm intensity (which correlates linearly with T3 oxidation).[21] This correlation and the fact that no signal is observed in deoxy T2D demonstrates that the new EPR signal is associated with the oxidized T3 sites. Dialysis of met-N_3^- eliminates the signal and restores the original 10 K EPR spectrum of only the T1 Cu(II) in met T2D. No anion other than N_3^- has been found to induce such signals.

The spectroscopic properties of the new N_3^- feature further indicate its binuclear cupric nature. Note that because the low-field portion of this signal overlaps the T1 Cu(II) spectrum, intensity measurements are of the high-field component and the signal is referred to as the "g = 1.86" signal. The relaxation[40] of the g = 1.86 signal in met-N_3^- compared to that of the T1 Cu(II) in both met and met-N_3^- is shown in Figure 13. The different saturation behavior of the intensity at g = 1.86 compared to that of the T1 Cu(II) in met-N_3^- T2D demonstrates that it is arising from a different paramagnetic center. Whereas $P_{1/2}$ for the mononuclear T1 Cu(II) signal at 8 K is 1–2 mW, the g = 1.86 signal only begins to saturate at ~50 mW. Parallel to hemocyanin chemistry[6] and consistent with the signal's absence in deoxy-N_3^- T2D laccase and powder pattern spectral shape (vide infra), we therefore assign this signal as the zero-field split triplet EPR signal characteristic of two dipolar Cu(II)'s. It should be noted that comparison (Figure 13) of the T1 Cu(II) saturation curves in met and met-N_3^- indicates that the T1 center is slightly harder to saturate in met-N_3^- T2D ($P_{1/2}$ = 2.2 vs. 0.7 mW). Since <20% of the protein molecules have uncoupled T3 sites (vide infra), the observable interaction of the uncoupled T3 site with the T1 site requires that the uncoupled T3 site be reasonably close to the T1 Cu(II) or that a significant structural distortion of the T1 Cu(II) occurs in the uncoupled molecules.

At 77 K, essentially no g = 1.86 signal intensity is observed. However, as the temperature is lowered, the EPR intensity follows Curie law behavior. From the temperature dependence of the signal intensity, a computer least-squares fit to the Bleaney Bowers[41] equation established that $|2J| < 10\ cm^{-1}$. Thus, the T3 cupric pair is effectively uncoupled in these sites, which requires that N_3^- must interfere with the endogenous bridge.

(40) Beinert, H.; Orme-Johnson, W. H. In *Magnetic Resonance in Biological Systems*; Ehrenberg, A., Malmstrom, B. G., Vanngard, T., Eds.; Pergamon: Oxford, 1967; pp 221–247.

(41) Bleaney, B.; Bowers, K. D. *Proc. R. Soc. London, Ser. A* **1952**, *214*, 451–465.

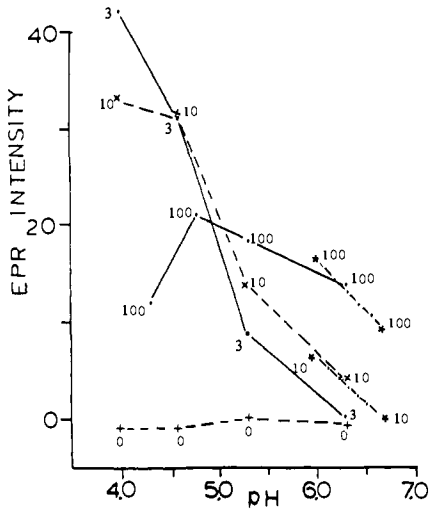
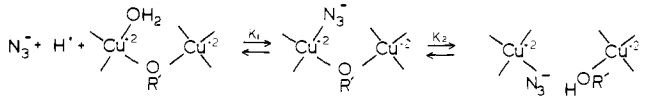


Figure 14. pH dependence of the 8 K EPR intensity at $g = 1.86$ for met T2D preincubated with (+---+) 0, (·—·) 3, (x---x) 10, and (·—·) 100 protein equivalents of NaN_3. The MES data points (10 and 100× N_3^-) are shown separately (*·····*). 0.1 M sodium acetate was used at pH 4.0, 4.6, and 5.3; 0.1 M potassium phosphate at pH 6.3; 0.1 M 2-*N*-morpholinoethanesulfonic acid (MES) at pH 6.0 and 6.7. Protein concentration, as determined by the 280-nm absorption for each sample, was used to scale the EPR intensities (0.2 mM protein for all samples).

Scheme I. N_3^- Interaction at the Type 3 Site in Met T2D Laccase



Accurate quantitation of the percentage of uncoupled T3 sites requires reasonable T1 and T3 signal intensities under nonsaturating conditions. At <20 K, low microwave powers will saturate the T1 Cu(II), while the uncoupled signal is not accessible at temperatures >40 K. From EPR studies at 35 K and 10 mW microwave power, the increase in double integrated EPR intensity of the met-N_3^- T2D relative to the oxidized T1 copper signal indicates that 13 ± 7% of the T3 sites are EPR detectable under optimum uncoupling conditions (pH 4.6, vide infra). However, from the 298 K binding constant for the 450 nm absorption feature at pH 4.6, approximately three times (or 40%) of the T3 sites should have N_3^- bound, and based on N_3^- binding studies of half met T2D (vide supra), this percentage should increase at lower temperatures. Thus, only a fraction of the N_3^--bound sites exhibit this $g = 1.86$ uncoupled EPR signal.

The intensity of the T3 EPR signal is dependent on pH as well as the N_3^- concentration. Figure 14 plots absolute signal intensity at $g = 1.86$ of met T2D treated with 0, 3, 10, and 100 protein equivalents of N_3^- over the pH range 6.7–4.0. In general, signal intensity increases with increasing $[N_3^-]$ and with increasing $[H^+]$, and for a fixed amount of N_3^-, it is larger at lower pH. However, at high $[N_3^-]$ and low pH, signal intensity maximizes and then decreases with increasing $[N_3^-]$.

Since under most circumstances H^+ and N_3^- together uncouple the T3 site, the new EPR signal is interpreted as an equilibrium involving competitive displacement by N_3^- of the protonatable exchange-mediating endogenous bridge (Scheme I). This chemistry strongly parallels hemocyanin met chemistry which we have recently reported.[6] As indicated in Figure 14, the concentration of uncoupled sites reaches a maximum value for a given pH and then decreases with increasing N_3^- concentration; thus, at high N_3^- concentrations, an additional N_3^- is binding to the uncoupled met-N_3^- T3 site and changing its spectral features. Parallel to studies on met hemocyanin, the data in Figure 14 can be used to calculate[6,42] apparent N_3^- binding constants at various pH values which provide an estimate of the pK_a of the endogenous

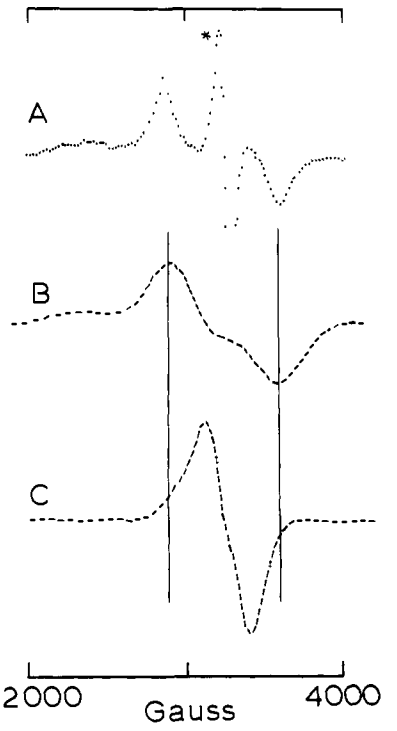


Figure 15. Met-N_3^- T2D and hemocyanin coupled binuclear cupric EPR signals. (A) Met-N_3^- minus met T2D difference EPR spectrum in 0.1 M NaOAc, pH 4.6 (12 K, 200 mW microwave power, $\nu = 9.39$ GHz), and (B) simulation of the triplet signal. The sharp peak at ~3200 G marked by an asterisk in the spectral subtraction is attributed to residual T1 Cu(II) whose saturation behavior changes with N_3^-. (C) Simulation of the met-N_3^- hemocyanin triplet EPR signal in 0.1 M succinate, pH 4.5 (~15 K, 10 mW, $\nu = 9.39$ GHz), adapted from ref 6. The vertical lines indicate the position of the maximum and minimum in the met-N_3^- T2D T3 signal.

bridge in the protein site. Such calculations indicate that the bridge is a protonatable residue with an intrinsic $pK_a > 6.7$.

The uncoupled signal contains structural information about the binuclear cupric site. As the temperature dependence has indicated that exchange coupling is small or negligible, dipolar interaction between the cupric ions is the dominant mechanism leading to zero-field splitting of their paramagnetic triplet spin state. This dipolar zero-field splitting is proportional to $1/r^3$, and the shape of the EPR spectrum should therefore reflect the distance between the cupric centers. By using an EPR spectral simulation calculation developed by Pilbrow,[31] the triplet signal in met-N_3^- T2D has been estimated and is compared to the met-N_3^- minus met T2D difference spectrum (which includes a residual contribution for the T1 Cu(II)) in Figure 15. A reasonable fit is obtained with $g_{\parallel} = 2.30$, $g_{\perp} = 2.06$, and $r = 4.0$ Å. The Cu–Cu distance, r, is dependent essentially only on the separation of the low-field maximum and high-field minimum and is fairly well defined; alternatively, full saturation of the T1 Cu(II) has not been obtained, which precludes elucidation of the detailed signal shape in this region and the angular information it contains. We note that as the relative intensity of the half-field $g = 4$ signal in randomly oriented samples is proportional[43] to $1/r^6$, the $\Delta m_s = 2$ signal for a 4-Å separation is calculated to be <1% the intensity of the $\Delta m_s = 1$ signal and this transition has not been observed.

Discussion

The chemical and spectral studies of the binuclear copper site derivatives presented here provide insight toward an effective model for the coupled binuclear copper T3 site in T2D laccase compared to that of the hemocyanins and tyrosinase. Like deoxy hemocyanin,[1] exogenous ligands bind to the reduced T3 copper in deoxy T2D laccase; yet, in strong contrast to the hemocyanins and

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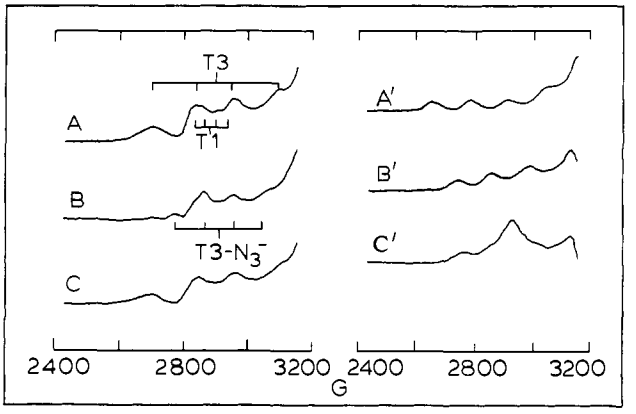

Figure 16. EPR spectral comparison ($g_{\parallel}$ region) at 77 K: (A) half met T2D laccase; (B) after reaction with excess N_3^-; (C) dialyzed; (A′) half met hemocyanin; (B′) after reaction with excess N_3^-; (C′) dialyzed.

tyrosinase, we have found that the binuclear cuprous site in deoxy T2D is stable to O_2 oxidation. This difference in dioxygen reactivity indicates that significant differences must exist between the T2D and hemocyanin and tyrosinase deoxy active sites and further suggests a strong role for the T2 copper[44,45] in the dioxygen reactivity of native laccase (vide infra).

In half met-aquo T2D, the ligand field transition energies and the EPR parameters of the T3 Cu(II) demonstrate that it has an approximately tetragonal structure. Upon reaction with azide, $N_3^- \rightarrow$ Cu(II) CT transitions appear at ~480 and 400 nm, which indicate that the N_3^- is binding equatorially to the Cu(II) center of the half met site. In competitive anion binding studies, NO_2^-, OAc^-, SCN^-, and Br^- compete with N_3^- and therefore also bind at the T3 Cu(II) center. CO is a reasonable ligand for Cu(I) but is not observed to perturb the EPR spectrum of half met-N_3^- (or half met-aquo) T2D, suggesting that the binding of anions to the half met T3 Cu(II) does not also involve the Cu(I) center.

A lack of exogenous ligand bridging between the half met Cu(I) and Cu(II) centers is strongly indicated by the low binding constant of anions at the site. For all anions investigated (N_3^-, SCN^-, Cl^-, Br^-, OAc^-, NO_2^-), only one ligand binds to the half met site ($K_{eq} \sim 10^2$ M^{-1} at 298 K) and it is readily removed by short-term dialysis. This behavior is in marked contrast to anion binding in half met hemocyanin and tyrosinase.[36] In these proteins, exogenous ligands were determined to bridge the cuprous and cupric ions of the binuclear site, which led to (1) an extremely tight anion binding at this site ($K_{eq} > 10^4$ M^{-1} at 298 K)[36a,46] and (2) a "group 1–group 2" ligand behavior. Group 1 ligands (Cl^-, Br^-, I^-, OAc^-, NO_2^-) bind tightly to the binuclear coppers but produce no change in EPR signal when present in excess concentrations, while group 2 ligands (N_3^-, SCN^-, CN^-) produce major EPR changes at high excess which are associated with a second group 2 ligand binding at the cupric center. This additional coordination position has been associated with the group 2 ligands competing with the endogenous bridge. Upon dialysis, a new EPR signal is observed, indicating that while the second lower affinity ligand is removed, the bridging exogenous ligand remains tightly bound. This difference in exogenous ligand reactivity is illustrated in Figure 16 for the group 2 ligand N_3^-. In T2D laccase (left, see also Figure 4), N_3^- decreases the parallel hyperfine splitting of the T3 Cu(II), but upon short-term dialysis, the original EPR spectrum of half met-aquo T2D is regained. Similarly, addition of N_3^- to half met hemocyanin (right, see also Figure 3, ref 36a) significantly changes its binuclear copper EPR spectrum. Extensive dialysis, however, further changes the EPR spectrum and does not return the original half met-aquo signal; one N_3^- remains tightly bound at the half met site. The lack of tight binding and group 1–group 2 behavior of anions in half met T2D laccase indicates that, in contrast to the hemocyanins and tyrosinase, exogenous ligands do not bridge the two coppers at the half met T3 site.

Table I summarizes the relative anion binding affinities of the half met T2D, met apo, and half met hemocyanin copper active sites. While both steric and electronic factors can contribute to this stability, the point to note is that T2D half met binding affinity parallels that of met apo, both of which are quite different from that of half met hemocyanin. As the half met hemocyanin differs from the met apo hemocyanin derivative in having the anion bridge to the adjacent Cu(I), the empirical correlation of half met T2D with met apo supports a similar nonbridging mode of exogenous ligand binding. Finally, no intervalence transitions are observed in optical studies of half met T2D and there is no delocalized, mixed valence character in the T3 Cu(II)-X^- EPR spectra. Both of these spectral properties are contrary to behavior in the hemocyanins and tyrosinase[1,4] and support a nonbridging mode for coordinating exogenous ligands to the Cu(II) center in half met T2D laccase.

The d–d transitions of met T2D together with its cupric XAS edge spectrum[19-21] demonstrate that it contains two tetragonal Cu(II) ions. The lack of T3 copper EPR signal then indicates that a superexchange pathway provides antiferromagnetic coupling between the Cu(II)'s. This pathway is confirmed by the observation in met-N_3^- T2D (uncoupled) at low pH of a broad, zero-field split triplet EPR signal (Figure 12). On the basis of its pH dependence and spectral properties, this signal is assigned to the two T3 Cu(II) ions which become paramagnetic upon protonation and competitive displacement by N_3^- of the exchange mediating endogenous bridge. A related EPR signal has been observed in N_3^- studies of met hemocyanin,[6] and a spectral comparison of the simulated uncoupled met-N_3^- T2D and hemocyanin signals is shown in Figure 15. Both signals are not observed at $T > 50$ K, follow a Curie law behavior at $T < 50$ K, and are much more difficult to saturate than is mononuclear copper(II). In met N_3^- T2D, the peak-to-peak spacing (marks indicated, Figure 15) is larger than in met hemocyanin and the simulation[6] of the met-N_3^- hemocyanin signal indicates $r \sim 5$ Å where it is only ~4 Å in met-N_3^- T2D. In both T2D laccase and hemocyanin, the exchange coupling is large ($2J \geq -550$ cm^{-1} in native laccase; $2J \geq -625$ cm^{-1} in oxyhemocyanin)[13] and the endogenous bridge ligands are calculated to exhibit similar intrinsic pK_a's (>6.7 in met T2D and >7.0 in met hemocyanin[6]). A similar endogenous bridging ligand therefore appears to provide the superexchange pathway in laccase and hemocyanin.

As demonstrated in the following paper,[21] the 330-nm band in met T2D laccase is assigned to endogenous ligand → Cu(II) CT at the binuclear cupric site with no significant contribution from peroxide → Cu(II) CT. The low-temperature optical spectrum of met T2D (Figure 8) demonstrates that several transitions contribute to the 330-nm band. While a strong S–Cu bond in a tetragonal cupric site can contribute significant CT intensity in the 300–350-nm spectral region,[47] EXAFS studies determined[32,48] that there is no sulfur ligation at <2.4 Å at the T3 site in laccase. Model studies by Schugar[49,50] show that copper imidazole complexes exhibit CT features at ~330 nm, with $\epsilon \sim$ 300–500 M^{-1} cm^{-1}. Further model studies[50] find that phenolate → Cu(II) CT occurs at ~420 nm, with alkoxide → Cu(II) CT

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to higher energy and, in the 330–360-nm region, hydroxide → Cu(II) CT. Each of these three RO^- ligands is capable of mediating strong superexchange and could also contribute to the 450–300-nm absorption in laccase. Reduction/reoxidation studies of met T2D show that the dominant transition intensity at 330 nm is associated with the dimer cupric unit. Dimer bands are often observed in binuclear metal complexes in spectral regions where the monomer shows little absorption. These bands have been considered to be simultaneous pair transitions where one photon excites d → d transitions in both metal centers. However, at least for Cu^{II}_2[51] and Fe^{III}–O–Fe^{III} [52] dimers, these bands can now be assigned as ligand-to-metal CT transitions which are stabilized in energy due to the binuclear interaction. In the case of $Cu_2Cl_6^{2-}$ and copper acetate, this stabilization results from a large excited state antiferromagnetism.[51]

Coordination of the exogenous ligand N_3^- to met T2D and met hemocyanin is compared optically in Figure 10B. In met-N_3^- T2D (coupled), two new features appear at 455 and 390 nm, with a concomitant loss in intensity in the 330-nm region. While the 455-nm band shows resonance enhancement enabling its assignment as an N_3^- → Cu(II) CT transition, protein damage at higher energy precludes resonance Raman study of the 390-nm feature, which however appears to be related to a shift to lower energy of the 330-nm band. On the basis of studies[39] of N_3^- → Cu(II) CT transitions in a wide variety of Cu(II) model complexes and proteins, the 455-nm band in met-N_3^- T2D is too low in energy and high in intensity to be consistent with a μ-1,1 bridging N_3^- geometry, while a μ-1,3 N_3^- bridge is expected to display a significantly more intense, higher energy ($\epsilon \sim 2000\ M^{-1}\ cm^{-1}$, $\lambda \sim$ 380 nm) CT feature than is observed. Alternatively, the indistinguishable absorption and CD N_3^- → Cu(II) CT transition energies at 455 nm support equatorial, nonbridging N_3^- coordination at the met T3 site. Azide bound to a single Cu(II) can have two CT transitions, N_3^- π_v^* → Cu($d_{x^2-y^2}$), and to higher energy with most intensity, N_3^- π_σ^* → Cu($d_{x^2-y^2}$). If N_3^- bridges to a second Cu(II) ion, each of these CT transitions splits in two to generate four transitions with different selection rules. As shown for met-N_3^- hemocyanin in Figure 10B, the coupling of the transition moment vectors to the two Cu(II)'s causes the maxima in absorption intensity (500, 380 nm) to be significantly split from the maximum in CD intensity (460 nm). Alternatively, for met-N_3^- T2D laccase, like met apo-N_3^- hemocyanin,[36] the coincidence of the N_3^- → Cu(II) CT absorption and CD maxima indicates no interaction between transition moments at the Cu(II) centers and thus N_3^- coordination to only one Cu(II).

In both met and half met T2D, N_3^- binds at room temperature with $K_{eq} \sim 150\ M^{-1}$. If N_3^- were bridging at these binuclear copper sites, the change in copper ion oxidation state would be expected to lead to significant changes in exogenous ligand affinity, as is observed in the hemocyanins and tyrosinase.[1] The similarity in the ligand field CD changes with coordination of N_3^- to met and half met T2D (Figure 9B) further suggests that at both protein sites only one copper is perturbed by azide.

A number of chemical and spectroscopic studies have generated spectral features which led to the concept of exogenous ligand bridging in the hemocyanins and tyrosinase. While one must be cautious in interpreting the lack of such features as evidence of nonbridging in T2D laccase, the strong correlation of features which are reproducibly observed[4] over a series of five arthropod and five mollusc hemocyanins, and fungal and mushroom tyrosinase,[4a,53] and which relate to a bridging geometry for small molecules, suggests that the marked differences in the results of parallel chemical and spectral studies of T2D laccase are associated with a nonbridging coordination mode for exogenous ligands.

From these results it appears that the inability of exogenous ligands to bridge the laccase T3 site is an intrinsic property of the binuclear copper unit in this T2D multicopper oxidase. This could result if exchangeable coordination positions (L_1 and L_2) on two Cu(II)'s are spatially separated such that bridging cannot occur (Chart I). A large bridging angle θ or the relative orientation (θ_1 and θ_2) of the exchangeable coordination positions could prevent exogenous ligand valence orbital overlap with the second Cu(II). In either case, two exogenous ligands could coordinate to the T3 site, unless binding of the first ligand sterically prevented binding of the second. Alternatively, there could be an exchangeable position on only one copper of the T3 site. The fact that for a wide range of ligands with different steric requirements and metal ion affinities only one ligand appears to bind to the met [Cu(II)Cu(II)], half met [Cu(II)Cu(I)], and deoxy [Cu(I)Cu(I)] forms suggests that exogenous ligands cannot bridge the binuclear copper site in T2D laccase because there is only one exchangeable position at the site.

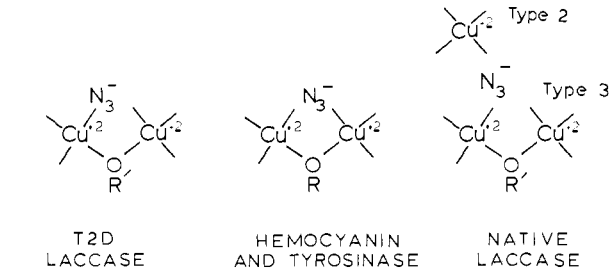
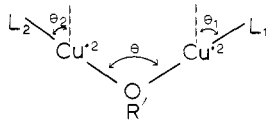


Figure 17. Spectroscopically effective models for N_3^- binding at the copper active sites in T2D laccase, the hemocyanins and tyrosinase, and native laccase. OR and OR′ represent the endogenous bridges in hemocyanin and laccase, respectively.

Chart I. Possible Orientations of Exogenous Ligand (L_1,L_2) Interactions at the Type 3 Site in T2D Laccase



This lack of exogenous ligand bridging in T2D laccase indicates that the T3 site is different from the coupled binuclear copper site in the hemocyanins and tyrosinase. This difference may correlate with the differences in chemical reactivity of these binuclear copper sites: reversible oxygen binding and two-electron[54] reduction in the hemocyanins and tyrosinase compared to no oxygen reactivity and one-electron reduction (to half met) in T2D laccase. Both the relative thermodynamic difficulty of the one-electron reduction of dioxygen to superoxide and the lack of exogenous ligand bridging which would stabilize the two-electron reduction could contribute to this difference in O_2 reactivity. The structural similarities for the coupled binuclear cupric sites in T2D laccase, the hemocyanins and tyrosinase, and native laccase, as well as the differences in their exogenous ligand binding mode for the competitive inhibitor N_3^-, are summarized in Figure 17.

In comparing T2D and native laccase, the T3 site in the native enzyme does, of course, react with dioxygen and functions as a two-electron acceptor.[14] The binding of anions and CO to deoxy T2D demonstrates that the lack of dioxygen reactivity in T2D is not due to an inaccessibility of the site to neutral or negatively charged small molecules. Further, the structural nature of the coupled binuclear cupric sites appears to be similar in T2D and native laccase. In both laccase forms, (1) the Cu(II) XAS edge spectra and ligand field features indicate two tetragonal Cu(II)'s at the site which are EPR nondetectable due to exchange coupling through an endogenous bridge; (2) this bridge is capable of being protonated and displaced by N_3^- at low pH and displays an intrinsic $pK_a > 6.7$;[44] and (3) there is a 330-nm dimer band in the absorption spectrum associated with this coupling. We note that there are limited quantitative differences in several spectral fea-

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tures. In met T2D, the zero-field splitting of the T3-N_3^- EPR signal is ~730 G, where it is only ~250 G in native laccase,[44] the intensity of the T2D 330-nm dimer band is ~2000 M^{-1} cm^{-1}, an ~44% decrease relative to the corresponding feature in native laccase,[21] and from EXAFS studies,[48] the Cu^{II}–Cu^{II} distance is suggested to be longer in T2D laccase.

Thus, the T2 copper appears to be required for the O_2 reactivity of native laccase. Other reports of T2D laccase forms state that T2D does react with dioxygen[23,24,55] and is a two-electron acceptor.[24,55] However, as demonstrated in the following article, it must be emphasized that only the T2D chemistry herein reported is documented with respect to copper ion oxidation state composition with use of the direct X-ray absorption edge spectroscopic probe. Absorption changes in the 330-nm region or redox titration studies for electron equivalents are very indirect methods for determining oxidation states of copper in proteins. For one series of T2D preparations (the T2D reported here and T2D derivatives prepared as in Morpurgo et al.[15-17] and Spiro et al.[48]) we found[56] using Cu K edges that what was originally assigned[16] as an oxidized T3 site is in fact quantitatively reduced. In the other most well-studied preparation,[24,55] the isolated T2D sample exhibits 330-nm absorption; however, from Cu K edge studies,[57] this T3 site contains a large 8984-eV peak that quantitates to ~70% reduced copper (the T1 copper is oxidized from EPR).

Exogenous ligands do not appear to bridge the T3 site in T2D laccase. From the above spectral correlations, the coupled binuclear copper sites are structurally similar in T2D and native laccase and it seems reasonable to extend this lack of exogenous ligand bridging to the T3 site in native laccase. A direct investigation of exogenous ligand interaction in native laccase is complicated by the presence of the T2 Cu(II). One key probe of exogenous ligand binding are the L → Cu(II) CT transitions associated with N_3^-. However, in absorption and low-temperature magnetic circular dichroism studies,[44] the dominant CT spectral features are associated with N_3^- bridging from the T3 to the T2 Cu(II) center, forming a trinuclear copper cluster (Figure 17). The inability of N_3^- to bridge the T3 site in native laccase would be consistent with N_3^- bridging between the T2 and T3 Cu(II) centers. This spectroscopically effective model of exogenous ligand binding to only one copper at the T3 site differs from the general assumption[12,58] of there being a μ-1,2 peroxide bridge at the T3 site. This assumption was at least partially based on the assignment[58] of the increase in 330 nm absorption intensity when native laccase reacts with peroxide as O_2^{2-} → Cu(II) CT, in analogy to oxyhemocyanin and oxytyrosinase. However, we have now determined (see following paper) that this 330-nm change is not related to exogenous peroxide binding but results from oxidation of a fraction (~25%) of reduced T3 sites present in the native enzyme. Further studies are now directed toward determining the importance of both this lack of bridging at the binuclear T3 copper and alternative three copper cluster coordination mode in the dioxygen reactivity of native laccase.

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Registry No. Cu, 7440-50-8; N_3, 14343-69-2; SCN^-, 302-04-5; CH_3COOH, 64-19-7; NO_2^-, 14797-65-0; F^-, 16984-48-8; I^-, 20461-54-5; Br^-, 24959-67-9; Cl^-, 16887-00-6; laccase, 80498-15-3.

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