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ANION BINDING TO OXIDIZED TYPE 2 DEPLETED AND NATIVE LACCASE: A SPECTROSCOPICALLY EFFECTIVE MODEL FOR EXOGENOUS LIGAND BINDING TO THE TYPE 3 - TYPE 2 ACTIVE SITE

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SUMMARY: Chemical and spectroscopic comparison of anion binding to the oxidized type 3 sites in type 2 depleted and native Rhus vernicifera laccase demonstrates that the type 3 - type 2 active site of laccase has an especially high affinity for exogenous ligands. N_3^-, O_2^{2-} and F^- binding and competition, and resonance Raman studies, indicate that this high affinity is not due to strong, equatorial type 2 ligation, but rather to a structurally non-specific role of the type 2 copper in stabilizing anion binding at the type 3 binuclear cupric site. These studies, combined with previous results on half met type 2 depleted laccase, generate a spectroscopically effective model for peroxide binding to the type 3 - type 2 active site.

INTRODUCTION

The active site of Rhus vernicifera laccase [1,2] contains one type 1 (blue, T1), one type 2 (normal, T2), and one type 3 (coupled binuclear, T3) copper site which couple four one-electron oxidations of various substrates to the four-electron reduction of dioxygen to water [3]. These copper sites must be in reasonably close proximity for this intramolecular electron transfer to occur. Evidence of a T1-T3 interaction has been demonstrated [4] in the T2 copper depleted (T2D) laccase derivative [5] (T1 oxidized, T3 reduced) wherein peroxide oxidation of the T3 cuprous ions (yielding oxidized T2D) [4] alters the resonance Raman and EPR parameters of the T1 copper site. A number of experiments have indicated that the T2 copper may be mechanistically involved in O_2 interaction at the T3 site [3,6-7]. We have recently shown that structurally, through a comparison of native and oxidized T2D laccase, the presence

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of the T2 copper strongly enhances ligand binding to the T3 site [4]. Here we further probe the nature of this T2-T3 interaction in binding exogenous ligands and propose a spectroscopically effective model for peroxide binding to the T2-T3 active site.

MATERIALS AND METHODS

Laccase was purified from the acetone powder of the Japanese lacquer tree (Rhus vernicifera) according to published procedures [8,9]. The T2 copper was selectively removed by the modified [10] method of Graziani [5], and the oxidized T2D derivative was prepared as reported in reference 4. All ligand binding studies were at equilibrium (4°C) in 0.1 M potassium phosphate buffer. Peroxide solutions were standardized by permanganate titration. Absorption spectra were recorded at room temperature on Cary 14 and Cary 17 spectrophotometers. Either a Varian E-9 or Bruker ER 220D-SRC EPR spectrometer was used on frozen solution samples at 77 K. Resonance Raman spectra were recorded using a UV CR18 Ar^+ laser, a Spex double monochromator, RCA C31034A photomultiplier, and Spex photon counter. CD spectra were taken on a Jasco J-500C spectropolarimeter.

RESULTS AND DISCUSSION

Azide binding to oxidized T2D laccase at pH 6.0 is characterized by a binding constant $K \sim 200\ M^{-1}$ and $N_3^- \rightarrow Cu(II)$ charge transfer (CT) at 450 nm ($\Delta\varepsilon = 500\ M^{-1}\ cm^{-1}$); a corresponding negative CD peak is also observed at 450 nm (Figure 1). The indistinguishable energies of the absorption and CD peaks directly contrast N_3^- binding to the met derivative of hemocyanin (a spectroscopic analogue of oxidized T2D) [11]; in the latter, three $N_3^- \rightarrow Cu(II)$ CT peaks are apparent from the splitting of the absorption and CD maxima and require N_3^- to bridge the coupled binuclear coppers [12,13]. The single CT peak in oxidized T2D is consistent, however, with the results of exogenous ligand binding studies on half met T2D laccase [14], in that these studies support a non-bridging azide coordination to one of the antiferromagnetically coupled binuclear cupric centers.

At pH 6.0, N_3^- binds to native laccase producing absorption bands at 410 nm and 500 nm ($K \sim 60{,}000\ M^{-1}$ at 500 nm, $\Delta\varepsilon_{410} = 630\ M^{-1}\ cm^{-1}$, $\Delta\varepsilon_{500} = 500\ M^{-1}\ cm^{-1}$) with no perturbation of the native EPR spectrum. These data are consistent with earlier reports of N_3^- binding at pH 4.5 [15] and suggest that the high affinity N_3^- is binding to the EPR non-detectable T3 cupric site. While $K \sim 200\ M^{-1}$ for N_3^- binding to oxidized T2D is similar to that found for N_3^- binding to met mollusc hemocyanin [12] and aqueous tetragonal cupric complexes

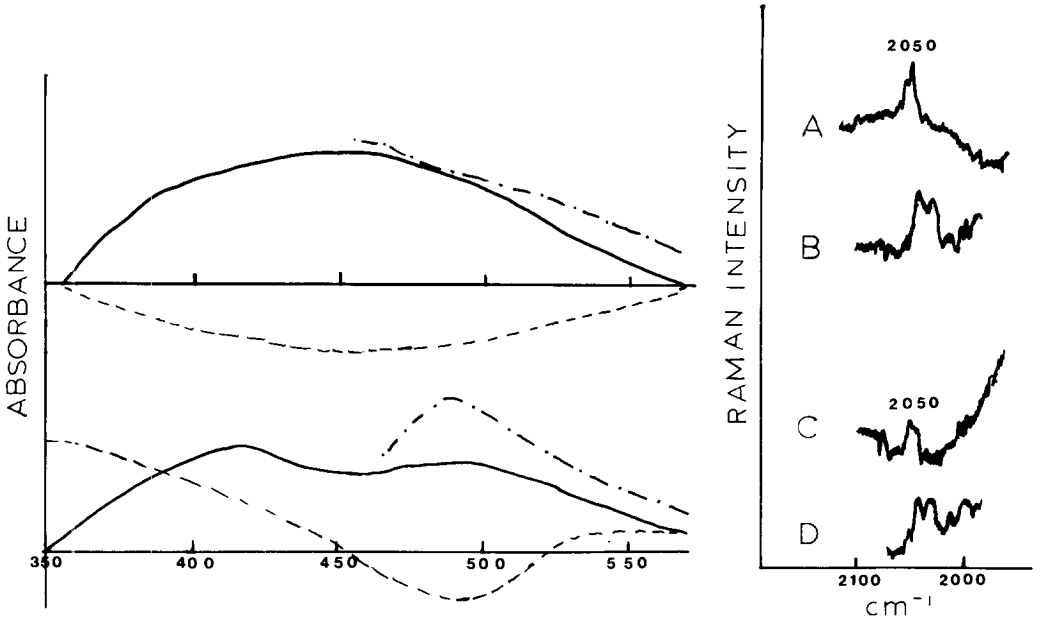

Figure 1. Left: Room temperature electronic absorption (———), CD spectra (– – –), and resonance Raman enhancement profiles (•—•—•) for oxidized T2D (top) and native (bottom) laccase.

Right: Resonance Raman isotope studies of the intra-azide stretching region (A) oxidized T2D + $^{14}N_3^-$, 476.5 nm excitation; (B) oxidized T2D + ($^{15}N^{14}N^{14}N$)$^-$, 488.0 excitation; (C) native laccase + $^{14}N_3^-$, 476.5 nm excitation, and (D) native laccase + ($^{15}N^{14}N^{14}N$)$^-$, 488.0 excitation. The isotopic splitting is 13 cm^{-1}. All studies at room temperature in a spinning cell with 100 mW (measured at the sample) laser irradiation; protein concentrations were ~ 0.3 mM.

[16], the K observed in native laccase is remarkably high and indicates that the presence of the T2 copper greatly stabilizes N_3^- at the T3 copper.

Resonance Raman excitation into the N_3^- absorption bands in both native and oxidized T2D laccase shows an intra-azide stretch at 2050 cm^{-1} (maximally enhanced at ~ 500 nm and 450 nm, respectively) and confirms the assignment of these features as N_3^- → Cu(II) CT. Binding of the mixed isotope, $^{15}N^{14}N^{14}N$, to oxidized T2D splits the intra-azide stretch into two peaks (Figure 1), demonstrating asymmetric coordination of N_3^- to the T3 site; no splitting of this peak would be observed if N_3^- bridged the two coppers in a 1,3 fashion. Moreover, parallel isotope studies on the native enzyme exhibit a splitting of the intra-azide stretch almost identical to that observed in oxidized T2D laccase. We conclude that the interaction of N_3^- with the T2 copper is not large enough to alter the inequivalent ^{15}N–^{14}N and ^{14}N–^{14}N distances of N_3^- bound to the T3

copper in oxidized T2D laccase. Thus, while the presence of the T2 copper greatly enhances N_3^- affinity at the T3 site, this added stability is not due to a strong, equatorial type bond to the T2 copper. The increased affinity could, however, result from increased positive charge due to the presence of the T2 Cu(II) in a low dielectric pocket; alternatively, the T2 Cu(II) may have a more structural role, aiding a protein residue in stabilization of the bound N_3^-, perhaps through hydrogen bonding. A structurally non-specific role of the T2 Cu(II) in high affinity exogenous ligand binding to this T3-T2 site is further indicated by unusually tight binding of F^- at the T2 Cu(II) and F^-/N_3^- and N_3^-/O_2^{2-} ligand competition (vide infra).

At pH 6.0, two fluorides bind with high affinity ($K_1 \sim 40{,}000\ M^{-1}$, $K_2 \sim 5000\ M^{-1}$ from 320 nm perturbations in the absorption spectrum) and coordinate equatorially to the T2 Cu(II) in Rhus laccase, as evidenced by triplet super-hyperfine splitting (^{19}F: $I_N = 1/2$) of the T2 EPR signal (Figure 2). At pH 7.0 and the same $[F^-]$, only doublets are seen in the EPR (Figure 2E), indicating that a single F^- binds to the T2 Cu(II) ($K \sim 2000\ M^{-1}$ at 320 nm). Thus, at the higher pH, an OH^- [17] or deprotonated protein residue appears to bind tightly to one of the F^- binding sites at the T2 copper, preventing the second F^- from competing effectively for that site.

Further evidence of T2-T3 interaction originates from N_3^-/F^- competitive binding experiments on native laccase. At pH 6.0, with two F^-'s bound to the T2 copper, the N_3^- binding constant decreases from $\sim 60{,}000\ M^{-1}$ to $\sim 10{,}000\ M^{-1}$. Also, at pH 7.0 in the presence of N_3^-, the binding constant for F^- decreases so that the EPR doublet normally seen at these $[F^-]$'s is barely discernible (Figure 2F). Thus, high affinity N_3^- bound at the T3 site reduces the affinity for F^- at the T2 copper.

Peroxide addition to native laccase at pH 6.0 ($K \sim 10^4\ M^{-1}$) causes a limited increase in the absorption and CD spectra at ~ 330 nm ($\Delta\varepsilon \sim 800\ M^{-1}\ cm^{-1}$, $\Delta(\Delta\varepsilon_{L-R}) < |0.7|\ M^{-1}\ cm^{-1}$) and small changes in the optical spectra at 614 nm with no perturbation of the EPR spectrum. A much larger binding constant ($K > 10^8\ M^{-1}$) [18] and CD feature ($\Delta(\Delta\varepsilon_{L-R})_{330} = -3.15\ M^{-1}\ cm^{-1}$) are

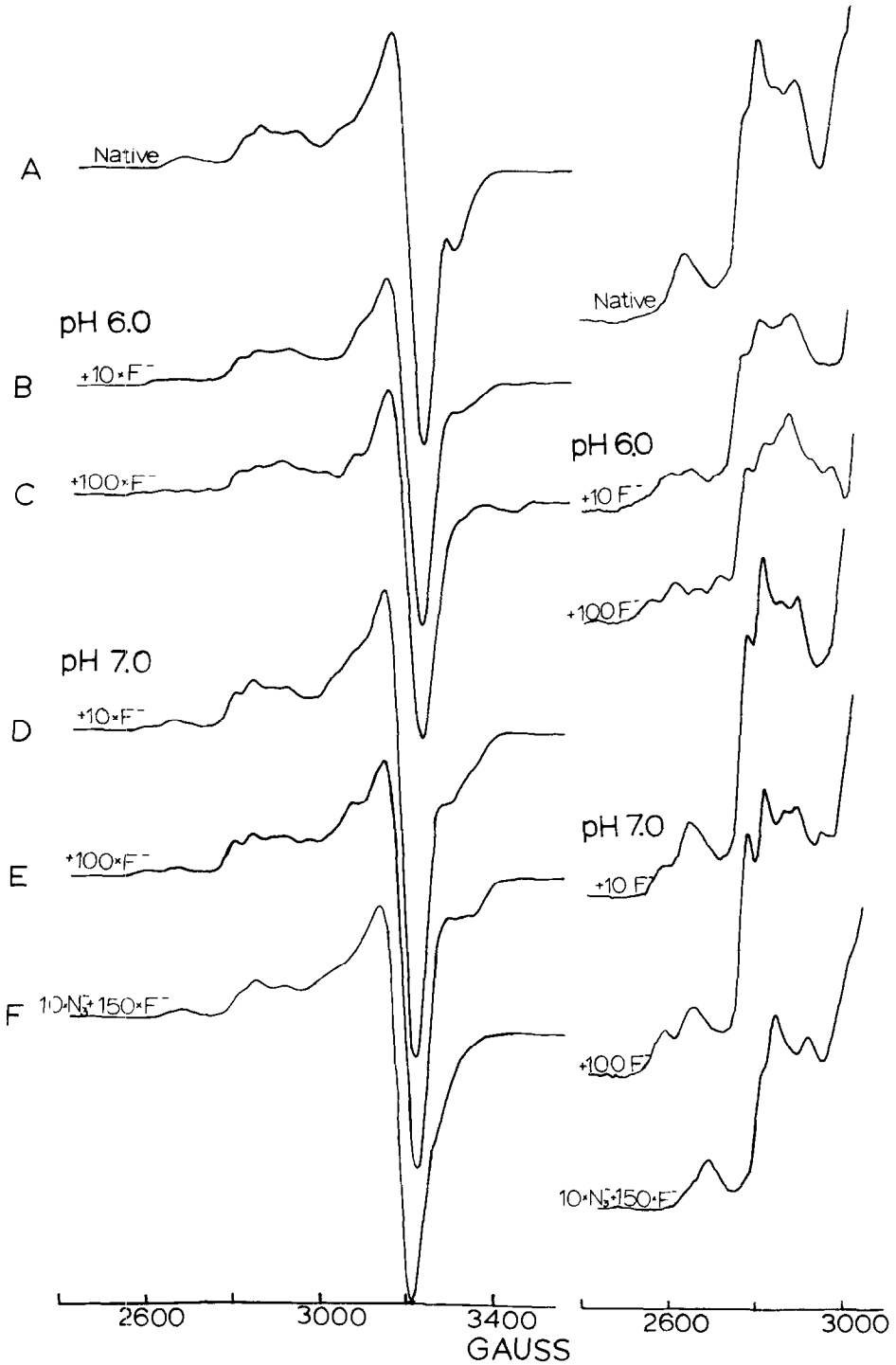

Figure 2. 77K EPR spectra of 0.15 mm Rhus laccase$_2$ at equilibrium in the presence of varying concentrations of F^-; the protein equivalents of F^- added, at the specified pH, are indicated for each spectrum. In (F), 10 equivalents of N_3^- are also present. Spectra to the right were recorded at ~ 5x higher gain. Microwave frequency = 9.08 GHz; microwave power = 10 mW.

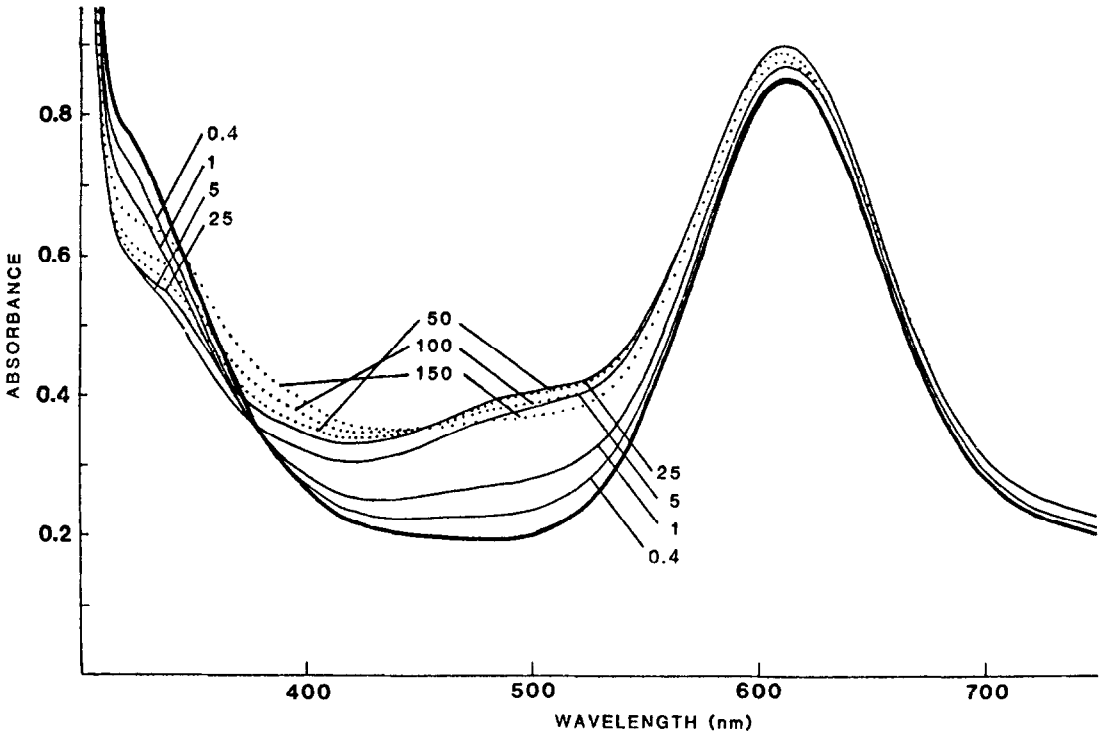

Figure 3. Room temperature absorption spectra of ~ .17 mM native laccase, pH 6.0, pre-incubated with 30 protein equivalents H_2O_2 (bold line) and titrated with N_3^-, at equilibrium; the protein equivalents of N_3^- added are indicated for each spectrum.

reported for the well-characterized peroxylaccase [19] at pH 7.0. In oxidized T2D laccase at pH 6.0, H_2O_2 exhibits a much lower binding constant ($K < 100$ M^{-1}) [20], again consistent with a significant role for the T2 copper in binding the exogenous peroxide to the T3 site [21].

When peroxylaccase, pH 6.0, is titrated with N_3^-, an absorption band grows in at ~ 500 nm ($\Delta\varepsilon \sim 1300$ M^{-1} cm^{-1}) with an accompanying decrease at 330 nm (Figure 3). The new 500 nm feature, shown [22] by resonance Raman spectroscopy to be N_3^- → Cu(II) CT, differs from the N_3^- → Cu(II) CT bands observed in native laccase at similar [N_3^-]'s; in addition, significant change in the T2 Cu(II) EPR signal is observed, in contrast to azide titration of native laccase. Thus, both N_3^- and O_2^{2-} can bind simultaneously with high affinity. Moreover, the altered CT features indicate a different mode of binding which, as judged by EPR, probably directly involves the T2 copper, in contrast to the structurally non-specific T2 requirement for high affinity N_3^- binding to

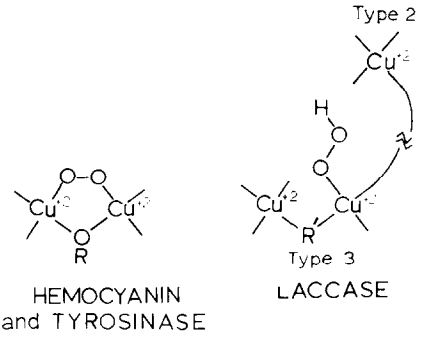

Figure 4. Spectroscopically effective T2-T3 active site of Rhus laccase and its interaction with the exogenous ligand peroxide. The oxyhemocyanin and oxytyrosinase active site is also shown for comparison. OR and R' denote the endogenous protein bridge in the hemocyanins and laccase, respectively.

native laccase. As $[N_3^-]$ is increased, the 500 nm absorption decreases while that at 330 nm increases and red-shifts; further investigation of this new binding geometry is underway.

The results presented in the preceding and present communications together provide a preliminary picture of the spectroscopically effective T3-T2 active site and its interaction with dioxygen (Figure 4). We first note that the optical features associated with H_2O_2 binding to native laccase are relatively small in comparison to the intense absorption changes ($\Delta\varepsilon_{345} \sim 20,000\ M^{-1}\ cm^{-1}$, $\Delta(\Delta\varepsilon_{L-R,350}) \sim -30\ M^{-1}\ cm^{-1}$) on binding H_2O_2 to met hemocyanin (oxyhemocyanin) [11,23]. Hence, while the μ-1,2 peroxo bridging geometry [23] characterizes bound peroxide in oxyhemocyanin, the contrasting spectral features in laccase suggest a different mode of peroxide coordination to the multicopper oxidase binuclear T3 site. The apparent inability of exogenous ligands to bridge the T3 copper site further suggests peroxide binds to a single copper of the T3 site in native laccase. While the μ-1,2 peroxo bridging geometry of oxyhemocyanin and oxytyrosinase allows the reversible binding of O_2 in these proteins, the different mode of peroxide binding (likely as hydroperoxide) in laccase may be important in the irreversible binding of this two-electron reduced dioxygen intermediate during its reduction to water. However, the hydroperoxide does not bind strongly in this geometry without the presence of the T2 copper, which apparently stabilizes the T3-hydroperoxide complex (perhaps through added positive charge in a low

dielectric pocket) for its further reduction to water. The nature of this added stability provided by the T2 copper requires further study.

ACKNOWLEDGMENTS

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20. While H_2O_2 is not a high affinity ligand of oxidized T2D, only an upper estimate of K is possible (based on lack of spectral perturbation at 30 protein equivalents) as higher $[H_2O_2]$'s damage the protein.
21. The $\Delta\varepsilon_{330}$ nm absorption feature may represent weak $O_2^{2-} \rightarrow$ Cu(II) CT or perturbation of the already existing 330 nm band; this feature, associated primarily with the T3 copper, is very sensitive to anion binding (e.g., F^-, N_3^-, SCN^-) in native laccase, and often distorts independent of $L \rightarrow$ Cu(II) CT. The N_3^-/O_2^{2-} competition experiments (vide infra) indicate direct evidence, however, that H_2O_2 is acting as a ligand to the T3 copper and not simply reoxidizing a small fraction of binuclear cuprous sites which may be present.
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