# Peroxide and Redox Titrations of Type 2 Copper Depleted Laccase

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*The chemistry of Type 2 copper depleted T2D Rhus laccase has been investigated with regard to the binding of peroxide, and the ability of the enzyme to undergo reduction and reoxidation. Although the peroxide affinity is diminished in the T2D enzyme ($10^4$ $M^{-1}$) relative to the holo-enzyme ($\geq 10^8$ $M^{-1}$) the actual mode of binding as a Type 3 μ-peroxo complex remains, as indicated by absorption and CD spectral measurements. Anaerobic reductive and reoxidative titrations with hydroquinone and hydrogen peroxide respectively revealed that the Type 3 copper pairwise interaction is disrupted during reduction but can be restored on reoxidation. The concept of separate Type 2 and Type 3 copper redox centers is suggested to be inadequate in view of the loss of functional integrity by the Type 3 site on removal of Type 2 copper.*

*(Abbreviations used in the Text: U.V.: ultraviolet, CD: circular dichroism, T2D laccase: Rhus laccase which has been depleted of Type 2 copper, $H_2Q$: hydroquinone, EPR: electron paramagnetic resonance.).*

The controlled catalytic utilization of ambient dioxygen in cool, dilute, neutral aqueous solutions ranks, along with nitrogen fixation, hydrogenation and photosynthesis, as one of the spectacular achievements of evolutionary invention. Among the biochemical constructs-enzymes, which manage and direct the powerful reactivity of dioxygen, considerable attention has been focused on the oxidases. In particular, cytochrome oxidase, a mammalian 2-iron/2-copper enzyme, and the 'blue' copper oxidases derived from both plant and animal sources have been extensively studied [1, 2].

Among the 'blue' oxidases *Rhus* laccase from the Japanese lacquer tree recommended itself to experiment since it contains but four copper ions; that is, the minimal functional unit for the four electron reduction of dioxygen. In addition the moderate oxidizing strength of the copper ions (350–450 mV) made *Rhus* laccase more tractable than the more oxidizing fungal protein varieties.

The four copper sites within the laccases include a single Type 1 site, with an intense absorption near 600 nm (hence the description 'blue'), and an axial epr spectrum showing an unusually narrow parallel hyperfine coupling constant. Additionally a single Type 2 copper is present which is essentially optically transparent but exhibiting a rhombic epr spectrum indicating a low symmetry ligation site. Finally the single Type 3 copper site consists of a pair of strongly antiferromagnetically coupled copper(II) ions [3, 4] associated with a strong electronic absorption band at 330 nm.

It is at the Type 3 site that the binding and initial reduction of dioxygen occurs [5]. The high affinity constant ($\geq 10^8$ $M^{-1}$) shown for the binding of hydrogen peroxide to the oxidized Type 3 site [6], as well as the spectroscopic similarities exhibited by the bound dioxygen 2-electron reduction intermediate and the Type 3 site–peroxide complex [5–8], indicated the high likelihood of similar structural forms. Other, further reduced oxygen (radical) species have also been reported and the Type 3 site copper ions suggested as the site of residence [9, 10].

The preparation of a derivative of *Rhus* laccase depleted of Type 2 copper was reported some time ago [11, 12] and has been subjected to considerable study [12–17]. The absence of Type 2 copper would allow the focus of experiments on the behavior of the Type 3 copper site, primarily as the dioxygen reduction center, manifested in the 'blue' oxidases.

In undertaking this work, our initial experiments were concerned with defining the resting spectroscopy and oxidation state, respectively of the Type 1 and Type 3 copper ions in T2D laccase [17]. Using a modification of the Lowry method [18] of protein quantitation, the extinction of the Type 1 copper at 615 nm was found to be $5620 \pm 570$ $M^{-1}$ $cm^{-1}$. This value is experimentally indistinguishable from that of the native enzyme. Anaerobic reductive titrations with $Ru(NH_3)_6^{2+}$ and Cr(II) aquo ions revealed that the Type 3 site copper ions are in the Cu(II) state in the resting T2D enzyme despite the loss of their diagnostic band at 330 nm on Type 2 copper removal. Variations in the intensity of the 280 nm band and the reversible formation of extra epr spectral features, observed on reduction-reoxidation of T2D laccase, indicated the unreliability of the former band for

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protein quantitation and led to the suggestion [17] that the Type 3 site in laccase has become labile upon loss of Type 2 copper.

Here we report the results of further investigations into the Type 3 site of T2D laccase. These include CD spectroscopically monitored titrations with hydrogen peroxide. The redox chemistry of copper in T2D laccase was explored in more detail by means of anaerobic equilibrium titration experiments using hydroquinone and hydrogen peroxide as reductant and oxidant respectively. The results showed that the Type 3 site in laccase is seriously perturbed on loss of Type 2 copper. This points up certain differences relative to those forms of the Type 3 copper site as found in hemocyanin (dioxygen transport) and tyrosinase (copper oxygenase).

## Experimental

*Materials and Methods*

*Rhus* laccase was prepared according to reported methods [19]. Depletion of Type 2 copper was carried out using a modification [20] of the method of Graziani *et al.* [11]. T2D protein samples were characterized by the Lowry method as well as Cu analysis, and UV-visible and epr spectra [17, 20–22].

Anaerobic procedures and techniques have been described [17, 20, 21]. Reagents and solutions were prepared and characterized using the methods published previously [17, 20].

Unless otherwise noted, reactions were thermostatted at 20 ± 0.5 °C. Electronic spectra were taken on a Cary 118 spectrophotometer. EPR spectra were recorded near liquid nitrogen temperature at about 9.05 GHz on a Varian E-3-spectrometer.

## Results

*Reaction with $H_2O_2$*

On addition to T2D laccase of a 70-fold excess of $H_2O_2$ spectral changes at 615 nm ($\Delta\epsilon = -550\ M^{-1}\ cm^{-1}$) and 330 nm ($\Delta\epsilon = 660\ M^{-1}\ cm^{-1}$) were observed (Fig. 1 and lower inset). After 2 hr incubation at 0 °C, the 615 nm extinction declined to a value of 4800 $M^{-1}\ cm^{-1}$ while the induced 330 nm band retained its initial intensity. In order to further characterize the source of the new 330 nm band in T2D laccase, two difference electronic spectra (native laccase minus T2D laccase and peroxy–native laccase minus peroxy–T2D laccase) were constructed from the data (Fig. 1, upper inset). These spectra are seen to be very similar in both structure and intensity over most of the near-uv region.

The circular dichroic spectrum of oxidized T2D laccase in the near UV region (Fig. 2, a) is similar to that of the native enzyme [8], in that negative (447 nm) and positive (375 nm) bands are present at nearly the same position and intensity as observed previously (cf. also ref. 12). The shorter wavelength features, though not as well resolved, are likewise similar. Upon addition of $H_2O_2$ [27], a prominent band appears at 330 nm, while that at 442 nm is unaffected (Fig. 2, b–d). The intensity of the 330 nm band increases with peroxide concentration, reaching a maximum value of 0.75 $M^{-1}\ cm^{-1}$ in the presence of a hundred fold excess of $H_2O_2$. This band bears close resemblance to the one produced when 1 mole equivalent of $H_2O_2$ is added to oxidized native laccase, and shown to be a peroxy–laccase complex [6, 7].

From several such titrations, done on two independently prepared T2D laccase samples, a Scatchard plot was constructed (Fig. 2, inset). This yielded a stability constant of 10 200 $M^{-1}$ for a 1:1 complex between the reactants. In comparison, the stability constant for the native laccase-peroxide complex was estimated as $\geqslant 10^8\ M^{-1}$ [6]. Hence, the affinity of laccase towards peroxide decreases drastically when the Type 2 copper is missing.

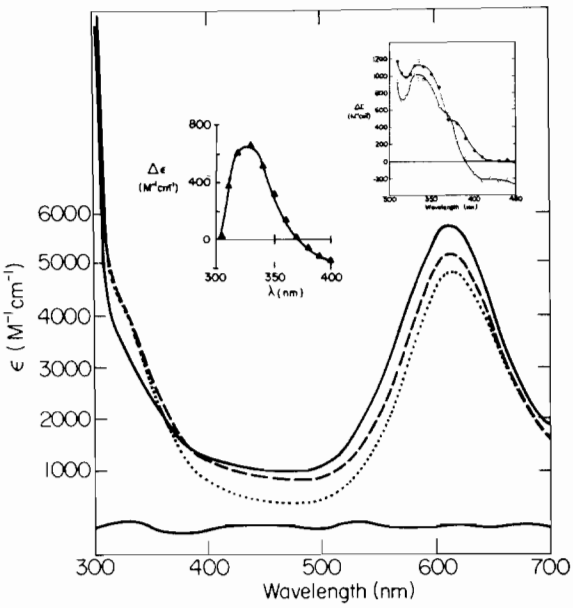


Fig. 1. (——): T2D laccase, 9.2 $\mu M$ in 0.05 $M$ NaAc pH 5.2 at 20 ± 0.5 °C. (- - -): The same, 20 min after treatment with 70× excess of $H_2O_2$. (· · ·): The same after a further 2 hr incubation at 0 °C. Lower inset: (Peroxy T2D minus T2D) laccase near-UV difference band. T2D protein samples treated as above showed no sign of oxidative damage as indicated by invariant epr and uv spectral line shapes. Evidence for oxidative damage was found in protein samples incubated for prolonged periods (1–2 hr) at 22 °C however. Upper inset: Near-uv difference spectra. ●: (Peroxy–laccase minus peroxy–T2D laccase); ○: (Native laccase minus T2D laccase). Peroxy-laccase was prepared by adding a 20× excess of $H_2O_2$ to 13 $\mu M$ laccase in 0.05 $M$ NaAc pH 5.2 at 20 ± 0.5 °C. The spectrum was recorded after 20 min.

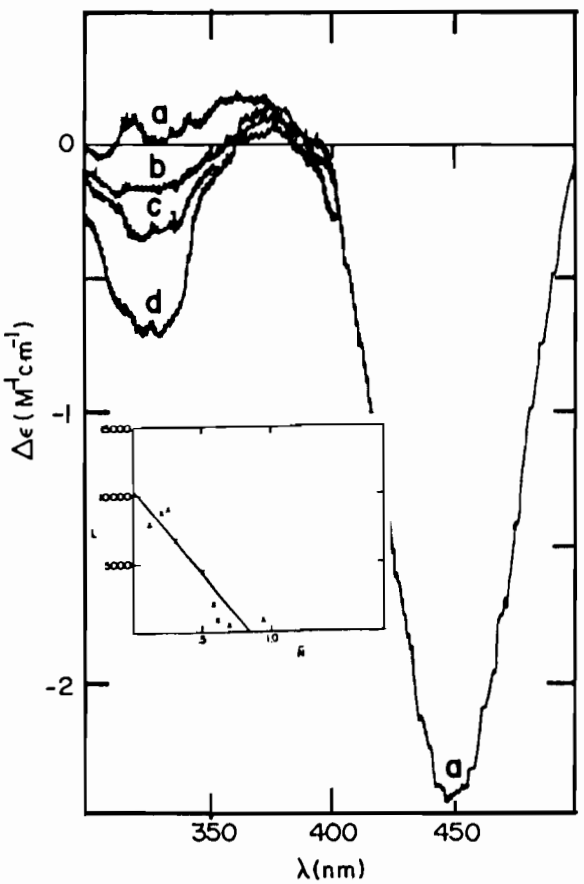


Fig. 2. CD spectra of T2D laccase (23 $\mu M$) titrated with hydrogen peroxide. a) no peroxide; b) 23 $\mu M$; c) 115 $\mu M$; and d) 1150 $\mu M$ of $H_2O_2$ added to solution a. CD spectra were measured in 1 cm cuvettes on a Roussel Jouan Dichrograph III. Data were collected by means of a Tektronix minicomputer averaging 200 measurements per nm. Inset: Scatchard plot derived from titrations of two independent preparations of T2D laccase with hydrogen peroxide. L is the concentration of free ligand ($H_2O_2$), and $\bar{N}$ is the fraction of complex divided by total enzyme concentration. $\bar{N}$ is determined at $(\Delta\epsilon_O - \Delta\epsilon_X)/\Delta\epsilon_O - \Delta\epsilon_N)$ where $\Delta\epsilon_O$ is the intensity of the 330 nm band for T2D laccase without peroxide, $\Delta\epsilon_N$ is the intensity of the same band after addition of a 100 fold excess of $H_2O_2$, and $\Delta\epsilon_X$ denotes the intensities at the various intermediate peroxide concentrations. Protein concentrations examined ranged from 23 to 70 $\mu M$.

Taken together however, the above absorption and CD spectral data clearly indicate that T2D laccase forms a Type 3 site peroxide complex having a strong structural homology with that of the native enzyme.

*Reductive Titrations with Hydroquinone*

When aliquots of hydroquinone solution were serially added to anaerobic solutions of T2D laccase a very slow decrease in the Type 1 A(615) was observed. Plots of the reduction kinetics with respect to log A(615) were linear over the entire range of reduction [21], indicating a first order dependence on T2D laccase. Reaction half-lives were in the range of 25 minutes, each reductive addition thus requiring approximately 4 hr to reach equilibrium.

In Fig. 3 is shown the progression of electronic spectra resulting from such a titration. The consecutive decline in A(615) during reduction of T2D laccase was not matched by a similar decline in the near UV spectrum. Rather, a small increase in absorbance intensity was noted, which was resolved after correction for benzoquinone absorbance [23] (Fig. 3, inset), as a small positive difference band, centered near 300 nm. Similar bands, ascribed to Cu(I) to ligand CT transitions, have been noted earlier in reduced single blue copper electron transport proteins [24].

As can be seen in Fig. 3, there are no intrinsic spectroscopic determinants indicating the extent of Type 3 copper reduction at each point in the titration. Therefore, calculation of the data points for Type 3 site percent reduction (Fig. 4) rested on the presumption that the hydroquinone unaccounted for by the observed reduction of the Type 1 site copper, reduced Type 3 site copper. This assumption was considered likely however since reductive titrations with both $Ru(NH_3)_6^{2+}$ and Cr(II) aq ions [17], as well as the above mentioned experiments with hydrogen peroxide together demonstrated that the Type 3 copper site is oxidized and redox active in resting T2D laccase. As will become clear below, the lack of a 330 nm band in T2D laccase has its roots in differences in Type 3 site conformation, rather than oxidation state, relative to native laccase.

The full lines through the titration data points in Fig. 4, as well as those of the Nernst plot (Fig. 4, inset) were calculated from a model in which the Type 1 site copper is in intramolecular redox equilibrium with a Type 3 site wherein the two copper ions are mutually independent and equipotential electron acceptors throughout the titration. Full thermodynamic equilibrium between the redox partners was included in the calculation and the close congruence of the lines with the data points to the correctness of the model. The suggestion of intermolecular redox equilibrium among the various T2D laccase reduction intermediates is reasonable since benzoquinone can serve as redox mediator during titration* [25, 26]. From the Nernst plot intercept, the Type 1 copper was found to have become 35 mV more oxidizing than the Type 3 site copper ions, and this finding was included in the above mentioned calculations. Thus the relative redox potentials of the Types 1 and 3 sites have inverted in T2D laccase compared to the holoenzyme [24, 25, 27, 28]. Morpurgo *et al.* [29] reported a similar trend although the experimental difference in the two site redox potentials was 14 mV (0.05 $M$ sodium acetate pH 5.3).

*At the experimental pH of 5.2, $E^\circ$ for the benzoquinone-hydroquinone couple is calculated to be 393 mV.

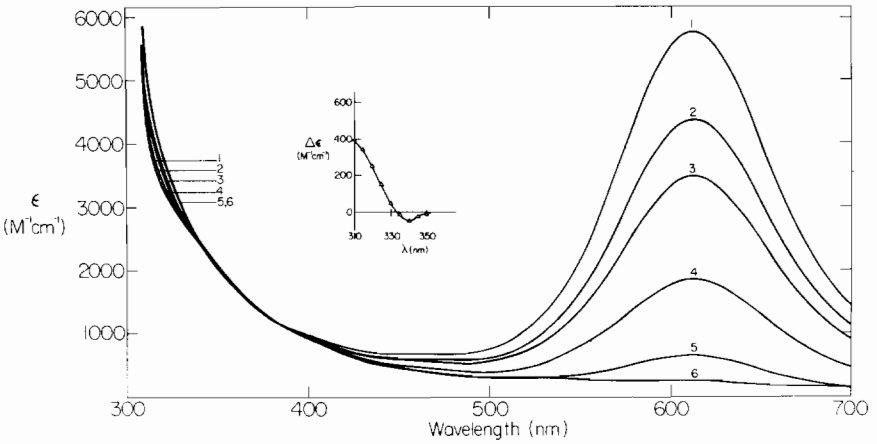


Fig. 3. (1) T2D laccase, 34.9 $\mu M$ in 0.05 $M$ NaAc pH 5.2, 20 ± 0.5 °C titrated with: (2) 0.35 equivalents, (3) 0.7 equivalents, (4) 1.4 equivalents, (5) 2.5 equivalents, (6) 3.2 equivalents of benzohydroquinone, 8.0 m$M$ in the above buffer. Inset: Near ultraviolet difference spectrum (6 minus 1, above) corrected for benzoquinone oxidation product [23].

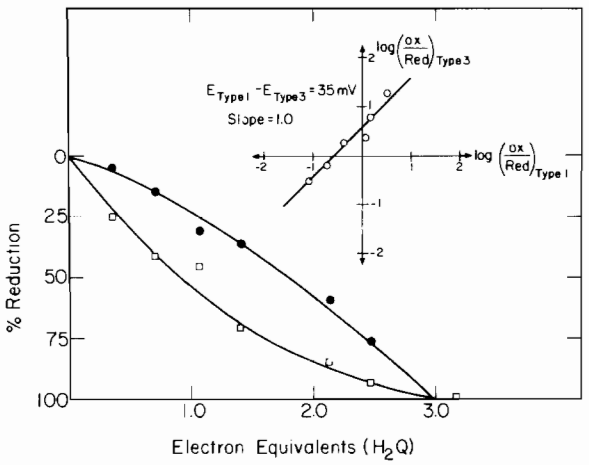


Fig. 4. Data points for two independent reductive titrations. The fraction of reduced Type 1 sites (□) was calculated as $(A_o615 - A615)/(A_o615 - A_f615)$ where $A_o615$, $A_f615$ are the initial and final absorbance values, respectively, at 615 nm. The fraction of reduced Type 3 sites (●) was calculated as (Equivalents $H_2Q$ added − Fraction Type 1 sites reduced)/2. The full lines were calculated for a three electron accepting system, wherein a single acceptor is in redox equilibrium with a set of two independent one electron acceptors. The latter two acceptors are each 35 mV more reducing than the former acceptor. Inset. Nernst plot of the titration data. The line is calculated from the model.

A prediction of the above model is that a population of half-reduced Type 3 sites be formed during reduction (Fig. 5, inset and legend). Since the Type 3 site Cu(II) ions were found to be 35 mV more reducing than the Type 1 copper ion, any emergent Type 3 site paramagnetism should dominate the epr spectrum of highly, but incompletely, reduced T2D laccase. In order to test this prediction, further reductive titration experiments were monitored by epr, in addition to absorption spectral methods (Figs. 5 and 6).

On anaerobic addition of 2.3 equivalents of hydroquinone to a solution of T2D laccase, the calculated distribution of reductant electrons within the Type 3 site (Fig. 5, inset) indicates that 29% of the Type 3 sites be half reduced, and constitute about 75% of the paramagentic Cu(II) present at equilibrium. In Figs. 5-2 and 6b-b″ are presented the visible electronic and frozen solution epr spectra respectively, of the partly reduced protein. The latter shows a new rhombic feature convolved with the Type 1 Cu(II) signal near 3240 G. Appearance of this signal on reduction reflects emergence of a previously undetectable Cu(II) ion; the Cu(II) of a half reduced Type 3 copper pair.

The partially reduced T2D laccase sample was found to have been significantly reoxidized upon freezing as indicated by the enhanced absorption intensity at 615 nm (Fig. 5-2′), observed upon thawing after acquisition of the epr spectrum. On standing, slow re-reduction of Type 1 Cu(II) commenced, tending to the prior position of equilibrium. Even after full reduction (Fig. 5-3) T2D laccase nevertheless displayed a rhombic epr spectrum (Fig. 6c) indicating the formation on freezing of an isolated Cu(II) within the reduced Type 3 site. On thawing, the absorption intensity at 615 nm was again found to have reached a level near that of Fig. 5-2′, providing an explanation for the observation of an epr signal from a 'fully reduced' copper enzyme.

In partly reduced T2D laccase, the observed reoxidation of Type 1 copper may possibly be ascribed to intramolecular electron redistribution induced by freezing. However, in the frozen solution of fully reduced protein, both Type 1 and Type 3 copper must clearly have been reoxidized by benzoquinone, given the rhombic epr spectrum. The reoxidation of reduced native laccase on freezing in the presence of other redox agents has been reported previous-

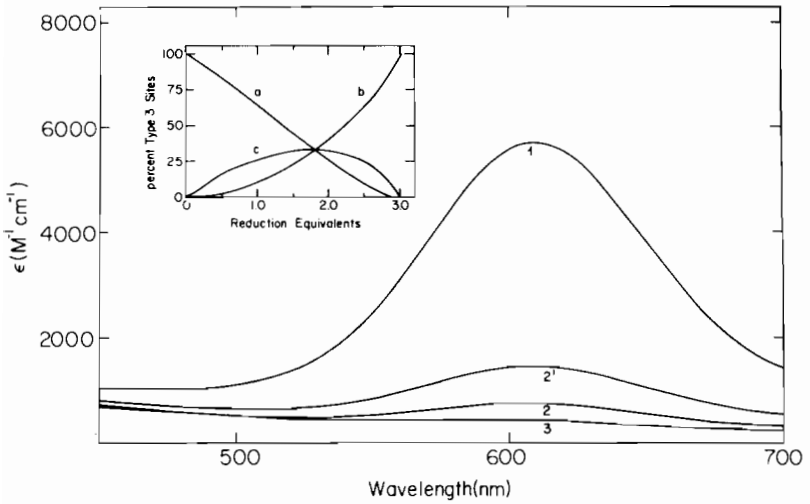


Fig. 5. Visible spectrum in 0.05 *M* sodium acetate, pH 5.2 of: 1. T2D *Rhus* laccase; 2. As in 1, after 2.16 equivalents of hydroquinone; 2′ As in 2 after freezing at about 100 K, then thawing; 3. As in 1, fully reduced with 4.3 equivalents of hydroquinone. Inset: Calculated equilibrium mole-fraction % of: a. fully oxidized; b. fully reduced; and c. half-reduced Type 3 sites within T2D laccase during reductive titration, based on the model described in the text, and in the Legend to Fig. 4.

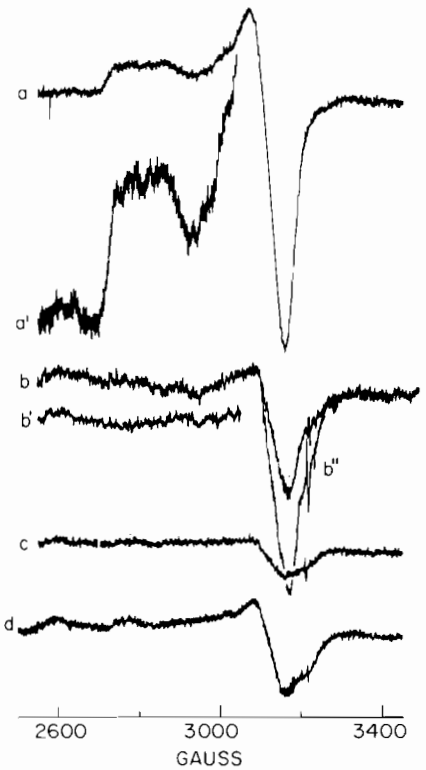


Fig. 6. EPR spectrum of: a. T2D laccase, 0.15 m*M* in 0.05 *M* sodium acetate pH 5.2, power 25 mW; lower trace, gain × 5; b. As in 1, after 2.2 equivalents of hydroquinone added; b′. cavity scan. b″. Power 50 mW, gain × 4; c. As in 1 after addition of 4.3 equivalents of hydroquinone. d. T2D laccase partially reoxidized with 1 mole-equivalent of $H_2O_2$ after full reduction with 4.8 equivalents of hydroquinone. The sharp line at g = 2 originates within the quartz EPR tube.

ly* [29, 30]. Interestingly, it was shown in the presence of NO [30] that the reoxidation phenomenon was not a function of freezing itself, but of lowered temperature. The suggested mechanism, involving electron transfer through Type 2 copper to a bound acceptor, is clearly untenable in the case of the T2D protein. However, the observed reoxidation of both Type 1 and Type 3 copper implies the possible utility of the latter in an alternative pathway. No obvious epr signal attributable to the presence of a trapped semiquinone radical [31] was observed.

After full reduction with 4.8 electron equivalents of hydroquinone, T2D laccase was partially reoxidized on addition of 1 mole-equivalent of $H_2O_2$. In the epr spectrum of the equilibrated product mixture (Fig. 6d), the Cu(II) $g_{//}$ hyperfine lines and assymetric lineshape near $g_{\perp}$ again indicate a paramagnetic, half reduced Type 3 site in the partly reoxidized protein**. Although the resolution of the experiment does not permit an unambiguous assignment, the epr spectrum in Fig. 6d does not appear to have the seven lines [32, 33] expected for a delocalized electron with a mixed valence half-oxidized dicopper site.

Thus the reductively emergent Cu(II) appears to be magnetically isolated within the half-reduced Type 3 site of T2D laccase. Furthermore, the rhombic epr

*From the data in ref. 29 a solution potential of 396 mV can be estimated for the laccase sample showing re-oxidation on freezing. In comparison, a potential of 388 mV can be calculated for the solution potential of the Fig. 5-3 sample prior to freezing.

**The hydroquinone was present in 61% equivalent excess, therefore the partly reoxidized T2D laccase molecules still contained 2.8 electrons on the average. From the final A(615), the Type 1 site was 9% reoxidized by this procedure.

spectrum implies that this copper occupies a low symmetry site. These data support the notion that half-reduction of the Type 3 site disrupts the pre-existing Cu(II) pairwise interaction. The outcome of such a process would be that the two Type 3 copper ions behave as two independent redox centers in the equilibrium sense. It can be seen that this behavior is precisely that predicted by the model described above.

*Reoxidation with $H_2O_2$*

T2D laccase which had been reduced anaerobically with hydroquinone was titrimetrically reoxidized with stoichiometric additions of $H_2O_2$. Each oxidative reaction was allowed to reach equilibrium, benzoquinone again serving as redox mediator. The reoxidation kinetics were followed only qualitatively but the relative rates of reoxidation of reduced protein by hydrogen peroxide were found to decrease in the order: 1 equivalent $\gg$ 3 equivalents, and 2 equivalents $\gg$ 4 equivalents. The final reoxidation step was sufficiently slow to require $>12$ hours anaerobic incubation at 4 °C to reach a stable state.

From the equilibrium values of A(615) (not shown) produced during these experiments it became clear that the reoxidation of reduced T2D laccase did not follow the reverse of the reduction curve. In all cases, the quantity of reoxidized Type 1 site copper showed an excess over the amount predicted by the reduction curve in Fig. 4 and the calculated average oxidation state of the enzyme. In addition to this, the induction of a new transition in the near ultraviolet spectrum was observed. The data obtained in two independent titrations are shown in Fig. 7 as (reoxidized minus oxidized) difference spectra. In these, the spectrum of the 'oxidized' T2D laccase sample is that of the original unreduced protein. Inspection of this figure reveals an isosbestic point near 380 nm, which is apparently maintained for more than 2/3 of the reoxidation reaction. Thus in at least the early stages of the reaction, reoxidation involves interconversion of only two species, neglecting any spectroscopically invisible intermediates.

The epr spectra of the starting oxidized and reoxidized T2D protein samples were essentially identical. However, for the 2/3 reoxidized protein intense new features were observed near 2950 G and 3400 G [20]. These transitions, possibly arising from within a dipole–and/or exchange coupled copper pair, indicate a weaker Cu(II) pairwise interaction within this oxidation intermediate, relative to the starting or ending protein.

The final difference spectrum in Fig. 7 derives from the fully reoxidized protein after oxidation with 4 equivalents (2 + 2) of hydrogen peroxide. It was expected that the final two equivalents of $H_2O_2$ would, by reaction with 2/3 reoxidized T2D laccase, produce the putative oxygen radical anion dioxygen

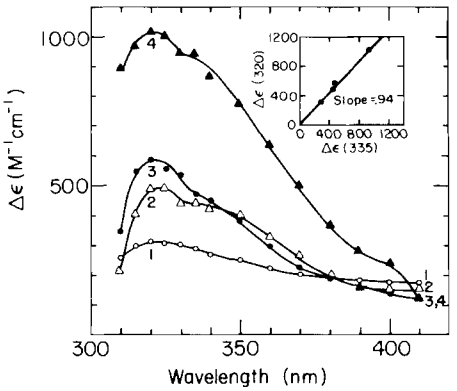


Fig. 7. Near UV (reoxidized minus oxidized) difference bands arising from reduced T2D laccase upon treatment with: 1. One equivalent, 2. Two equivalents, 3. Three equivalents, 4. Four equivalents of $H_2O_2$. These spectra are taken from data obtained in two independent titrations, producing spectra 1 and 3, and 2 and 4, respectively. Spectra 1, 2 and 4 represent equilibrium positions, spectrum 3 represents a reaction position prior to equilibrium in which the Type 1 site was only 40% of the intensity signifying full reoxidation of T2D laccase. Inset. Spectral data plotted as $\Delta\epsilon$(320 nm) *vs* $\Delta\epsilon$(335 nm). Spectra are corrected for absorbance by benzoquinone and residual reduced protein.

reduction intermediate [10, 27, 34]. However, that this did not occur is indicated by the linear plot of $\Delta\epsilon(320)$ *vs.* $\Delta\epsilon(335)$ (Fig. 7, inset). The points representing equilibrated samples, (1, 2 and 4) show a constant ratio of extinctions at these two maxima, indicating that the new near UV transition arises from a single chromophore throughout the entire course of the reoxidation process. Therefore to invoke a radical oxygen intermediate for this band would require that 2/3 reoxidized T2D laccase react with $H_2O_2$ faster than does the fully reduced T2D enzyme. If such was indeed the case, the new near UV absorption would always represent the terminal oxidation product, and the ratio of $\Delta\epsilon(320)$ to $\Delta\epsilon(335)$ would be a constant regardless of the apparent equilibrium position of the reoxidation reaction. Since this criterion is not met (*i.e.* the point and trace for addition 3, cf. Legend, Fig. 7), an oxygen radical can be discounted as an equilibrium (though not as a transitional) oxidation intermediate species.

Consideration of the chemistry involved leads to a similar conclusion, since redox mediation by benzoquinone, especially over the long reaction times involved, would result in relaxation of any electrochemically non-equilibrium state. An oxygen radical, bound to Type 3 Cu(II) would undoubtedly be a powerful oxidant. These considerations likewise exclude the possible formation of a T2D peroxylaccase molecule as the source of the new 320 nm transition. Alternatively, a non-equilibrium mixture of bound dioxygen reduction intermediates and partially reoxidized protein would not show an isosbestic

point or a linear extinction ratio over the course of the reoxidation reaction. The near UV transition can thus be assigned to the reoxidized Type 3 copper site.

The extra (*i.e.* fourth) oxidizing equivalent, or the oxygen radical intermediate if formed, was undoubtedly quenched by an endogenous reductant (*e.g.* a pendant saccharide). Consequently, reduced T2D laccase can be fully reoxidized by essentially the minimal equivalence stoichiometry of hydrogen peroxide. More importantly, the emergent near-UV band in reoxidized T2D laccase relative to freshly prepared (oxidized) T2D laccase implies that Type 3 site reoxidation by peroxide is accompanied by a conformational transition which is more than a reversal of the reductive decoupling of the Type 3 site copper ions.

## Conclusions

The experiments described herein reveal certain similarities as well as profound differences in the Type 3 site of T2D laccase relative to that of the native enzyme. With regard to the binding of hydrogen peroxide, both the electronic absorption and CD spectral results indicate that the mode of binding is similar in T2D and native laccase. Thus the Kuhn anisotropy factor for the near-UV transition $\gamma = |\epsilon_L - \epsilon_D|/\epsilon$ (where $\epsilon = \epsilon$(Peroxy T2D laccase) – $\epsilon$(T2D laccase) at 330 nm; the latter value being the same as in reduced T2D laccase) can be estimated from this data to be $\gamma \approx 1.1 \times 10^{-3}$ for peroxy T2D laccase. This is very close to that of the peroxide complex of native laccase [8] as well as those of hemocyanin [35, 36] and tyrosinase [37]. In these latter proteins, peroxide is known to bind as a binuclear bis-Cu(II)–$\mu$-peroxo complex [38] yielding a CD transition of position, sign and shape similar to the peroxylaccase, but more intense (as are the absorption bands). However, intensity in these transitions is undoubtedly sensitive to the microscopic ligation symmetries as well as to the local protein dielectric.

The lesser binding constant in T2D relative to native *Rhus* laccase ($10^4\ M^{-1}$ vs $\geqslant 10^8\ M^{-1}$) cannot then be viewed as reflecting a difference in bonding within the complex itself. Rather, this disparity may reflect a higher entropy of association if the peroxide binding step involved the organization of a more flexible Type 3 site in T2D laccase (vide infra). Alternatively, the change to a less positive dielectric field (as has been suggested [39, 40]) on loss of Type 2 Cu(II), or to a less hydrophobic pocket might have similar consequences.

The profound differences in redox behavior manifested by T2D laccase vis-a-vis the native protein reveal that the Type 2 copper ion must be intimately associated with the management of electrons at the Type 3 copper site in the native protein. The close correspondence in the reductive titration between the data points and the calculated line along with the emergence during reduction of a new magnetically isolated Cu(II) support the idea that the copper-copper interaction within the Type 3 site of T2D laccase is disrupted on uptake of an electron. The recent report of an epr signal from a half reduced Type 3 site as a kinetic intermediate trapped at very low temperature in native [41] and T2D [42] laccases implies a transient existence for such species during turnover. Reductive uncoupling of the Type 3 copper pair had been previously predicted to occur following experiments which showed that the distribution of electrons within *Rhus* laccase was reductant dependent [25]. However, its observation at equilibrium, as shown here, demonstrates a Type 3 site unable to maintain its integrity as a binuclear center during reduction as a consequence of the absence of Type 2 copper.

Although the data indicate that peroxide can be reduced at the Type 3 site in the absence of Type 2 copper, this peroxide reduction –Type 3 copper reoxidation is accompanied by a conformational transition within the site. Therefore, with regard to the contribution of Type 2 copper to the chemistry of the Type 3 site, the following may be inferred:

1. During reduction of laccase the presence of Type 2 copper is required to prevent the equilibrium disruption of the Type 3 binuclear pair. It seems reasonable that this could be best accomplished by a Type 2 copper(II), in view of the improved ligand binding ability of divalent *vs.* monovalent copper. If so, the relatively low oxidizing strength of Type 2 copper undoubtedly ensures its remaining divalent during reduction of Types 1 and 3 copper in laccase [25, 43].

2. Although reduced T2D laccase will reduce $H_2O_2$, the reaction is slow, with respect to Type 1 copper oxidation and involves a conformational change at the Type 3 site. Evidence for a similar conformational change on reoxidation of reduced T2D laccase by dioxygen has also been obtained [17, 21]. Therefore Type 2 copper contributes to the reduction of dioxygen at least to the extent of facilitating electron transfer from reduced Type 1 copper, and either maintaining, or promoting the rapid re-establishment of, the dicopper pairwise interaction of the Type 3 site.

Indeed, the slow reduction and reoxidation rates of T2D laccase may reflect the difficulty of direct electron transfer between the Types 1 and 3 copper ions. If so, it may be that reduced Type 2 copper experiences a rapid oxidation-re-reduction cycle in the native protein during turnover with dioxygen. Thus after contributing its reductant electron to the reduction of a bound peroxide intermediate at the Type 3 site, the Type 2 Cu(II) could rapidly accept the fourth electron from reduced Type 1 copper, and

participate in the final reduction of bound oxygen to water. This notion is not inconsistent with the current experimental data [9, 10, 44] or current models [1, 2, 5, 8] of laccase reaction.

3. With regard to peroxide binding, it seems clear from the data presented here and elsewhere, as discussed above, that both native and T2D laccase form Type 3 $\mu$-peroxo complexes in analogy with hemocyanin and tyrosinase. In the case of redox chemistry however it now appears necessary to include as a single functional unit, the Type 2 and Type 3 copper site, rather than to speak in terms of two interactive but otherwise autonomous protein subassemblies.

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