Reactivity, Electrochemical, and Spectroscopic Studies of Type 2 Copper-Depleted *Rhus vernicifera* Laccase†

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ABSTRACT: Spectroelectrochemical, circular dichroism (CD), and kinetic measurements have been performed on native and type 2 copper-depleted (T2D) tree laccases to determine the effect of type 2 copper removal on the tertiary structure, blue copper reduction potential, and electron-transfer reactivity of the metalloenzyme. The midpoint potentials (E^0) of type 1 Cu in $T2D_r$ (reduced type 3 center) and native laccases, determined with (hydroxyethyl)ferrocene (HEF) as mediator, are essentially identical (430 and 429 mV, respectively; 25 °C, pH 7.0, I = 0.5 M). Comparisons of the 300–700-nm CD spectra of native, $T2D_r$, and $T2D_{ox}$ (oxidized type 3 center) laccases reveal no detectable structural change at the type 1 copper center associated with removal of the type 2 Cu atom and only small effects of the oxidation state of the type 3 center on the environment of the type 1 site. Stopped-flow kinetic studies of electron transfer to $T2D_r$-type 1 Cu(II) from HEF and seven hydroquinones were performed to verify the importance of type 2 copper in the binding of polyphenolic substrates and to determine the effect of type 2 copper removal on the intrinsic electron-transfer reactivity of the blue copper atom. Removal of type 2 copper has remarkably little effect on the overall electron-transfer reactivity of substituted hydroquinones with laccase type 1 Cu(II), as measured by a second-order rate constant (k). The reduction rate of type 1 copper is slightly sensitive to the oxidation state of the type 3 center, as k values for electron transfer to $T2D_r$ and $T2D_{ox}$ from hydroquinone are 5.2×10^2 and 3.2×10^2 M^{-1} s^{-1}, respectively, at 25 °C, pH 7.0, and I = 0.5 M. Detailed comparisons of rate–substrate concentration profiles show that ES complex formation constants are much smaller in $T2D_r$ than in native laccase. The role of type 2 copper as a substrate binding site in the blue copper reduction pathway therefore is confirmed. The intrinsic electron-transfer reactivity of laccase type 1 copper increases substantially upon removal of the type 2 copper atom, as shown by rate constants for the HEF reduction of $T2D_r$-type 1 Cu(II) (1.9×10^4 M^{-1} s^{-1}) and native type 1 Cu(II) (4.8×10^3 M^{-1} s^{-1}), at 25 °C, pH 7.0, and I = 0.5 M. Apparent blue copper self-exchange electron-transfer rate constants estimated from the application of relative Marcus theory to these HEF rate data are 1.6 and 2.5×10^1 M^{-1} s^{-1} for the native and T2D proteins, respectively.

Proposed functions of the type 2 copper atom in *Rhus vernicifera* laccase include the binding of polyphenolic substrates (Holwerda & Gray, 1974; Clemmer et al., 1979a), concerted two-electron reduction [type 1 and type 2 Cu(I)] of the binuclear type 3 copper center (Andréasson & Reinhammar, 1976, 1979), and a cooperative role, with the reduced type 3 site, in the rapid reduction of O_2 to H_2O (Bränden & Deinum, 1977; Farver et al., 1980). Kinetic studies of the reduction of laccase by substituted hydroquinones (Clemmer et al., 1979a), 3,4-dihydroxyphenylalanine, and other catechol derivatives (Wynn et al., 1983a) have clarified the mechanism of electron transfer to the blue (type 1) copper atom and the mechanism of substrate binding in enzyme molecules with reduced type 3 sites. Coordination of singly ionized polyphenols to type 2 Cu(II) is thought to occur, with further stabilization of the enzyme–substrate complex arising from the interaction of lone-pair-bearing substituents on the aromatic ring with the type 3 cuprous atoms. Electron transfer to type 2 Cu(II) is not a prerequisite to reduction of the blue copper site (Holwerda & Gray, 1974; Andréasson & Reinhammar, 1979; Reinhammar & Oda, 1979). Furthermore, the thermodynamic oxidizability of most hydroquinone substrates has little bearing on the rate of electron transfer to type 1 Cu(II) within the enzyme–substrate complex (Clemmer et al., 1979a).

Until recently, mechanistic conclusions regarding the role of laccase type 2 copper were based solely on electron paramagnetic resonance (EPR) measurements (Malmström, 1982) or inhibition experiments with anions (i.e., N_3^-, SCN^-, F^-, and CN^-) known to ligate this site (Holwerda et al., 1982). The availability of tree laccase specifically depleted of the type 2 copper atom (T2D) (Graziani et al., 1976) has inspired numerous new approaches to understanding the behavior of the type 2 and 3 centers (Lubien et al., 1981; Spira et al., 1982; Winkler et al., 1982; Morpurgo et al., 1980a,b; Avigliano et al., 1981; Farver et al., 1982; Wynn et al., 1983b; Reinhammar, 1981, 1983). Nevertheless, only a single rate study of T2D laccase redox reactions has appeared (Reinhammar & Oda, 1979).

We report mechanistic studies of the reduction of type 1 Cu(II) in T2D laccase by seven hydroquinones and (hydroxyethyl)ferrocene. In this work, the primary objective was to confirm the importance of type 2 Cu(II) in the binding of polyphenolic substrates and to determine the extent to which removal of the non-blue copper atom perturbs the intrinsic electron-transfer reactivity of the blue copper site.

We also report circular dichroism and spectroelectrochemical measurements on T2D laccase performed to determine the effect of type 2 copper removal on the tertiary structure and blue copper reduction potential of the metalloenzyme. As mediator concentration-dependent E^0 values have resulted from the use of $Fe(CN)_6^{4-/3-}$ in previous electrochemical studies of laccase (Reinhammar, 1972; Morpurgo et al., 1980b), a neutral mediator, (hydroxyethyl)ferrocene (HEF), was employed in our comparison of the native and T2D laccase reduction potentials. The results of these titrations represent the first reported mediator concentration-independent values of E^0 for laccase.

Experimental Procedures

Preparation of Native and Type 2 Copper-Depleted Lac-

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case. Rhus vernicifera laccase was isolated from lacquer tree acetone powder and purified by the method of Reinhammar (1970). Type 2 copper-depleted laccase was prepared and reconstituted with cupric sulfate according to the method of Graziani et al. (1976), with minor modifications. Nonspecifically bound Cu(II) was removed on a Chelex-100 column following the reconstitution procedure. Type 2 copper-depleted laccase prepared by the above method contains oxidized type 1 copper and a reduced type 3 site (designated $T2D_r$; see below). T2D laccase containing oxidized type 1 and type 3 sites ($T2D_{ox}$) was prepared by treatment of $T2D_r$ with H_2O_2 (LuBien et al., 1981).

Reagents and Solution Preparation. Reagent grade chemicals were used throughout. Hydroquinone, bromohydroquinone, hydroquinonesulfonic acid (potassium salt), 2,5-dihydroxybenzoic acid, hydroxyhydroquinone, methoxyhydroquinone, and 2,5-dichlorohydroquinone were obtained as previously described (Clemmer et al., 1979a). (Hydroxyethyl)ferrocene was used as supplied by Strem Chemical Co. The disodium salt of (2,9-dimethyl-1,10-phenanthroline-4,7-diyl)bis(benzenesulfonic acid) (Na_2dpmp) was supplied by Sigma Chemical Co. Anaerobic reductant and laccase solutions for kinetic measurements were prepared in pH 7.0, I = 0.5 M sodium phosphate buffer (Clemmer et al., 1979a). An anaerobic saturated solution of HEF in this buffer (25 °C) was standardized by mixing an aliquot with excess $Cu(dpmp)_2^{2-}$ [prepared in situ from $Cu(OAc)_2 \cdot H_2O$ and Na_2dpmp], quantitatively producing $Cu(dpmp)_2^{3-}$ and the (hydroxyethyl)ferricenium ion. The $Cu(dpmp)_2^{3-}$ concentration was then determined spectrophotometrically (ϵ_{483} = 1.225×10^4 M^{-1} cm^{-1}) (Blair & Diehl, 1961), correcting for the small background absorbance of HEF^+ and excess $Cu(dpmp)_2^{2-}$ at 483 nm.

Kinetic Measurements. Kinetic data were obtained from a Durrum D-110 stopped-flow apparatus thermostated at 25.0 ± 0.1 °C (Clemmer et al., 1979a). Aerobic ferrocyanide turnover rates of native, T2D, and reconstituted laccase samples were measured by a literature method (Holwerda & Gray, 1975). Reduction of laccase type 1 copper was followed under anaerobic conditions at 614 nm, maintaining the enzyme concentration at ca. 10 μM and using sufficient excesses of the reductants (10-fold minimum) to permit a pseudo-first-order kinetic analysis. As T2D laccase is not active toward the catalytic reduction of O_2 (see below), complications encountered with the native enzyme in the analysis of post-steady-state A_{614}-time traces (Holwerda & Gray, 1974) were largely circumvented in the present study. Thus, exponential decay curves were observed from the time of mixing of all reductants with T2D laccase. Pseudo-first-order rate constants (k_{obsd}) were derived from the least-squares slopes of ln ($A_t - A_\infty$) vs. time correlations which were linear over at least 90% of the *entire* 614-nm absorbance change.

The presence of some residual native laccase in T2D preparations was evident from a secondary small decrease in A_{614} subsequent to the initial exponential decay. This secondary process, corresponding to the ultimate reduction of native laccase following the consumption of trace O_2, did not interfere with the measurement of A_∞ for the much faster initial absorbance change.

Instrumental Methods. Ultraviolet and visible spectra were acquired on an Aminco Model DW-2a spectrophotometer at a spectral resolution of 3 nm. A Metrohm/Brinkmann pH-103 meter was used to monitor pH. Circular dichroism measurements were performed on a JASCO Model J-20 recording spectropolarimeter equipped with phase-sensitive detection and a PAR model 121 lock-in amplifier.

EPR spectroscopy was performed at the Department of Biophysics of the University of California, Berkeley, on a modified JEOL X-band spectrometer with an Air Products cryogenic system for temperature control. Potentiometric titrations were performed as described previously (Smith et al., 1981) by using a minor modification of the procedure of Hawkridge & Ke (1977). The oxidation state of native and T2D laccase type 1 Cu was monitored by following the absorbance at 614 nm and correcting for contributions to the absorbance from the (hydroxyethyl)ferrocene used as a mediator. All titrations were performed at 25 °C, where the metalloproteins and redox mediator both are stable over the 5–6-h time period required for the titrations. All titrations were fully reversible. Midpoint potentials and n (electron equivalents transferred) values were calculated from least-squares fits of the data to the Nernst equation.

Results

Properties of Type 2 Copper-Depleted Laccase. The UV–visible spectra of our T2D laccase preparations were in excellent agreement with those reported by Lubien et al. (1981). The absence of any absorbance feature at 330 nm in our T2D preparations confirmed the reduced state of the type 3 center. The 330-nm absorbance feature of the fully oxidized native enzyme could be restored in our samples either by oxidation with H_2O_2 (Lubien et al., 1981) or by reconstitution of the $T2D_r$ protein with Cu (Graziani et al., 1976).

The EPR spectra of native and $T2D_r$ laccase are in good agreement with those reported previously (Reinhammar & Oda, 1979). Double integration of these EPR spectra showed that >70% removal of type 2 copper was achieved in all T2D laccase preparations. Excellent correspondence was also observed between the EPR spectra of the native and reconstituted T2D enzymes.

Ferrocyanide turnover rates were evaluated from the initial slopes of A_{420} vs. time traces in air-saturated enzyme solutions (13.5 μM) containing 10 mM $K_4Fe(CN)_6$ (25 °C, pH 7.0, I = 0.5 M). Turnover rates of native, $T2D_r$, and reconstituted laccase solutions were 6.9×10^{-6}, 1.2×10^{-6}, and 6.0×10^{-6} M s^{-1}, respectively. These results confirm the absence of O_2 reductase activity in $T2D_r$ laccase, as the turnover rate of the $T2D_r$ preparation (17% of the native enzyme activity) correlated well with its residual type 2 copper content (ca. 20% by EPR). The reversibility of type 2 copper removal is verified by the restoration, to 87% of the original value, of turnover activity in reconstituted $T2D_r$ laccase.

Kinetics of the Reduction of T2D Laccase by Substituted Hydroquiones and HEF. Excellent first-order analytical plots were derived from anaerobic, 614-nm stopped-flow studies of electron transfer to $T2D_r$-type 1 Cu(II) from substituted hydroquinones (H_2Q-X) and (hydroxyethyl)ferrocene. The substrate concentration dependences of k_{obsd} (Table I, H_2Q-X; Figure 1, HEF) indicate a simple rate law in each case:

$$-\frac{d[\text{T2D-T1 Cu(II)}]}{dt} = k[\text{reductant}][\text{T2D-T1 Cu(II)}] = k_{obsd}[\text{T2D-T1 Cu(II)}] \quad (1)$$

Second-order rate constants (k) derived from the linear least-squares analysis of k_{obsd}–concentration profiles are given in Table II and compared with analogous rate parameters associated with reduction of the native enzyme. The k values for type 1 Cu(II) reduction in $T2D_r$ and native laccases are remarkably similar, with the modified protein enjoying a small

Table I: Observed Rate Constants for the Reduction of Type 1 Cu(II) in Type 2 Copper-Depleted Laccase[a,b]

reductant	substrate concn (mM)	k_{obsd} (s^{-1})
H_2Q	0.20	0.118
	0.50	0.258
	1.0	0.482
	5.0	2.51
	10.0	5.19
H_2Q[c]	0.20	0.0568
	1.0	0.295
	10.0	3.17
H_2Q-Br	0.02	0.178
	0.05	0.454
	0.10	0.904
	0.50	3.51
	1.0	5.81
	5.0	29.1
	10.0	60.6
H_2Q-SO_3^-	0.20	0.0153
	0.50	0.0350
	1.0	0.0638
	5.0	0.334
	10.0	0.497
H_2Q-COO^-	0.20	0.00446
	0.50	0.00832
	1.0	0.0133
	5.0	0.0508
	10.0	0.102
H_2Q-OH	0.25	14.2
	0.50	42.9
	1.0	74.5
	5.0	303
H_2Q-OCH_3	0.02	0.136
	0.05	0.355
	0.10	0.874
	0.50	3.84
	1.0	6.96
	5.0	40.1
	10.0	66.7
H_2Q-2,5-Cl_2	0.50	10.6
	1.25	30.0
	2.50	68.0
	4.00	122

[a] pH 7.0, I = 0.5 M, and 25.0 °C. All values correspond to the reduction of laccase with a reduced type 3 site, except where noted. [b] In most runs, the average deviation from the mean was within ±5% of k_{obsd}. [c] The type 3 site was oxidized by pretreatment with H_2O_2 (see text).

reactivity advantage in most cases. The blue copper reduction rate in T2D laccase is moderately sensitive to the oxidation state of the type 3 site, as electron transfer from H_2Q to $T2D_r$-T1 Cu(II) is 63% faster than transfer to $T2D_{ox}$-T1 Cu(II).

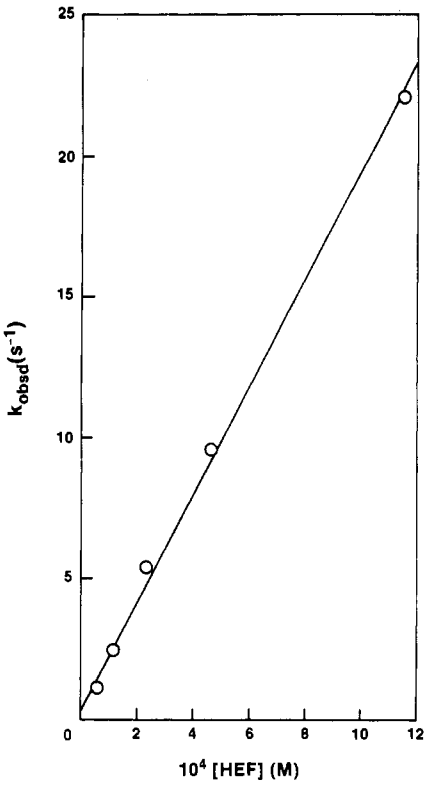

FIGURE 1: Plot of k_{obsd} vs. [HEF] for the reduction of $T2D_r$ laccase type 1 Cu(II): 25.0 °C, pH 7.0, and I = 0.5 M.

In spite of the similarity in rate constants, a significant difference should be noted in the rate laws for blue copper reduction in native and T2D laccases. Reactions of polyphenols with native laccase follow a two-step mechanism: rapid ES complex formation (formation constant Q_p), followed by rate-limiting electron transfer to type 1 Cu(II) (rate constant k_2) (Clemmer et al., 1979a; Wynn et al., 1983a). On this basis, eq 2 describes the relationship between k_{obsd} and

$$k_{obsd} = \frac{k_2 Q_p[H_2Q\text{-}X]}{1 + Q_p[H_2Q\text{-}X]} \quad (2)$$

substrate concentration. The rate law found in the present study is a limiting form of this general expression, applicable when ES binding is sufficiently weak that $Q_p[H_2Q\text{-}X] \ll 1$ and $k = k_2Q_p$. In marked contrast to their reactions with $T2D_r$ laccase, five of the hydroquinones under consideration (X = Br, COO^-, OH, OCH_3, and 2,5-Cl_2) bind so strongly to native laccase that k_{obsd}–$[H_2Q\text{-}X]$ correlations are distinctly nonlinear

Table II: Comparison of Rate Parameters for the Reduction of Type 1 Cu(II) in Native and T2D Laccase by Substituted Hydroquinones and HEF[a]

substrate	T2D	native[d]		
	k (M^{-1} s^{-1})[b]	k (M^{-1} s^{-1})	k_2 (s^{-1})	Q_p (M^{-1})
H_2Q	5.2 (0.1) × 10^2	4.5 × 10^2		
	3.2 (0.1) × 10^2 [c]			
H_2Q-Br	6.0 (0.1) × 10^3	5.9 × 10^3	30	1.93 × 10^2
H_2Q-SO_3^-	5.1 (0.5) × 10^1	4.0 × 10^1		
H_2Q-COO^-	9.9 (0.2)	7.2	0.25	2.84 × 10^1
H_2Q-OH	5.9 (0.2) × 10^4	2.7 × 10^4	27	1.00 × 10^3
H_2Q-OCH_3	6.9 (0.3) × 10^3	5.3 × 10^3	43	1.21 × 10^2
H_2Q-2,5-Cl_2	3.2 (0.2) × 10^4	3.9 × 10^4	20	1.92 × 10^3
HEF	1.9 (0.1) × 10^4	4.8 (0.2) × 10^3 [e]		

[a] 25.0 °C, pH 7.0, and I = 0.5 M. Standard deviations are given in parentheses. [b] Rate constants correspond to the reduction of $T2D_r$, unless otherwise stated. [c] Reduction of $T2D_{ox}$. [d] All data from Clemmer et al. (1979a). Ratc parameters correspond to the post-steady-state reduction of type 1 Cu(II) in laccase with a reduced type 3 site. [e] Calculated as $k_{obsd}/[HEF]$; k_{obsd} = 5.51 s^{-1} at 1.15 mM HEF. First-order HEF dependence established from data not shown.

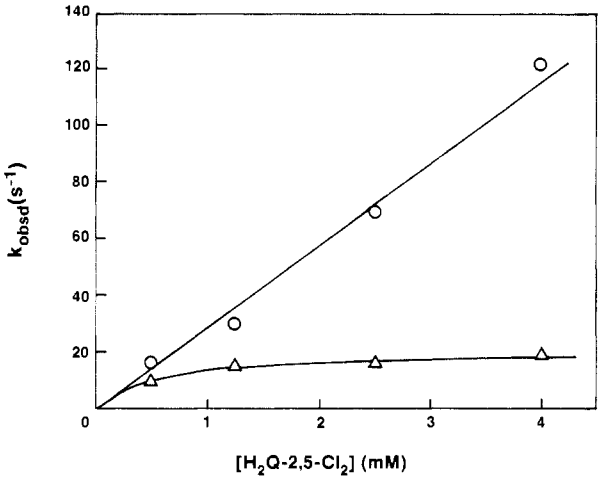

FIGURE 2: Substrate concentration–k_{obsd} profiles for the reduction of native (Δ) and $T2D_r$ (O) laccase type 1 Cu(II) by H_2Q-2,5-Cl_2: 25.0 °C, pH 7.0, and I = 0.5 M.

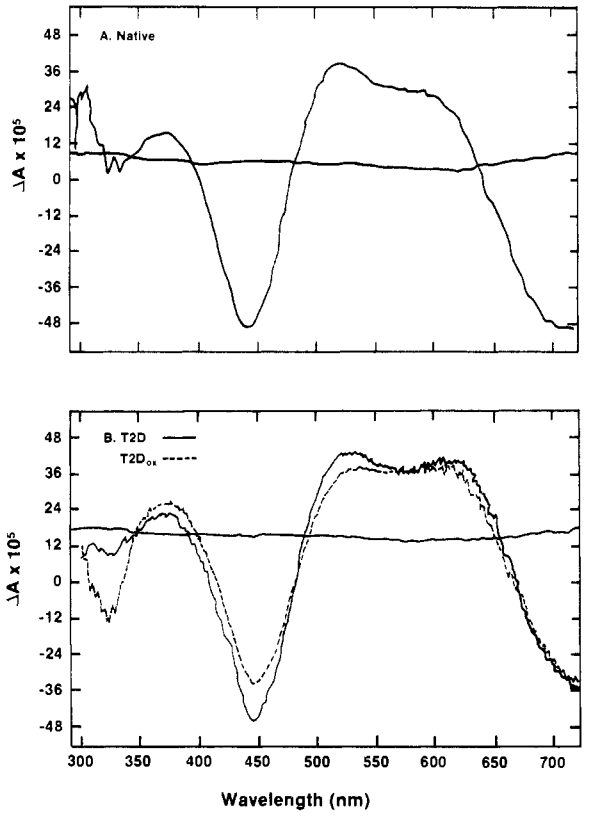

FIGURE 3: (A) Circular dichroism spectrum of native laccase (200 μM) in pH 7.0, I = 0.5 M phosphate buffer. Optical path length = 1 cm. (B) Circular dichroism spectra of $T2D_r$ (—) and $T2D_{ox}$ (---) laccases at 34 μM concentration in pH 7.0, I = 0.5 M phosphate buffer. Optical path length = 5 cm.

(Clemmer et al., 1979a). Under these conditions, both k_2 and Q_p values may be evaluated (Table II). This contrast is particularly vivid in the reactions of H_2Q-2,5-Cl_2 with native (strong-binding, Q_p = 1.92 x 10^3 M^{-1}) and $T2D_r$ (weak-binding, $Q_p < 5 \times 10^1$ M^{-1}) enzymes (Figure 2).

Circular Dichroism Measurements on Native, $T2D_r$, and $T2D_{ox}$ Laccases. A comparison of the CD spectra of native, $T2D_r$, and $T2D_{ox}$ laccases is shown in Figure 3. The native laccase spectrum is in good accord with the results of Dooley et al. (1979), Farver et al. (1978), and Morpurgo et al. (1980a). The latter workers report that the CD spectra of native and T2D laccases are essentially identical in the 400–650-nm region. Our results support this conclusion and extend

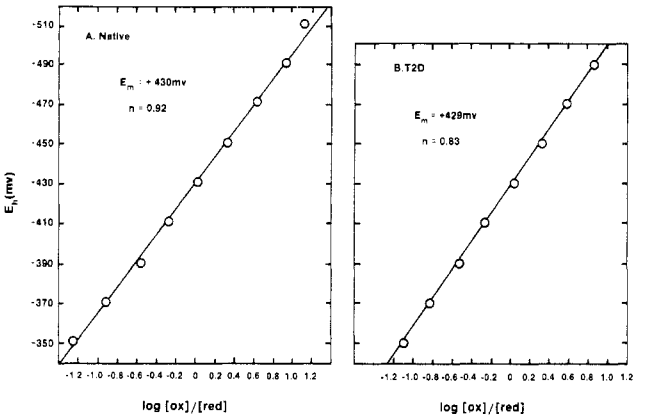

FIGURE 4: Nernst plots supporting the calculation of E^0 for type 1 Cu(II) in native (A) and $T2D_r$ (B) laccases: 25.0 °C, pH 7.0, I = 0.5 M, and 614-nm monitoring wavelength. (A) [Laccase] = [HEF] = 100 μM. Least-squares line drawn on the basis of n = 0.92 and E^0 = +430 mV vs. SHE. (B) [$T2D_r$] = 75 μM, and [HEF] = 100 μM. Least-squares line drawn on the basis of n = 0.83 and E^0 = +429 mV vs. SHE.

it by demonstrating that the CD spectrum of T2D and native laccases are also virtually identical in the region from 650 to 730 nm. Since all of the CD features in the region from 400 to 800 nm can be attributed to the type 1 Cu of laccase by comparison with the CD spectra of proteins that contain only type 1 Cu (Sjöholm & Stigbrand, 1974; Dooley et al., 1979), we can conclude that removal of the type 2 Cu from laccase produces no large-scale conformational change at the type 1 site. There do appear to be some effects of the oxidation state of the type 3 Cu center on the environment of the type 1 Cu. Figure 3 shows that there are some differences in the 448-nm CD feature, attributable to type 1 Cu, in $T2D_r$ compared to $T2D_{ox}$.

Figure 3 does reveal one obvious major difference between the CD spectra of native and $T2D_{ox}$ laccases. The latter exhibits a large negative CD feature centered at 330 nm. In this, our results agree with those of Farver et al. (1982), who first observed the negative 330-nm CD band in H_2O_2-treated T2D laccase and ascribed it to a T2D laccase–peroxy complex. The CD spectrum of H_2O_2-treated T2D laccase was unaffected by exhaustive dialysis against fresh phosphate buffer.

Redox Titrations of the Type 1 Cu(II) Centers of Native and $T2D_r$ Laccases. Spectroelectrochemical titrations of the type 1 Cu centers of native and $T2D_r$ laccases were carried out with HEF as a mediator at 25 °C, pH 7.0, and I = 0.5 M [$E^0(HEF^+/HEF)$ = 402 mV] (Szentrimay et al., 1977). Titrations were performed at two mediator concentrations in each case, in order to establish the dependence, if any, of midpoint potentials on [HEF]. Figure 4 shows Nernst plots of the oxidation–reduction titration data for both forms of the enzyme at 100 μM mediator concentration. The data are consistent with an n = 1 redox process; E^0 values of 430 ± 5 mV (native) and 429 ± 5 mV ($T2D_r$) were obtained. Titrations at lower mediator concentrations of 25 μM (native) and 50 μM ($T2D_r$) (data not shown) yielded essentially identical results (E^0 = 434 ± 5 mV for both proteins). The present $T2D_r$ E^0 values agree well with that of 430 mV [independent of $Fe(CN)_6^{4-}$ concentration] measured by Morpurgo et al. (1980b) under slightly different conditions (pH 7.0, 0.05 M phosphate buffer). Our mediator-independent E^0 value for native laccase type 1 Cu(II) corresponds closely to that determined by Reinhammar (1972) at high concentrations of the mediator $Fe(CN)_6^{4-}$ (434 mV) but deviates considerably from his result at less than stoichiometric concentrations of

the same mediator (394 mV at pH 7.5).

Discussion

Conflicting reports exist concerning the optical and electrochemical characteristics of type 2 copper-depleted laccase prepared by the method of Graziani et al. (1976) (Reinhammar & Oda, 1979; Morpurgo et al., 1980a,b; LuBien et al., 1981; Reinhammar, 1983). The absence of significant 330-nm absorption in our T2D laccase preparations supports the conclusion of Solomon and co-workers (LuBien et al., 1981) that the type 3 site remains reduced, even after exposure to O_2. X-ray absorption edge measurements on $T2D_r$ and $T2D_{ox}$ laccase preparations have conclusively shown that these contain Cu(I)–Cu(I) and Cu(II)–Cu(II) type 3 centers, respectively (LuBien et al., 1981). Comparisons of the fluorescence and phosphorescence spectra of native and $T2D_{ox}$ laccases recently performed in our laboratories (Wynn et al., 1983b) further support this conclusion. Protein denaturation did not accompany type 2 copper removal in the present study, as the optical, EPR, and ferrocyanide turnover characteristics of native laccase were restored through the addition of Cu(II) to the T2D derivative. Valid comparisons may therefore be made of the spectral, electrochemical, and reactivity properties of the native and T2D enzymes.

Removal of type 2 copper has little effect on the overall electron-transfer reactivity of substituted hydroquinones with tree laccase type 1 Cu(II), as measured by the rate constant k. To be precise, this similarity pertains between laccase molecules with reduced type 3 centers [in $T2D_r$ and the post-steady-state reduction of native laccase blue copper (Clemmer et al., 1979a)]. The rate constant comparisons in Table II therefore are consistent with the report (Reinhammar & Oda, 1979) that blue copper reduction rates by ascorbate are identical in T2D and native laccases.

The similarity between native and $T2D_r$ laccase blue copper reduction rate constants is somewhat misleading in that k is a composite quantity, dependent upon both the ES binding constant Q_p and the redox rate constant k_2. The first-order reductant dependences observed in the reactions of H_2Q-Br, H_2Q-OH, H_2Q-OCH_3, and H_2Q-2,5-Cl_2 with $T2D_r$-T1 Cu(II) contrast strikingly with the saturation of k_{obsd} at high [H_2Q-X] found in the analogous reactions with native laccase (Clemmer et al., 1979a). Substrate binding to laccase therefore is thermodynamically less favorable (Q_p < ca. 50 M^{-1}) in the T2D derivative. By implication, intracomplex electron-transfer rate constants in $T2D_r$ laccase must be significantly *larger*, relative to the native enzyme, in order for $k = k_2Q_p$ values to be comparable in the two proteins.

The present and previous (Reinhammar & Oda, 1979) kinetic investigations of T2D laccase clearly show that electron transfer to type 2 Cu(II) is not a prerequisite to reduction of the blue copper atom. Thus, this common feature of several proposed mechanisms of laccase action (Holwerda & Gray, 1974; Clemmer et al., 1979a; Andréasson & Reinhammar, 1976, 1979) is confirmed. The dramatic decrease in Q_p values resulting from the removal of type 2 Cu(II) fully supports the proposed role of this copper atom in the binding of polyphenolic substrates (Holwerda & Gray, 1974; Clemmer et al., 1979a). It should also be noted, however, that type 2 copper–polyphenol binding clearly is not essential to activate the bound substrate towards electron transfer.

Although extensive justification for the intermediate complex mechanism of laccase reduction by polyphenols has been given previously (Holwerda & Gray, 1974; Clemmer et al., 1979a; Wynn et al., 1983b), kinetically indistinguishable alternatives should be considered. In particular, a mechanism involving parallel outer-sphere (k_1) and intermediate complex (k_2) type 1 Cu(II) [L-Cu(II)] reduction pathways (eq 3) would

$$\begin{gathered} H_2Q\text{-}X + \text{L-Cu(II)} \xrightarrow[\text{slow}]{k_1} \text{products} \\ H_2Q\text{-}X + \text{L-Cu(II)} \underset{\text{fast}}{\overset{Q_p}{\rightleftharpoons}} \text{complex} \xrightarrow[\text{slow}]{k_2} \text{products} \end{gathered} \tag{3}$$

$$k_{obsd} = \frac{(k_1 + k_2Q_p)[H_2Q\text{-}X]}{1 + Q_p[H_2Q\text{-}X]} \tag{4}$$

result in a rate expression (eq 4) quite similar to that which pertains when only the k_2 pathway contributes. On this basis, the second-order rate constant k would be equivalent to $k_1 + k_2Q_p$ rather than k_2Q_p.

Since this mechanism cannot be completely ruled out on kinetic grounds alone, it should be noted that our principal mechanistic conclusion for the intermediate complex pathway is valid regardless of the possible contribution of k_1 to k_{obsd}. Thus, the Q_p parameter could still be obtained by our method (intercept/slope in a k_{obsd}^{-1} vs. $[H_2Q\text{-}X]^{-1}$ plot), such that attenuation in Q_p associated with type 2 copper removal is unambiguous. Furthermore, a meaningful distinction between outer-sphere and intermediate complex redox pathways is not possible when only simple first-order oxidant and reductant dependences are observed, as in the reactions of H_2Q-X with T2D-T1 Cu(II). Specifically, weak precursor complex formation between the redox partners is a well-documented feature of outer-sphere redox processes (Holwerda et al., 1980).

Outer-sphere electron transfer from hydroquinones to many transition metal oxidants has been demonstrated, including the bis(2,9-dimethyl-1,10-phenanthroline)copper(II) ion (Clemmer et al., 1979b). Nevertheless, the importance of the hypothetical k_1 outer-sphere laccase reduction pathway is doubtful. The strong correlation between rate and thermodynamic driving force characteristic of outer-sphere hydroquinone oxidations (Clemmer et al., 1979b) is absent in the reactions of H_2Q-X with both native and T2D laccases. Furthermore, the similarity between k values for native and T2D laccase reductions is not readily understood in terms of the two-pathway mechanism (eq 3), as it cannot be assumed that differences in the k_1 and k_2Q_p terms would cancel to achieve invariance in $k = k_1 + k_2Q_p$. Finally, potent inhibition by type 2 Cu(II)-binding anions (Holwerda & Gray, 1974) effectively rules out simple outer-sphere electron transfer to type 1 Cu(II) in native laccase.

Type 1 copper reduction potentials of native and $T2D_r$ laccases are identical, to within experimental error, under our conditions. It should be pointed out that the E^0 values determined in this study with HEF as a mediator satisfy all the criteria usually set for valid E^0 determinations. The titrations gave good fits to $n = 1$ Nernst curves, were reproducible (to within ±5 mV), were fully reversible, and were independent of mediator concentration (over a 4-fold range). This is in distinct contrast to earlier measurements using the ferricyanide/ferrocyanide couple as a mediator in which E^0 values for native laccase were reported to depend significantly on mediator concentration (Reinhammar, 1972; Morpurgo et al., 1980b). As E^0 for laccase should be an intrinsic property of the protein, true determinations of E^0 must be independent of mediator concentration. The results from other laboratories suggest that laccase interacts with ferri- and/or ferrocyanide and indicate that this couple is inappropriate as a mediator for use in determining E^0 values for laccase.

As the E^0 values of the type 1 Cu centers are identical in native and T2D laccase, apparent increases in k_2 redox rate

constants in $T2D_r$ relative to native laccase cannot be attributed to enhancements in thermodynamic driving force. The implication, based on the Marcus electron-transfer theory (Marcus, 1968), is that the intrinsic electron transfer reactivity of laccase type 1 copper increases substantially upon removal of the type 2 copper atom. The equation of Marcus outer-sphere electron transfer theory is (for reactions with small driving force at 25 °C)

$$k_{12} = [k_{11}k_{22} \exp[38.94(\Delta E)]]^{1/2} \quad (5)$$

where k_{11} and k_{22} are the self-exchange electron-transfer rate constants of the metalloprotein and its redox partner, respectively. The cross-reaction rate constant and corresponding standard cell potential (in volts) are designated by k_{12} and ΔE, respectively.

The hypothesis that k_{11}[T2D-T1 Cu(I,II)] exceeds k_{11}-[native-T1 Cu(I,II)] was checked through the measurement of k_{12} values for the reduction of native and T2D proteins by (hydroxyethyl)ferrocene, an outer-sphere redox agent (Pladziewicz & Espenson, 1973). A comparison of the rate constants in Table II shows that the $T2D_r$ blue copper center is more reactive with HEF, by a factor of 4, than that of native laccase. Apparent k_{11}'s of 1.6 and 2.5×10^1 M^{-1} s^{-1} (25 °C) may be calculated from eq 5 for the blue copper centers of native and $T2D_r$ laccases.[1] While these figures are approximate and may differ significantly from the actual k_{11} parameters, the intrinsic redox reactivity advantage of T2D-T1 Cu(II) is clearly confirmed.

While there is some correlation between rate constants and the thermodynamic oxidizability of hydroquinone substrates, the Marcus relationship is of little use in understanding the complex dependence of k values for either T2D or native laccases on hydroquinone substitutents. Similarly, k_{12} values predicted from eq 5 for the HQ^- reductions of laccase blue copper (native or T2D) are in very poor agreement (3–4 orders of magnitude too large) with the actual rate constants.[2] Consistent with our previous findings on the native enzyme (Clemmer et al., 1979a), the reduction of type 1 Cu(II) in $T2D_r$ laccase appears to be governed for the most part by protein-dependent activation requirements.

The reduction rate of type 1 copper in T2D laccase is slightly sensitive to the oxidation state of the type 3 center, as evidenced by the rate constants for electron transfer from hydroquinone to $T2D_{ox}$ and $T2D_r$. An earlier conclusion (Holwerda & Gray, 1974) that the oxidation state of the type 3 site has no influence on the reactivity of the blue copper atom and vice versa must therefore be revised. Exchange of H_2O and anions between the bulk medium and the enzymatic cavity containing the type 2 and type 3 copper centers is substantially slower in fully oxidized native laccase than in the partially reduced enzyme (Goldberg et al., 1980; Holwerda et al., 1982). Similarly, a larger contribution to the activation free energy from ES complex formation could be partially responsible for the slower rate of $T2D_{ox}$-type 1 Cu(II) reduction. Spectroscopic studies of T2D laccase have shown that the oxidation state of the type 3 site has a more direct impact on the structure and physical properties of the blue copper atom, however. Thus, small but distinct differences between the resonance Raman, EPR, and optical characteristics of blue copper in $T2D_{ox}$ and $T2D_r$ laccases suggest that reduction of the type 3 center induces a structural change at type 1 Cu(II) involving the Cu(II)–N (histidine imidazole) bonds (LuBien et al., 1981). The CD spectrum of native tree laccase in the 350–800-nm range has been attributed to the type 1 site, as single copper blue proteins such as azurin, plastocyanin, umecyanin, and stellacyanin exhibit similar spectra (Dooley et al., 1979; Sjöholm & Stigbrand, 1974). Our CD data (see Figure 3), which reveal small but significant differences in the 448-nm negative CD feature of $T2D_r$ vs. $T2D_{ox}$ laccase, are consistent with the idea that the oxidation state of the type 3 center affects the environment of the type 1 Cu. In contrast, any structural changes in the type 1 copper coordination sphere associated with type 2 copper removal must not be large, as all prominent CD bands in the 500–750-nm region of native, $T2D_r$, and $T2D_{ox}$ laccases are essentially identical.

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Registry No. H_2Q, 123-31-9; H_2Q-Br, 583-69-7; H_2Q-SO_3H-K, 21799-87-1; H_2Q-CO_2H, 490-79-9; H_2Q-OH, 533-73-3; H_2Q-OCH_3, 824-46-4; H_2Q-2,5-Cl_2, 824-69-1; HEF, 1277-49-2; Cu, 7440-50-8; laccase, 80498-15-3.

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[1] In performing these calculations, k_{22} was assumed to be identical with that of (hydroxymethyl)ferrocene (4.2×10^6 M^{-1} s^{-1}) (Pladziewicz & Espenson, 1973). The applicability of the Marcus equation to redox reactions of ferrocene derivatives is strongly supported in this reference.

[2] See Clemmer et al. (1979b) for a tabulation of Q-X/H_2Q-X reduction potentials and equations relevant to these Marcus calculations.

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Fluorescence Anisotropy Changes in Platelet Membranes during Activation†

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ABSTRACT: Dynamic changes in platelet membrane components were evaluated by two fluorescent probes, the anion channel blocker 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) and the membrane-impermeant stachyose derivative of pyrenebutyryl hydrazide (SPBH). Fluorescence anisotropy, r, was measured in intact platelets treated with either fluorophore. Activation of platelets by thrombin, arachidonic acid, and ADP under nonaggregating conditions increased the anisotropy values of DIDS within 60–120 s. A slow return to base-line values occurred after 8–10 min. Thrombin produced an initial transient reduction of r during the first 60 s. Its effect was specific as inactivated enzyme did not induce any changes. The latter could also be prevented by omitting Ca^{2+} from the platelet suspension. Treatment of platelets with SPBH, a fluorophore inserted into the lipid leaflet of membranes, revealed an activation-induced increase of its fluorescence anisotropy during the first 120 s. It was followed by a 6–8 min lasting decline of r when thrombin and ADP were the stimulants. Preexposure of platelets to colchicine did not change significantly the fluorescence anisotropy pattern of either fluorophore, but cytochalasin B inhibited such changes almost completely. The findings are interpreted as demonstrating greater motional freedom in the lipid bilayer but a decrease in this parameter in membrane proteins upon stimulation of platelets.

Platelet aggregation is a complex process (Marcus, 1969) which is initiated by the interaction of certain agents with their specific receptor sites on the platelet surface. This sets in motion a series of events in the course of which the discoid platelet is changed to a contracted spherical cell with many pseudopodial projections. Concomitantly the platelets become sticky and adhere to each other. An internal redistribution of subcellular granules is associated with a change in the state of polymerization of structural membrane proteins and is eventually followed by release of granule contents to the surrounding medium. The conversion of the platelet from a cell with nonadhesive character to one of extreme stickiness is an amazing phenomenon with respect to both the profoundness and the rapidity of the change. We believe that the platelet transformation to a spiny sphere is not solely responsible for the change in adhesivity. Dynamic changes in platelet membrane components have been reported during platelet activation induced primarily by thrombin (Nathan et al., 1979, 1981). In these studies, however, platelets were stimulated by the aggregating agent while being vigorously agitated through continuous stirring. The resultant clumping of platelets makes it virtually impossible to determine whether changes in fluorescence polarization are due to platelet–platelet interaction or due to a positional redistribution of membrane components. For this reason, we have designed our experiments to expose platelets to aggregating agents without con-

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