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Absorbance and Fluorescence Stopped-Flow Kinetics of *Rhus* Laccase and the Catalytic Reaction Sequence†

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ABSTRACT: Anaerobic reduction of *Rhus* laccase causes a 1.76-fold increase in protein fluorescence [Goldberg, M., & Pecht, I. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4684]. Parallel stopped-flow kinetic absorbance and fluorescence measurements were made to determine with which reduction step(s) the fluorescence changes were associated. Anaerobic reduction data obtained at pH 6.0, 7.4, and 8.5 with and without fluoride showed that fluorescence increases are either slower than (e.g., pH 6.0, 0.25 mM ascorbate) or faster than [e.g., pH 6.0, 0.1 mM $Ru(NH_3)_6^{2+}$] net type 1 Cu(II) reduction. Fluorescence increases are usually slower than type 3 reduction. However, type 3 reduction and fluorescence enhancement profiles are congruent in the presence of fluoride or at pH 8.5; the fluorescence changes are never faster than type 3 reduction. The forms of the fluorescence kinetic profiles invariably are similar to the type 3 reduction profiles and not the type 1. The oxidation of reduced copper sites by O_2 and the associated fluorescence decrease occur with the same rate constant. Satisfactory simulations are only obtained by assuming that two fluorescence changes occur which are associated with two separate reduction steps. The data are consistent with a first fluorescence increase occurring upon type 3 reduction and a second with the *final* type 1 Cu(II) reduction. This suggests that a change in protein conformation occurs when the type 3 site is reduced which in turn makes fluorescence sensitive to the redox state of the type 1 copper. pH-jump experiments indicate that the active and inactive forms evident at pH 7.4 and 8.5 [Andréasson, L.-E., & Reinhammar, B. (1976) *Biochim. Biophys. Acta 445*, 579] are not in equilibrium in the fully oxidized enzyme.

Laccases catalyze the oxidation of a variety of reductants and use oxygen as the sole electron acceptor to form water. The enzymes isolated from the Japanese lacquer tree *Rhus vernicifera* and the fungus *Polyporus versicolor* are the most extensively characterized to date (Malmström et al., 1975; Holwerda et al., 1976; Reinhammar & Malmström, 1981; Farver & Pecht, 1981). Four tightly bound Cu(II) atoms are essential for catalytic activity in both laccases. These are distinguished by their spectroscopic parameters into three types. Extensive transient kinetic studies indicate that both type 1 Cu(I) and type 2 Cu(I) are required for the reduction of the type 3 Cu(II) pair (Andréasson et al., 1973b; Brändén & Reinhammar, 1975; Pecht & Farraggi, 1971; Pecht, 1979; Andréasson & Reinhammar, 1976, 1979; Dawson et al., 1972; Holwerda & Gray, 1974, 1975; Wherland et al., 1975; Holwerda et al., 1978). Current kinetic schemes propose that type 1 Cu(II) reduction precedes type 2 Cu(II) reduction before type 3 reduction occurs via intramolecular electron transfer from the type 1 and type 2 sites (Andréasson & Reinhammar, 1976, 1979; Malmström, 1982). The type 3 pair acts as a cooperative two-electron acceptor, at least under some conditions (Malkin et al., 1969; Reinhammar & Vänngård, 1971; Reinhammar, 1972; Farver et al., 1978). An added complexity in the kinetics of anaerobic reduction of *Rhus* laccase is that two forms of the oxidized enzyme in slow exchange have been postulated to account for biphasic reduction profiles at pH 7.4 under rapid reducing conditions (Holwerda & Gray, 1974; Andréasson & Reinhammar, 1976, 1979).

Reoxidations of types 1 and 3 Cu(I) by O_2 are very rapid (Andréasson et al., 1973a, 1976), while reoxidation of type 2 Cu(I) by O_2 is much more sluggish (Andréasson et al., 1973a; Brändén & Deinum, 1978). The reduced type 3 site

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is thought to deliver the first two electrons during oxygen reduction (Malmström et al., 1975; Holwerda et al., 1976; Reinhammar & Malmström, 1981; Farver & Pecht, 1981). This leads to H_2O_2 as an intermediate (Goldberg et al., 1980). Reactions involving oxygen prior to type 3 reductions may also occur (Farver et al., 1980).

Goldberg & Pecht (1974) found that the intrinsic fluorescence of *Rhus* laccase is significantly enhanced when the protein is reduced anaerobically. A linear dependency of fluorescence intensity on type 1 Cu(II) reduction was apparent from anaerobic reductive titrations. Morpurgo et al. (1980) found that fluorescence was enhanced when the type 2 Cu(II) was selectively removed from native *Rhus* laccase. Since removal of the type 2 Cu(II) was accompanied by a large decrease in absorption at 330 nm, it was suggested that the fluorescence increase occurring when the native enzyme is reduced is correlated to type 3 reduction.

Parallel absorption and fluorescence stopped-flow kinetic experiments were undertaken in an attempt to clarify which Cu(II) reduction steps were associated with the fluorescence increases and to determine if the rates of the fluorescence changes are consistent with their reflecting kinetically competent protein conformation changes associated with the Cu(II) reduction reaction sequence. A modified reduction scheme based largely on the reaction sequence proposed by Andréasson & Reinhammar (1976, 1979) accounts for the absorption and fluorescence data under a variety of conditions. The fluorescence increases are apparently associated with reduction of both the type 3 and type 1 Cu(II) sites with the latter requiring prior reduction of the type 3 site.

Experimental Procedures

Reagents. $Ru(NH_3)_6^{2+}$ as the dichloride was prepared according to the method of Lever & Powell (1969) and recrystallized once under argon following treatment with zinc dust. All other materials were reagent grade and used without further purification.

Laccase Purification. Laccase was purified from an acetone powder extract of *Rhus vernicifera* latex (Saito Co., Tokyo, Japan) according to a modified procedure from Reinhammar & Oda (1979). After fractionation on a 2.5 × 40 cm CM-Sephadex (Pharmacia, Uppsala) column, the laccase fraction was dialyzed against three changes of 10 mM sodium phosphate, pH 6.8, and applied to a 5 × 30 cm Biorex 70 (Bio-Rad) 400-mesh column equilibrated against the same buffer. The sample was eluted by using a linear sodium phosphate gradient (10–75 mM) at pH 6.8. An overlapping green fraction preceded the blue fraction, and cuts were made at $A_{330}/A_{615} \leq 0.8$. This step eliminates a contaminant associated with an absorbance transition at 420 nm. The protein was concentrated on a 2.5 × 15 cm Biorex 70 (200–400 mesh) column equilibrated as described above, eluted with 100 mM sodium phosphate, pH 6.8, further concentrated to (3–4) × 10^{-4} M by pressure filtration, and stored at −70 °C. The A_{280}/A_{615} ratios were routinely 16.5, and the copper content was four Cu per mole, assuming E_{615nm} = 5700 M^{-1} cm^{-1} (Malmström et al., 1970). Type 2 depleted laccase was prepared according to published procedures (Reinhammar & Oda, 1979) and contained 3 mol of Cu/mol of laccase. Copper analyses were by flameless atomic absorption (Perkin-Elmer Model 360 spectrophotometer with an HGA 2100 graphite furnace).

Spectra. Absorption spectra were recorded on a Cary 17 spectrophotometer. Fluorescence emission spectra were obtained on an SLM 8000 fluorometer interfaced with a Cromemco Z-2D microcomputer. Several fluorescence spectra were averaged, and a blank was subtracted. The resultant observed spectrum (305–450 nm) was corrected and integrated. A 100-mL tonometer equipped with a 1-mL cuvette was used to obtain anaerobic absorption and fluorescence emission spectra of oxidized laccase and enzyme reduced with 4 equiv of ascorbate.

Stopped-Flow Kinetics. Absorbance and fluorescence kinetic measurements were performed in two Gibson-Durrum stopped-flow instruments (Gibson & Milnes, 1964) equipped with all-stainless-steel valve blocks. The latter were necessary to minimize oxygen leaks. The two instruments were identical except for the flow cells. For absorption measurements, a standard 2-cm flow cell was used, monitoring type 1 Cu(II) absorbance at 615 nm and type 3 absorbance at 340 nm. A 2-mm flow cell equipped with top and side quartz windows was constructed for the fluorescence measurements. A 0.5 × 2.5 in. quartz rod wrapped in several layers of black paper guided fluorescent and scattered light to the detector. An Oriel G-774-4000 band-pass filter was placed in front of the detector to isolate the fluorescence emission. Excitation light was provided from a 150-W xenon lamp with a Durrum 13000 monochromator directed through the side window of the flow cell. An Oriel G-774-3300 band-pass filter was placed in front of the side window, and experiments were carried out in subdued light to eliminate any extraneous light that might enter. A Nova 820 computer, equipped with an OLIS 3620 interface, was used to acquire 500 data points per reaction which were averaged from several consecutive runs. The stopped-flow was initially flushed with a dithionite solution, left for more than 30 min to reduce any residual oxygen, and then thoroughly rinsed with anaerobic buffer. To confirm that the mixed products from the two valve blocks were identical, the valve blocks and mixing jets were exchanged. This had no effect on the kinetic constants obtained.

The nature of the reductants had to be varied in the kinetic experiments because varying the concentration of any one reductant could not provide both a wide range of reduction rates and minimal fluorescence interference. Ascorbate was used to obtain relatively slow rates. $Ru(NH_3)_6^{2+}$ was used for relatively high reduction rates with minimal fluorescence interference.

The possible influence of changes in the reductant absorbance on the measured fluorescence was estimated from aerobic steady-state experiments. A small slow increase in fluorescence is observed during the oxygen turnover period. From this, it was calculated that interference from reducing substrates would never exceed 5% of the total fluorescence change in anaerobic reduction kinetic experiments.

Preparation of Solutions for Kinetics. Protein solutions were introduced into a 500-mL tonometer and flushed with ultrapure nitrogen (Union Carbide) for 40 min on ice and gently shaken for the last 20 min. Glass syringes filled with buffer were bubbled with ultrapure nitrogen for 10 min before appropriate volumes of anaerobic protein solutions were withdrawn from the tonometer. Protein concentrations prior to mixing were (1.8–2.5) × 10^{-5} M. In reoxidation experiments, the protein-containing syringe was capped with a serum stopper, and a small volume (1%) of anaerobic sodium ascorbate corresponding to 5–6 electron equiv was injected. The protein solution was left to reduce at room temperature for 3 h. Fluoride-inhibited laccase was prepared by incubation for 12 h at the appropriate pH and peroxylaccase by incubating with a 3–4-fold excess of peroxide while monitoring the increase in absorption at 325 nm (Farver et al., 1976).

Data Handling. The parameter optimization techniques of Fletcher & Powell (1963) were used to extract rate constants

Table I: Rate Constants for Types 1 and 3 Cu(II) Reduction and Fluorescence Increase[a]

reductant	concn (mM)	pH	NaF concn (mM)	obsd parameter[b]	k_{fast} (s^{-1})[c]	k_{slow} (s^{-1})[c]	% fast phase[d]
ascorbate	0.25	6.0	0	1	1.70×10^{-2} [e]		
				3	3.23×10^{-2}		
				F	1.80×10^{-2}		
ascorbate	2.5	6.0	0	1		1.64×10^{-1}	
				3	3.95×10^{-1}		
				F	1.67×10^{-1}	4.18×10^{-2}	72.2
$Ru(NH_3)_6^{2+}$	1.0	6.0	0	1	2.73×10^{1}	1.74	30.1
				3	1.39×10^{1}	1.90	16.5
				F	2.19	7.06×10^{-2}	32.8
$Ru(NH_3)_6^{2+}$	10	6.0	0	1	9.91×10^{1}	6.28	51.6
				3	7.99×10^{1}	6.02	47.9
$Ru(NH_3)_6^{2+}$	1.0	6.0	100	1	7.19	1.12	59.1
				3	1.82	5.9×10^{-2}	33.6
				F	8.28×10^{-1}	5.29×10^{-2}	33.7
ascorbate	1.0	7.4	0	1	1.32×10^{-1} [e]		
				3	1.52×10^{-1}		
				F	8.73×10^{-2}		
$Ru(NH_3)_6^{2+}$	0.1	7.4	0	1	3.80	2.84×10^{-1}	40.1
				3	4.71×10^{-1}	1.49×10^{-1}	73.6
				F	3.42×10^{-1}	1.15×10^{-1}	66.5
$Ru(NH_3)_6^{2+}$	1.0	7.4	0	1	2.54×10^{1}	1.81	70.2
				3	2.46×10^{1}	5.64×10^{-1}	36.8
				F	3.62	3.00×10^{-1}	39.7
$Ru(NH_3)_6^{2+}$	0.1	7.4	100	1	2.02		
				3	2.3×10^{-1}	6.40×10^{-3}	21.7
				F	1.8×10^{-1}	6.67×10^{-3}	25.8
$Ru(NH_3)_6^{2+}$	0.1	8.5	0	1	2.83×10^{1}	3.63	57.8
				3	1.9×10^{1}	8×10^{-2}	40
				F	1.1×10^{1}	1×10^{-1}	40

[a] Reactions were carried out under the indicated conditions at 25 °C. [b] 1 denotes type 1, 3 denotes type 3, and F denotes fluorescence. [c] The data were fit to $ae^{-k_1 t} + c$ or $ae^{-k_1 t} + be^{-k_2 t}$ where k_1 and k_2 are the rate constants of the observed fast and slow phases, respectively. [d] $(a \times 100)/(a + b)$. [e] Only the last part of the multiphase reaction profile was fit.

from mono- and biphasic reaction profiles. The number of data points was reduced from 500 to 50 by a second-order polynomial fit prior to application of the non-linear-fitting algorithms. These data were then fitted to expressions containing either one- or two-exponential terms. Two-exponential fits were only accurate if the ratio k_{fast}/k_{slow} was at least 4. In cases where this ratio was smaller than 4, yet significantly larger than 1, the data were fit to an expression containing one exponential plus a base-line term. This may lead to a small underestimate in the rate constant of the faster process.

The properties of complex kinetic models were examined on an EAI-48 analog computer (Electronics Associates Inc.). In each case, one experimentally determined rate constant (Table I) was fixed. Semiquantitative fits were then obtained by placing tracings of the experimental data over the display screen and varying the potentiometer settings for all other rate constants, fractional fluorescence emission intensities, and the distribution of active/inactive forms until a visual best fit was attained. The rate constants equivalent to the potentiometer settings are given in Table II.

Results

Fluorescence Controls. Fluorescence emission spectra were recorded for oxidized and reduced laccase during excitation at 299 nm as controls to correlate with the stopped-flow kinetic data. This excitation energy was necessary to avoid excessive inner filter effects at the protein concentrations used in the parallel stopped-flow kinetic absorbance experiments. The fluorescence intensity of the reduced enzyme obtained by integrating corrected spectra was 1.76-fold higher than the fluorescence of the oxidized enzyme. This equals the effect of reduction previously reported for excitation at 305 nm (Goldberg & Pecht, 1974). Identical values were obtained at pH 6.0 and 7.4, and the same quantum yields were observed in the kinetic experiments. The fluorescence emission maximum of both oxidized and reduced laccase is at 328 nm rather than at 324 nm when exciting at 280 nm.

Kinetics of Copper Reduction and Fluorescence Enhancement at pH 6.0. In all the figures of this paper, fluorescence enhancements are shown in reverse direction for clarity of comparison to the Cu absorbance profiles. At 0.25 mM ascorbate, type 1 reduction is characterized by a small initial rapid phase followed by a pronounced turnover phase. This is followed by a final monophasic reduction (Figure 1A). Note the term turnover is used to reflect the probable reoxidation of the type 1 Cu(I) by the type 3 center (Andréasson & Reinhammar, 1976, 1979). Similar type 1 Cu(II) reduction profiles were reported with ≥1 mM hydroquinone at pH 6.0 (Andréasson & Reinhammar, 1976). The fluorescence increase associated with reduction under these relatively slow reducing conditions occurs faster than the overall rate of reduction of the type 1 Cu(II). Note, however, that the final rate of type 1 reduction and the rate of the fluorescence increase are equal (Table I). Both the fluorescence increase and the type 3 Cu(II) reduction are monophasic (Figure 1A, Table I). Under faster reducing conditions [2.5 mM ascorbate or 0.1 mM $Ru(NH_3)_6^{2+}$], similar patterns are observed, but the fluorescence increases occur slower than net type 1 Cu(II) reduction. At ≥1 mM $Ru(NH_3)_6^{2+}$, the reduction profiles of type 1 and type 3 centers closely parallel one another, and the increase in fluorescence is markedly slower than either copper reduction (Figure 1B). All three kinetic patterns are biphasic. In contrast to what is observed at pH ≥7.0 (Andréasson & Reinhammar, 1976, 1979; Holwerda & Gray, 1974), the slow phases under these conditions are *substrate dependent*. Similar results were reported when laccase was reduced with chromous

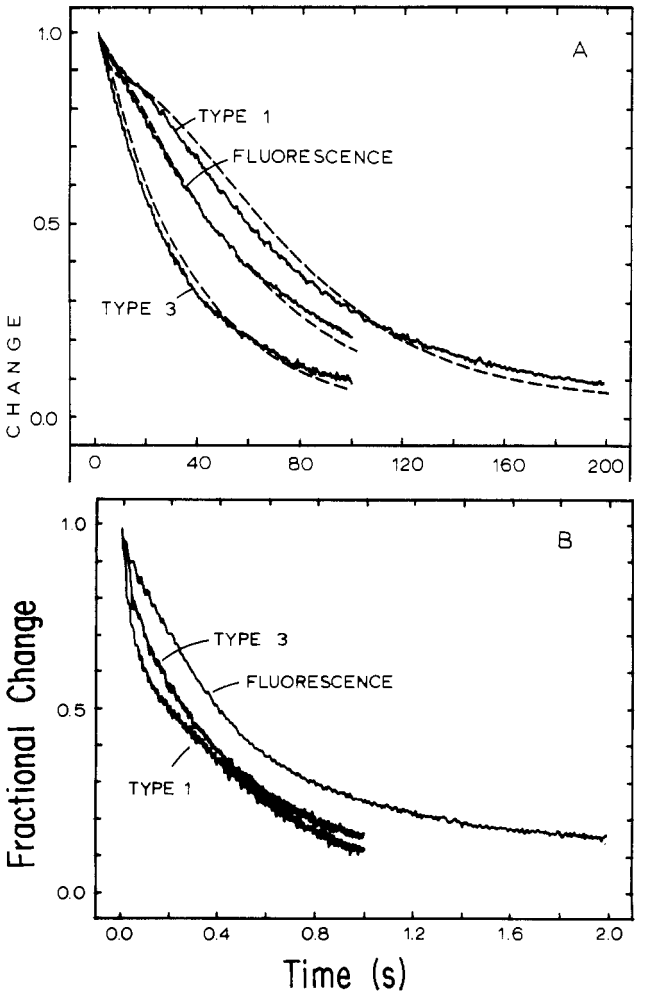


FIGURE 1: Anaerobic reduction in 0.1 M sodium phosphate buffer at pH 6.0, 24.5 °C. (A) Laccase (9.6 μM) was reduced by 0.25 mM ascorbate. The type 1 curve represents one accumulation, the type 3 curve is three accumulations, and the fluorescence curve is the average of five accumulations. The dashed curves are simulated profiles assuming Scheme I and the parameters given in Table II (see Discussion). (B) Laccase (8.5 μM) was reduced by 1.0 mM Ru$(NH_3)_6^{2+}$. The curves are averages of 7 (type 1), 10 (type 3), and 9 accumulations (fluorescence).

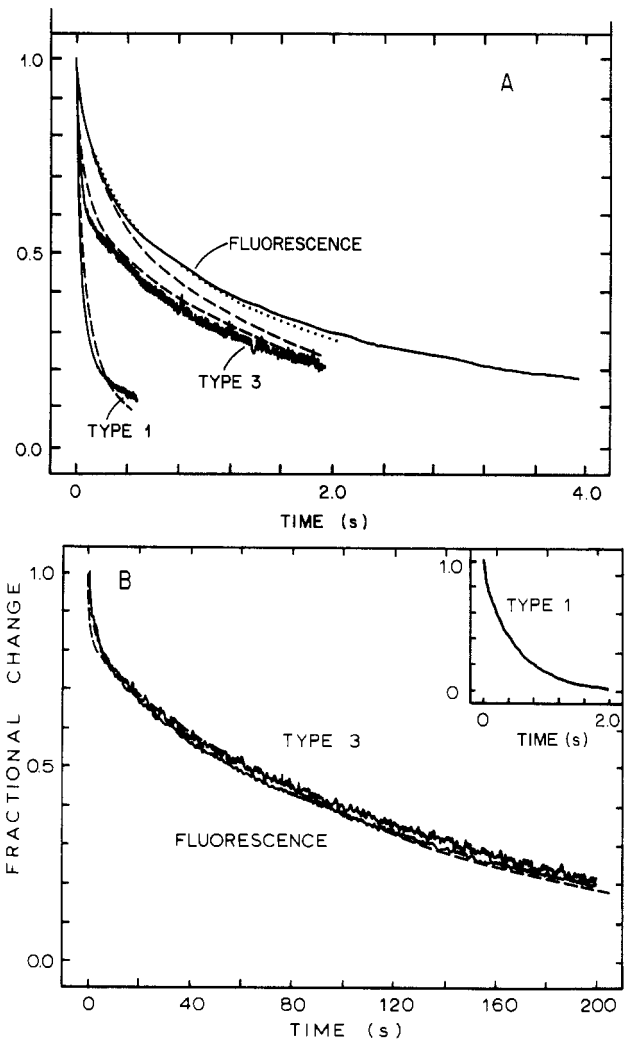


FIGURE 2: Anaerobic reduction in 0.1 M sodium phosphate buffer at pH 7.4, 25 °C. (A) Laccase (11 μM) was reduced by 1.0 mM Ru$(NH_3)_6^{2+}$. All curves are averages of 10 accumulations. (B) Laccase was reduced by 0.1 mM Ru$(NH_3)_6^{2+}$ in the presence of 0.1 M fluoride. The reduction curves are averages of six (type 1), three (type 3), and four accumulations (fluorescence). Note the very rapid net reduction type 1 Cu(II) reduction (insert). The dashed curves are simulated profiles assuming Scheme I and the parameters given in Table II (see Discussion). The dotted fluorescence profile in (A) was obtained by assuming that $Q_{active}/Q_{inactive} = 0.75$ as explained under Discussion. The simulated spectrum of the type 1 profile in (B) was congruent to the observed curve.

ion at pH 4.8 (Dawson et al., 1972). The substrate dependency and parallel type 1 and type 3 reduction profiles are not accounted for by the scheme proposed below, or any reported in the literature; i.e., the reduction mechanism is apparently different under these conditions.

Effects of Fluoride at pH 6.0. Fluoride (100 mM) has no effect on the static fluorescence of oxidized *Rhus* laccase at pH 6.0. In the presence of F^-, the observed net rate of type 1 Cu(II) reduction by 1.0 mM Ru$(NH_3)_6^{2+}$ is high. This reflects a marked inhibition of its reoxidation by type 3 reduction (Table I). Type 3 reduction and fluorescence enhancement show nearly congruent, parallel biphasic kinetics. The slow phases which are *substrate independent* have the same rate constant (Table I). The 100 mM F^- was close to saturating as shown by no significant concentration dependence from 2 to 100 mM F^-. A F^- inhibitory effect on post-steady-state reduction at pH 6.0 was reported earlier (Holwerda & Gray, 1974).

Kinetics of Copper Reduction and Fluorescence Enhancement at pH 7.4 and 8.5 with or without Fluoride. At relatively slow rates of reduction (e.g., 1 mM ascorbate, pH 7.4), type 1 Cu(II) reduction is characterized by a fast initial phase followed by a pronounced turnover phase as reported earlier (Andréasson & Reinhammar, 1976). The fluorescence enhancement, like type 3 reduction, is monophasic. As at pH 6.0, the rate of type 3 reduction is about twice as fast as the fluorescence change (Table I). The half-time of the fluorescence increase is slightly higher than the half-time for net type 1 reduction. Rapid reduction by 1.0 mM Ru$(NH_3)_6^{2+}$ gives biphasic kinetics of both type 3 reduction and fluorescence increase (Figure 2A). The slow phases are *substrate independent* (Table I; Holwerda & Gray, 1974; Andréasson & Reinhammar, 1976). The initial (fast) rate of the fluorescence change is significantly slower than the initial rate of type 3 reduction while the rate constants for the slow phases are equal (Table I). Biphasic Cu(II) reduction and fluorescence increase profiles associated with F^- inhibition are coincident at pH 7.4 (Figure 2B). The rate constant for the substrate-independent phase is much lower than that at pH 6.0 (Table I).

Kinetic results at pH 8.5 are potentially complicated by slow changes in absorbance (Holwerda & Gray, 1974) and fluorescence. Therefore, the kinetic experiments at this pH

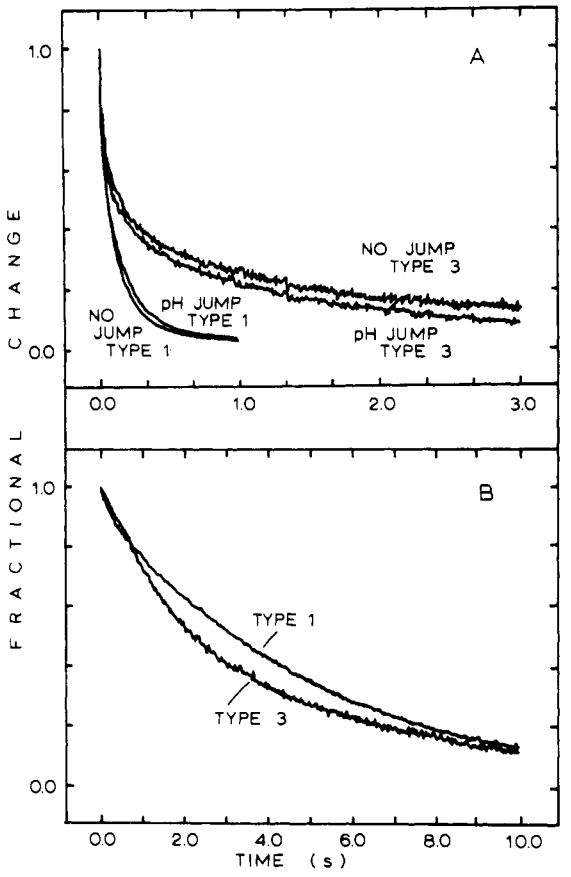


FIGURE 3: (A) Effect of a rapid pH change on the anaerobic reduction of *Rhus* laccase at pH 7.35, 25 °C, by 10.0 mM hydroquinone. (No jump) The protein (9 μM) and the reductant were in 0.1 M sodium phosphate, pH 7.35. The curves are averages of 5 (type 1) and 10 (type 3) accumulations. (pH jump) The protein (10 μM) was in 0.1 M sodium phosphate buffer, pH 5.94, and the reductant in 0.1 M sodium phosphate, pH 11.5 (dissolved anaerobically to eliminate rapid oxidation by oxygen at this pH). Mixing equal volumes of these buffers gave pH 7.35. The kinetic curves are averages of 5 (type 1) and 10 (type 3) accumulations. (B) Anaerobic reduction of *Rhus* laccase (9.5 μM) at pH 5.94, 25 °C, by 10.0 mM hydroquinone. The kinetic curves are averages of five (type 1) and eight (type 3) accumulations.

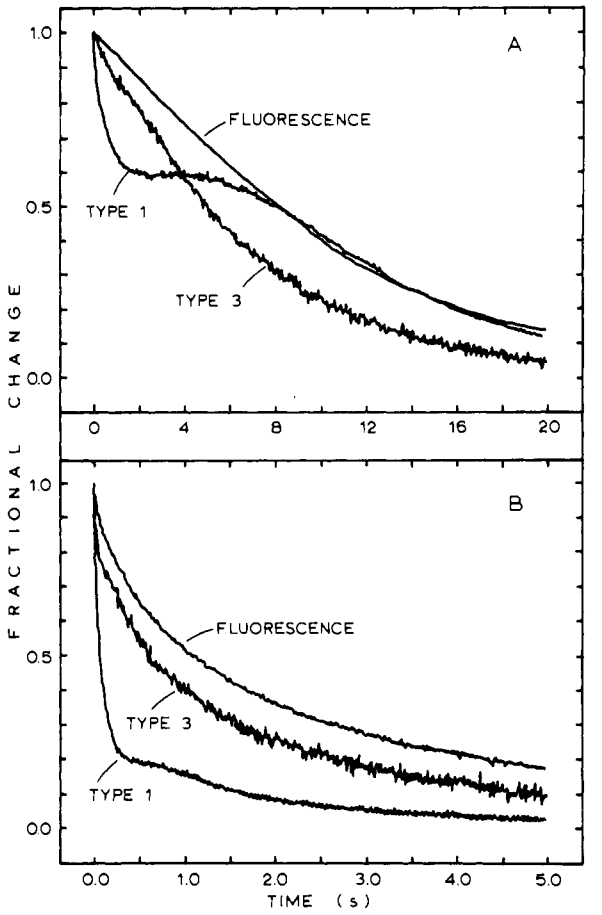


FIGURE 4: Anaerobic reduction of peroxylaccase in 0.1 M sodium phosphate buffer at pH 7.4, 25 °C. Laccase (20 μM) was incubated with 67 μM hydrogen peroxide for 3 h at room temperature. A crystalline suspension of catalase was added to give a final concentration of 33 ng/mL, and the solution was left for 25 min on ice before the protein was deoxygenated (cf. Experimental Procedures). (A) Anaerobic reduction by 1.0 mM sodium ascorbate. The kinetic curves are averages of one (type 1), four (type 3), and 1 (fluorescence) accumulation(s). (B) Anaerobic reduction by 1.0 mM $Ru(NH_3)_6^{2+}$. The kinetic curves represent averages of five (type 1 and type 3) and one (fluorescence) accumulations.

were performed after overnight dialysis, i.e., after completion of the slow decrease in the 615-nm absorbance (~45%) and the increase in fluorescence intensity (~59%). The further increase in fluorescence upon reduction was comparable to the increase at pH 6.0. The reduction results at pH 8.5 without F^- are qualitatively similar to what was obtained with F^- at the lower pH value. Anaerobic reduction by 0.1 mM $Ru(NH_3)_6^{2+}$ gives biphasic patterns for type 3 Cu(II) reduction and fluorescence enhancement which are virtually superimposable (Table I).

Kinetics following a pH Jump on Mixing. Biphasic type 3 reduction at pH 7.4 was ascribed to a slow conversion of an inactive to an active enzyme form (Holwerda & Gray, 1974; Andréasson & Reinhammar, 1976, 1979). The two forms were proposed to be present in roughly equal amounts at pH 7.4, but at pH 6.5 or below, more than 90% was proposed to be in the active form. Since the rate of interconversion at pH 7.4 was postulated to be 0.5 s^{-1}, uninhibited type 3 Cu(II) reduction should be observed at pH 7.4 when laccase is predominantly in the active form immediately prior to reduction. This was tested by mixing anaerobic *Rhus* laccase at pH 6.0 with an anaerobic reductant solution at high pH such that the resultant mixture had a pH of 7.4. Unexpectedly, biphasic rather than monophasic profiles were obtained which were virtually identical with what was obtained with the resting enzyme at pH 7.4 (Figure 3). Note that the reduction of type 1 Cu(II) has a half-time of 20 ms at pH 7.4. This is much faster than the slow equilibrium (half-time for interconversion would be about 1 s) which was proposed to account for the slow substrate-independent reduction of type 3 Cu(II) at pH 7.4 (Figure 2A). Additional pH-jump experiments (from pH 6.0 to 7.4, from pH 6.0 to 8.5, and from pH 7.4 to 6.5) using 1.0 mM $Ru(NH_3)_6^{2+}$ as reductant yielded similar results; i.e., the pH effects are very rapid.

It should be noted that potential ionic strength effects in the pH-jump experiments with hydroquinone are precluded by the fact that the controls and the pH-jump samples after mixing had identical ionic strengths. Moreover, recent results suggest that specific ionic interactions between hydroquinone and laccase are not a factor in the reduction kinetics (Holwerda et all., 1982). Also, similar pH-jump results were obtained with $Ru(NH_3)_6^{2+}$.

Reduction and Fluorescence Kinetics of Peroxylaccase. Anaerobic titrations of peroxylaccase showed that 6 electron equiv is required to fully reduce this enzyme form (Farver et al., 1976). Peroxide results in an increase in absorbance at 325 nm and a decrease at 615 nm as well as a small decrease in fluorescence emission intensity. When peroxylaccase is reduced with 1.0 mM ascorbate at pH 7.4, the turnover phase in type 1 Cu(II) reduction is markedly accentuated (Figure

4A). This probably reflects the requirement for more reducing equivalents to fully reduce the laccase and H_2O_2. A similar, but much smaller, turnover phase is also obtained when peroxylaccase is reduced more rapidly by 1.0 mM $Ru(NH_3)_6^{2+}$ (Figure 4B).

Peroxide appears to have only small effects on the observed kinetics of type 3 Cu(II) reduction (Figure 4A,B). The overall form for the fluorescence increase is again more similar to the type 3 than the type 1 reduction profile.

Reduction of Type 2 Depleted Laccase. In agreement with Morpurgo et al. (1980), selective removal of the type 2 Cu(II) from *Rhus* laccase is associated with a large decrease in the 330-nm absorbance and a large increase in fluorescence in our hands. The decrease in the 330-nm absorbance has been shown to be due to type 3 reduction (Lubien et al., 1981). No shift in the emission maximum is detected, and the integrated emission intensity of type 2 depleted laccase is 1.69 times higher than that in the native enzyme. Interestingly, a further 1.21-fold increase in fluorescence occurs when the type 1 Cu(II) is reduced in the type 2 depleted enzyme with ascorbate at pH 6.0. This is associated with an approximate 3-nm shift in the emission maximum. That the total fluorescence increase from native oxidized laccase to reduced type 2 depleted enzyme is greater than the increase from reducing the holoenzyme may be due to additional effects of the type 2 removal.

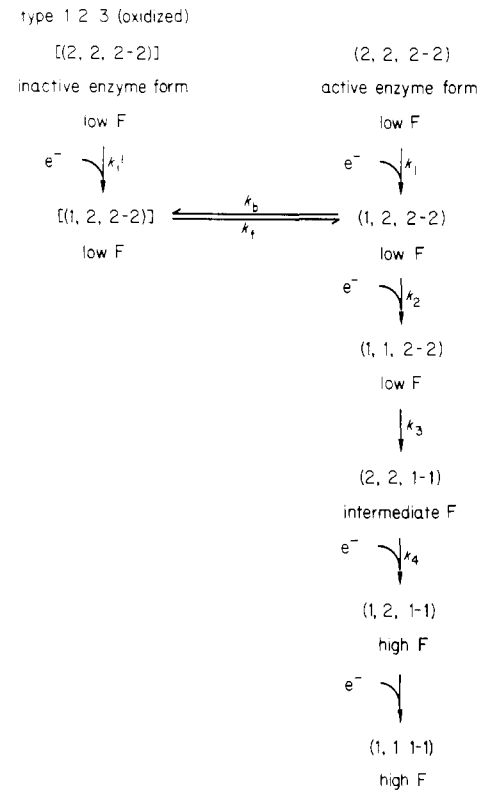
Reoxidation Kinetics. When laccase which is reduced with 5 equiv of ascorbate is mixed with 1.2–1.4 equiv of dioxygen, very rapid changes are observed in copper absorbances and fluorescence. Second-order rate plots are linear; the increases in type 1 and type 3 absorbances and the decrease in fluorescence all occurred with approximately the same rate constants (type 1 oxidation, $3.18 \times 10^6 \pm 5.77 \times 10^3$ M^{-1} s^{-1}; type 3 oxidation, $4.48 \times 10^6 \pm 1.62 \times 10^4$ M^{-1} s^{-1}; fluorescence decrease, $4.5 \times 10^6 \pm 3.5 \times 10^5$ M^{-1} s^{-1}). The rate constants for the copper site oxidations were in good agreement with reported values (Andréasson et al., 1976; Petersen & Degn, 1978).

Discussion

The results of the pH-jump experiments reported here show that the pH-dependent step(s) involving the putative inactive form must be very rapid since biphasic kinetics are obtained within the stopped-flow mixing time when the pH is concurrently rapidly changed. These results can be interpreted in terms of the reduction scheme proposed by Andréasson & Reinhammar (1976, 1979). If the type 1 reduction data at 1 mM ascorbate, pH 7.4, are simulated, assuming rapid interconversions between inactive and active forms, adequate fits can only be obtained when the rate of type 1 Cu(II) reduction in the inactive, fully oxidized form is at least 5 times greater than that in the active form. For the same rapid equilibrium assumption to apply at high reduction rates, at least 80% of the fully oxidized enzyme pool should be driven toward the inactive form, and 80% slow phase in the type 3 reduction should be detected. However, only 50–60% slow phase is detected under rapid reducing conditions [Table I, 1 mM $Ru(NH_3)_6^{2+}$, pH 7.4; Andréasson & Reinhammar, 1976; Hansen et al., 1984]. Thus, neither a slow nor a fast equilibrium among oxidized enzymic forms is consistent with the pH-jump experiments and the Cu(II) reduction data under slow and fast reducing conditions.

The previously proposed scheme (Andréasson & Reinhammar, 1976, 1979) can account for the pH-jump results if the proposed slow equilibrium between the two fully oxidized forms is deleted as indicated in Scheme I. In the less active enzyme form, a slow, substrate-independent step ($k = 0.6$ s^{-1}) precedes

Scheme I: Two-Form Model



reduction of the type 2 Cu(II) at pH 7.4 as previously proposed. In the simplest form of such schemes, all rate constants from type 2 Cu(II) reduction forward are assumed equal for the two forms. A more stringent requirement is that the initial type 1 Cu(II) reduction rate must be equal in the two forms at pH 6.0 since type 3 reduction is monophasic at low reduction rates and is dependent on the rate of type 1 Cu(II) reduction. The observed pH dependence for detecting the two forms is attributed to rapid equilibrium with a protonated enzymic form for which the substrate-independent step is rapid. It should be noted that the two nonequilibrium forms proposed in Scheme I may correspond to distinct isoenzyme species since isoenzymes have been reported with *Rhus* laccase (Reinhammar, 1970). Two charged isoenzymes were detected by isoelectric focusing with the enzyme preparations used here.

The following three fluorescence kinetic characteristics must be stressed before the fluorescence kinetics are considered in terms of Scheme I: (i) The increase in fluorescence can occur faster or slower than the overall rate of type 1 Cu(II) reduction, and the fluorescence kinetic profiles are never coincident with changes in the oxidation state of this copper site. (ii) The fluorescence changes apparently never occur faster than type 3 Cu(II) reduction. Under the partially inhibiting conditions of high pH or the presence of fluoride with fast reduction of the type 1 Cu(II), the kinetics of type 3 reduction and fluorescence enhancement are coincident. For all other conditions, the fluorescence changes occur slower than the rate of type 3 reduction and can be considerably slower. (iii) The overall form with respect to monophasic vs. biphasic or possible steady-state plateaus for fluorescence kinetics is invariably similar to the pattern of type 3 reduction and not type 1 reduction.

The Cu(II) reduction steps which are associated with the fluorescence increases can be inferred from the kinetic data and Scheme I. First, it is instructive to consider possibilities which are precluded by the kinetic data. The fluorescence increases cannot be associated exclusively with the initial type

Table II: Parameters for Simulations Shown in Figures 1 and 2[a]

condition	k_1[b]	k_f	k_2[e]	k_3	k_4	% fast phase
0.25 mM ascorbate, pH 6.0	3.2×10^{-2} [c]	4[d]	3.2×10^{-1}	4[d]	2.4×10^{-2} (1.7×10^{-2})[f]	100[g] (100)
1 mM $Ru(NH_3)_6^{2+}$, pH 7.4	10 (25)	0.56[c]	100	very fast[h]	6[i]	42 (37-40)
0.1 mM $Ru(NH_3)_6^{2+}$ and 100 mM F^-, pH 7.4	2 (2)	6×10^{-3} [c]	20	very fast[h]	1[i]	20 (22-26)

[a] Simulations were carried out according to Scheme I as described under Experimental Procedures. [b] Rate constants (s^{-1}) are numbered according to Scheme I. [c] Fixed parameter from Table I (see Experimental Procedures). Note the best experimental estimate of k_1 is the k_{fast} of type 3 Cu(II) reduction (Table I). [d] Substrate-independent rate constant taken from steady-state kinetic data at pH 6.0 (unpublished results). [e] The rate constant of type 2 Cu(II) reduction was taken as $k_2 = 10k_1$ (Andréasson & Reinhammar, 1979). [f] Experimentally determined values (Table I) are in parentheses. [g] At pH 6.0, the type 3 Cu(II) and fluorescence kinetic profiles remain monophasic because $k_f \geq k_1, k_1', k_2$. [h] For substrate-independent steps, $k \geq 560\ s^{-1}$ (Peterson & Degn, 1978). [i] A complex function of k_f and k_4.

1 reduction step as this would make the fluorescence changes faster than the rate of type 3 reduction under inhibited conditions. The fluorescence increases cannot solely be associated with the final type 1 Cu(II) reduction step either since if this were the case, long lags would be detected for the fluorescence changes and the type 1 Cu(II) reduction and fluorescence increase profiles would be coincident toward the end of the reduction process. Obviously, the fluorescence changes cannot be associated with only all type 1 reduction steps since there is no evidence for turnover phases in the fluorescence profiles. The fluorescence increase also cannot exclusively be associated with type 3 reduction because the fluorescence increase usually occurs more slowly and can occur much more slowly than the 330-nm absorbance changes. Three results also suggest that the fluorescence changes are not closely associated with changes in the oxidation state of the type 2 Cu(II). These are the following: (1) At pH 6.0, its reduction profile is identical with that exhibited by the type 1 Cu(II) (Andréasson & Reinhammar, 1979), whereas the fluorescence profile is not. (2) Although complete reduction of the type 2 Cu(II) at pH 7.4 under rapid reducing conditions is much slower than reduction of the type 3 Cu(II) (Andréasson & Reinhammar, 1979), the kinetics of the fluorescence enhancement tend to coincide with type 3 reduction under these conditions. (3) Upon reoxidation, the decrease in fluorescence is very rapid, whereas the reoxidation of type 2 Cu(II) has a half-time of more than 1 s (Brändén & Deinum, 1978).

At least two separate increases in fluorescence which are associated with at least two different reduction steps seem to be required to account for the fluorescence data in terms of Scheme I. This restriction is particularly evident when one attempts to fit the data while accounting for the magnitude of the separation between the fluorescence and 330-nm profiles. The reaction intermediates that require intermediate fluorescence intensities to simulate the data are indicated in Scheme I. The (2, 2, 1-1)[1] state is assigned a fluorescence which is less than that of the fully reduced protein but greater than that of the fully oxidized protein (approximately half of the total increase); the remaining fluorescence increase is assigned to the final type 1 Cu(II) reduction step. The assumption that a partial fluorescence change occurs during the last reduction of type 1 Cu(II) implies that the rate constants for the fluorescence change and the final type 1 reduction should be comparable under slow reducing conditions. This is observed (Table I). The observed fluorescence increase associated with type 1 Cu(II) reduction in the type 2 depleted enzyme is also consistent with this assignment.

Although several models were assessed, the two-form model, shown in Scheme I, was the simplest kinetic scheme which could account for both the fluorescence and absorbance data as well as pre-steady-state data (Hansen et al., 1984). That the fluorescence kinetics can be accounted for by Scheme I substantiates the scheme. Moreover, the fluorescence changes are apparently associated with changes in protein conformation that occur at comparable rates to the Cu(II) reduction steps.

The properties of the modified two-form model were analyzed by computer simulations (see Experimental Procedures). It was assmed that all extinction coefficients and the fluorescence quantum yields were the same for the putative two forms of the enzyme. It was also assumed that after conversion of the inactive to the active (1, 2, 2-2) species, the rate constants for the subsequent reduction steps were identical for the two forms. Simulations are given in Figures 1 and 2 which illustrate the experimental extremes of slow reduction at pH 6.0, moderately rapid reduction at pH 7.4, and conditions under which the fluorescence and type 3 kinetic patterns are congruent. The simulations fit the Cu(II) reduction data reasonably well. The adequacy of this model can also be assessed by comparing predicted and observed rate constants and percent fast phase values (Table II). The fluorescence kinetic data at pH 6.0, pH 8.5 (not shown), or pH 7.4 with F^- are also approximated well by the scheme when the relative quantum yields given above are assumed (Figures 1 and 2). In the specific case of intermediate rates of reduction at pH 7.4, in the absence of F^- [e.g., 1.0 mM $Ru(NH_3)_6^{2+}$], the biphasic form and the relative rate of the fluorescence profile and copper reduction profiles are correctly predicted by Scheme I, but the quantitative fit toward the end of the reduction process is relatively poor. This may be accounted for by a protic equilibrium involving a protein quenching group such that the quantum yield of the active form is three-fourths the quantum yield of the inactive form (Figure 2A). In summary, while more complex reduction schemes may be valid, Scheme I seems to represent the simplest scheme reasonably consistent with the data available thus far.

The apparent kinetic relationships between copper reduction and fluorescence enhancement have molecular implications for the fluorescence effects. Increases in fluorescence apparently cannot be *exclusively* coupled to either type 1 Cu(II) or type 3 Cu(II) reduction by direct fluorescence, energy transfer, or heavy-metal quenching mechanisms for the reasons stated above. According to the kinetic model, the anaerobic reduction pathway of *Rhus* laccase is associated with at least three distinct protein states sensed by fluorescence. Some change in protein conformation and/or a decrease in fluorescence quenching apparently occurs as the type 3 center is reduced which in turn makes laccase fluorescence sensitive to the oxidation state of the type 1 Cu(II).

The fluorescence kinetics are consistent with the reported equilibrium fluorescence results cited in the introduction. Morpurgo et al. (1980) reported that the fluorescence of type 2 Cu(II)-depleted (partially) laccase was 45% higher than the

[1] The notation of Farver et al. (1980) for the reduction states of the three types of copper sites is used here; e.g., (1, 2, 1-1) denotes an enzyme form with reduced type 1 and type 3 sites and an oxidized type 2 site.

fluorescence of hololaccase. Goldberg & Pecht (1974) reported a linear correlation between the oxidation state of the type 1 Cu(II) and the fluorescence enhancement at equilibrium. Simulations of anaerobic reductive titrations assuming three fluorescence states as in the two-form model gave reasonable fits to the reported data when the (2, 2, 1-1) state was given a fluorescence intensity about 1.15 times the fluorescence of the resting, fully oxidized enzyme. However, no combination of relative contributions to the total fluorescence change gave a strictly linear relation between type 1 reduction and fluorescence increase.

The reduction of peroxylaccase reported here may provide additional insights into steps in the laccase mechanism after oxygen binds. Reduction of peroxylaccase gives a prolonged turnover phase in type 1 Cu(II) reduction (Figure 4A). Pronounced turnover is evident even at very fast type 1 Cu(II) reduction rates (Figure 4B). Since the complete reduction of peroxylaccase requires 6 reducing equiv (Farver et al., 1976), these results suggest that the type 1 site is involved in the reduction of peroxide to water in agreement with earlier proposals (Andréasson & Reinhammar, 1979; Malmström, 1982). The kinetic results also imply that H_2O_2 interacts with the oxidized type 3 site as suggested earlier (Farver et al., 1976).

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Registry No. $Ru(NH_3)_6^{2+}$, 19052-44-9; Cu, 7440-50-8; L-ascorbic acid, 50-81-7; laccase, 80498-15-3.

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