Effects of a Single Reduction–Reoxidation Cycle on the Kinetics of Copper Reductions in *Rhus* Laccase†

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ABSTRACT: Absorbance and fluorescence stopped-flow kinetic experiments were designed to examine the effects that catalytic turnover has on *Rhus* laccase kinetics. Biphasic type 3 Cu reduction kinetics are observed under anaerobic reducing conditions at pH 7.4 or 8.5. This suggests kinetically active and inactive oxidized enzyme forms. Inhibited type 3 reduction phases are abolished by a single reduction–reoxidation cycle. The kinetic profiles associated with types 1 and 3 Cu rereduction and the concurrent fluorescence enhancement all slowly return to those exhibited by the resting enzyme as rereduction is initiated at increasing times after the single turnover. The calculated rate of this transformation from activated to resting enzyme equals the rate of type 2 Cu(I) oxidation when minimally reduced enzyme is mixed with O_2. In contrast, the rate of type 2 Cu(I) oxidation is much faster when excess reductant is present during oxidation by O_2 as expected if the type 2 Cu(I) has a role in the reduction of the type 3 copper pair. The results are consistent with a steady-state kinetic reaction sequence in which the unliganded, fully oxidized enzyme does not participate. A pivotal role for an intermediate in which the type 2 Cu is reduced is proposed. To satisfactorily account for the 30% reduced types 1 and 2 Cu(I) during steady-state turnover, it is also suggested that reduction of the oxygen radical intermediate occurs only after reduction of both type 1 and type 2 Cu(II).

Laccases, like other multi-copper oxidases, catalyze the reduction of dioxygen to water (Fee, 1975; Malmström et al., 1975). The fundamental properties of the three types of copper sites found in *Rhus vernicifera* and *Polyporus versicolor* laccase are well characterized (Fee, 1975; Malmström et al., 1975; Reinhammar & Malmström, 1981; Farver & Pecht, 1981). Possible catalytic reaction sequences have also been proposed on the basis of a variety of spectral and stopped-flow kinetic data (Andréasson et al., 1973a,b, 1976; Brändén & Reinhammar, 1975; Andréasson & Reinhammar, 1976, 1979; Aasa et al., 1976a,b; Brändén & Deinum, 1978; Farver et al., 1980; Goldberg et al., 1980; Goldberg & Pecht, 1978; Hansen et al., 1984; Malmström, 1982). In particular, at least two relatively long-lived oxygen intermediates are apparent, H_2O_2 (Farver et al., 1980; Goldberg et al., 1980; Goldberg & Pecht, 1978) and an oxygen radical species whose exact chemical nature remains unknown (Andréasson et al., 1973a, 1976; Aasa et al., 1976a,b; Brändén & Deinum, 1978; Malmström, 1982). Current schemes propose functional roles for both types 1 and 2 Cu sites in reducing the type 3 Cu(II) pair, which in turn is proposed to be the primary reaction site for O_2 (Andréasson & Reinhammar, 1976, 1979; Malmström, 1982). Additional possible reaction steps involving O_2 have also been proposed (Farver et al., 1980; Goldberg et al., 1980). The type 1 Cu has been proposed to have a role in the reduction of the peroxide and oxygen radical intermediates to H_2O (Andréasson & Reinhammar, 1976, 1979; Malmsröm, 1982), and a possible role for the type 2 site in dioxygen cleavage has been suggested (Farver & Pecht, 1981; Farver et al., 1980; Goldberg et al., 1980).

One complexity that has been noted in anaerobic reduction kinetic studies with *Rhus* laccase is that the kinetic profiles for type 3 reduction are biphasic under fast reducing conditions at pH 7.4 or 8.5 (Holwerda & Gray, 1974; Andréasson & Reinhammar, 1976, 1979; Hansen et al., 1984). This has been attributed to the presence of both active and inactive forms in the resting enzyme (Holwerda & Gray, 1974; Andréasson & Reinhammar, 1976, 1979; Hansen et al., 1984). However, biphasic type 3 reduction profiles are not observed following catalytic turnover in the presence of excess reductant and O_2 (Andréasson & Reinhammar, 1976; Holwerda & Gray, 1974). In addition to these immediate effects, catalytic turnover also leads to quasi-stable effects on the copper spectral properties of *Rhus* laccase (Farver et al., 1980; Goldberg et al., 1980). Thus, a catalytically active "C state" was proposed (Farver et al., 1980; Goldberg et al., 1980). In this paper, we report absorbance and fluorescence stopped-flow kinetic data aimed at characterizing the kinetic properties of laccase activated by turnover and the mechanism for reverting to the resting enzyme. The data are consistent with a steady-state reaction sequence which does not include participation of the fully oxidized enzyme.

Materials and Methods

Reagent sources and the general methods used for laccase purification, anaerobic solution preparations, absorbance and fluorescence stopped-flow measurements, and data treatment were as reported previously (Hansen et al., 1984).

Reoxidation–Rereduction Stopped-Flow Experiments. Protein solutions for experiments involving one turnover cycle in the presence of excess reductant were prepared by reducing the protein in a reservoir syringe with 20 mM sodium ascorbate for 90 min. Preliminary experiments were performed to determine the concentration of oxygen necessary to fully oxidize the reduced copper sites without excess oxygen. Various concentrations of oxygen were mixed with the reduced protein, and the optical changes at 340 and 615 nm were recorded in the stopped-flow instrument. An oxygen concentration was chosen so that a steady-state lag period prior to reducton of the type 3 site was absent, and the optical change at 615 nm

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remained maximal. The final concentrations of laccase and oxygen were 1×10^{-5} M.

Somewhat higher initial protein concentrations were used for experiments involving a waiting period between reoxidation of minimally reduced enzyme and rereduction with excess reductant. Laccase (40 μM) in 0.1 M sodium phosphate, pH 7.4, was reduced with 4 reducing equiv of sodium ascorbate for 3 h. Protein solution (2 mL) was introduced into one of the anaerobic drive syringes (2.5 mL); the other drive syringe contained anaerobic-buffered $Ru(NH_3)_6^{2+}Cl_2$ solutions. Air-equilibrated buffer was injected into a capped 2.5-mL glass syringe containing 200 μL of deoxygenated buffer to give 1 equiv of O_2. This syringe was connected to the drive syringe containing the reduced protein. The contents of these syringes were then mixed manually to give freshly oxidized laccase. Half the contents of the drive syringes were then flushed through the flow system before the first rereduction reaction was recorded. The time from oxidation to initiation of the first rereduction was from 10 to 20 s. Subsequent reactions were initiated after indicated waiting periods.

Rates of Disappearance of Reduced Type 2 and the Oxygen Radical. Anaerobic laccase (0.4 mM, 0.1 M sodium phosphate, pH 7.4) was reduced with 4 reducing equiv of sodium ascorbate for 2 h. Reduced protein was rapidly mixed with oxygen-saturated sodium phosphate buffer (1.9 mM O_2) and delivered into an electron spin resonance (ESR) tube kept at 5 °C. The contents were frozen at various times thereafter by rapidly submerging the tubes into liquid isopentane kept at −150 °C. Freezing occurred in about 1 s. ESR spectra were recorded at 13 K on a Varian E9 spectrometer operating at X-band frequency. The relative amounts of type 2 Cu(II) and oxygen radical were estimated from the areas of the low-field type 2 Cu(II) signal (2450–2700 G) and the broad trough of the radical signal (3400–4500 G). Rate constants were estimated by nonlinear least-squares fits (Hansen et al., 1984) of the area vs. time data to an exponential relation as described in Table I.

Steady-State Concentrations of Type 1 and Type 2 Cu(II). Anaerobic *Rhus* laccase (0.4 mM, 0.1 M sodium phosphate, pH 7.4) was reduced with approximately 5 mM anaerobic hydroquinone for 2 h. Reduced protein solution (200 μL) was introduced into a nitrogen-flushed ESR tube and capped. Oxygenated buffer (200 μL) was squirted into the ESR tube, and the mixture was mixed vigorously for a few seconds. The reaction was performed at 5 °C. The contents were frozen by rapid immersion into an acetone–salt mixture kept at −90 °C. Freezing occurred in about 5 s. ESR spectra of the samples were recorded at approximately 100 K on a Varian E9 instrument operated at X-band frequency.

Results

Approaches to Steady State from Oxidized and Reduced Laccase. When oxidized laccase was mixed with excess hydroquinone (0.4–10 mM) and excess dioxygen (125–600 μM) at pH 7.4, 25 °C, a pre-steady-state overshoot in the fraction of reduced type 1 copper was detected followed by a slow back-decay toward a lower concentration of reduced type 1 Cu(II) at steady state as reported earlier (Andréasson & Reinhammar, 1976). A limiting value of approximately 50% initial type 1 reduction was detected in the pre-steady-state phase over this concentration range of hydroquinone. The substrate-independent rate constant of the back-decay was 0.5 $\pm$ 0.1 s^{-1}, which is in excellent agreement with the observed rate constant for the slow, substrate-independent phase of type 3 reduction by 10 mM hydroquinone under anaerobic conditions (Andréasson & Reinhammar, 1976, 1979). Similar results were obtained with ascorbate or $Ru(NH_3)_6^{2+}$ as reductants.

The pre-steady-state type 1 Cu(II) reduction pattern is strikingly different when laccase is reduced with excess reductant before being mixed with dioxygen. Oxidation to steady-state levels is virtually complete within the mixing time of the instrument (the calculated half-time for oxidation under these conditions is 0.3 ms). A small undershoot is observed, i.e., a transiently higher than steady-state concentration of oxidized type 1 Cu(II). The steady state is established very rapidly even at high reductant concentrations (e.g., 10 mM hydroquinone) in marked contrast to the approach to the steady-state levels of reduced type 1 Cu when one begins with oxidized laccase. The kinetic changes observed at 340 nm for the type 3 site were unaffected by beginning with oxidized or reduced enzyme.

It is important to note that the steady-state concentration of reduced type 1 copper is significantly different than zero, as has also been noted from rapid-freeze ESR results (Andréasson & Reinhammar, 1979). A steady-state concentration of reduced type 1 Cu(I) of 10–12% was found to be independent of reductant concentration over the range 0.4–10 mM hydroquinone. Similar values were observed from either approach to steady state.

Anaerobic Rereduction of Reoxidized Laccase. According to current schemes which have been proposed to account for laccase kinetics (Andréasson & Reinhammar, 1976, 1979; Hansen et al., 1984), the overshoot described above corresponds to reduction of the type 1 Cu(II) in an inactive form, and the back-decay reflects a slow conversion to an active form of laccase. Since a slow transition is not observed when fully reduced protein is mixed with excess O_2, the reduced enzyme apparently behaves as being fully active. These results prompted an examination of the rereduction kinetics of laccase immediately following a single reduction–reoxidation cycle. Laccase was fully reduced with excess reductant and then mixed with 1 equiv of dioxygen. The rereducing condition of 1 mM ascorbate allowed essentially complete reoxidation of the type 1 and type 3 sites prior to the subsequent rereduction at pH 6.0, 7.4, or 8.5 (panels A, C, and E, respectively, of Figure 1). The copper reoxidation rates and the rate of the accompanying decrease in fluorescence are comparable and pH independent over this range (Hansen et al., 1984) (Figure 1A,C,E). (Note all fluorescence changes are shown in the reverse direction for comparisons to the absorbance changes.) Following the rapid reoxidation phase, a slower rereduction of the type 1 and type 3 sites and a fluorescence increase occur (Figure 1A,C,E). At pH 6.0, there is a pronounced turnover phase in type 1 Cu(II) reduction which is more pronounced than that with the resting enzyme (Figure 1A,B). Both type 3 Cu(II) reduction and the fluorescence enhancement are more notably faster than the net reduction of type 1 Cu(II) in comparison with the native oxidized protein (Figure 1B). The reduction rate constants were about double relative to those of the resting enzyme (Table I).

The turnover phase in the type 1 Cu(II) rereduction profile is less pronounced at pH 7.4 (Figure 1C). However, the overall disappearance of the type 1 Cu(II) relative to the type 3 Cu(II) or the fluorescence increase is faster at this pH than at pH 6.0. Under these conditions, with the resting enzyme, an inactive form is evident in the type 1 Cu(II) reduction profiles, but not in the type 3 reduction profiles (Figure 1D). At pH 8.5, the biphasic type 3 Cu(II) reduction and fluorescence increase observed with the resting enzyme are typical of the partially inhibited native protein (Figure 1F). These profiles

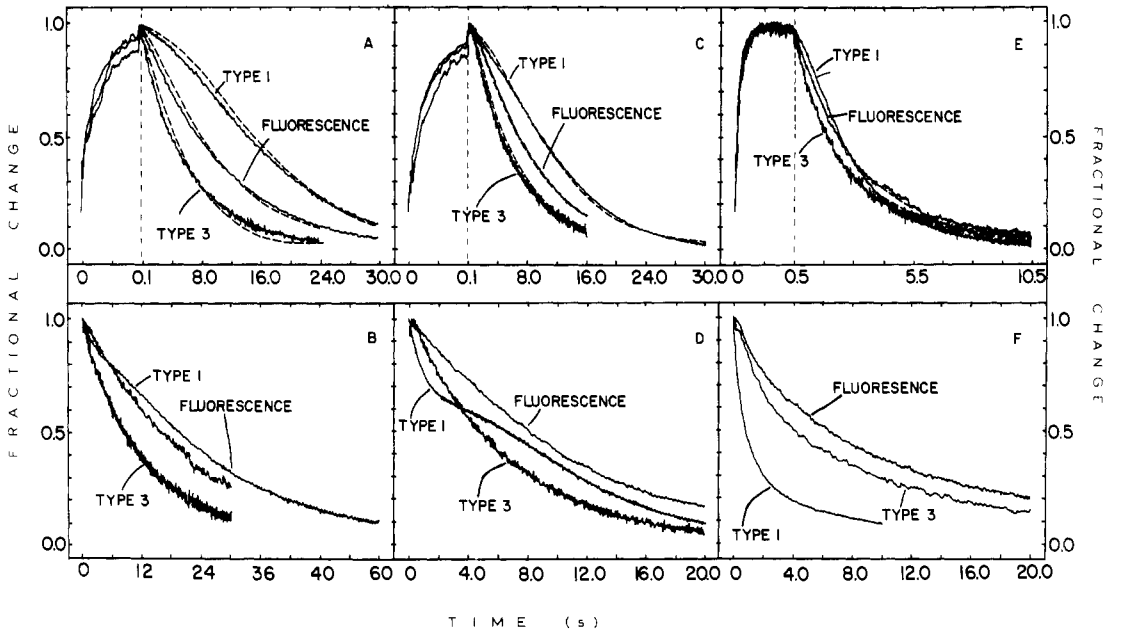


FIGURE 1: Effect of reoxidation on the anaerobic reduction of *Rhus* laccase by 1.0 mM sodium ascorbate at 25 °C. The protein (8.3–12.7 μM) was in sodium phosphate, pH 6.0 (A, B) or pH 7.4 (C, D), or in 0.1 M Tris–phosphate, pH 8.5 (E, F). (A, C, E) Anaerobic protein was incubated with excess reductant for 1–2 h at room temperature, and the anaerobic reduced protein was mixed with equimolar O_2 (cf. Materials and Methods). Note the differences in the time scale between the oxidation and reduction parts of the reaction. (B, D, E) As controls, the anaerobic *oxidized* protein was mixed with the anaerobic reductant. The types 1 and 3 kinetic curves are averages of 3 (A), 6 (B), 5 (C), 10 (D), 3 (E), and 5 accumulations (F). The fluorescence kinetic curves are averages of five (A), one (B), five (C), eight (D), four (E), and three accumulations (F). The dashed profiles in (A) and (C) were simulated according to Scheme I beginning with the (2, 1, $[2\text{-}2{:}O_n]^{1+}$) intermediate. The parameters used (numbered according to Scheme I) were the following: pH 6.0; $k_1 = 0.36\ s^{-1}$, $k_7 = 0.085\ s^{-1}$; pH 7.4; $k_1 = 0.33\ s^{-1}$, $k_7 = 0.22\ s^{-1}$, pH 8.5; $k_1 = 0.8\ s^{-1}$, $k_7 = 4\ s^{-1}$. The simulated patterns at pH 8.5 were congruent to the observed patterns.

Table I: Anaerobic Reduction or Reduction after Reoxidation of *Rhus* Laccase by 1.0 mM Sodium Ascorbate[a]

pH	obsd parameter[b]	k_1 (s^{-1})[c] after reoxidation	control k_1 (s^{-1})[c]	control k_2 (s^{-1})[c]	% fast phase[d]
6.0	1	7.23×10^{-2} [e]	4.32×10^{-2} [e]		
	3	1.62×10^{-1}	8.51×10^{-2}		
	F	9.3×10^{-2}	4.67×10^{-2}		
7.4	1	1.1×10^{-1} [e]	1.32×10^{-1} [e]		
	3	1.77×10^{-1}	1.52×10^{-1}		
	F[f]	1.07×10^{-1}	8.73×10^{-1}		
8.5	1	4.68×10^{-1} [e]	1.42	1.35×10^{-1}	63.1
	3	4.63×10^{-1}	5.35×10^{-1}	7.67×10^{-2}	32.0
	F	5.23×10^{-1}	3.63×10^{-1}	6.35×10^{-2}	30.1

[a] Reactions were performed in 0.1 M sodium phosphate buffer (pH 6.0 and 7.4) or 0.1 M Tris–phosphate (pH 8.5) at 25 °C as described under Materials and Methods (cf. Figure 1). [b] 1, 3, and F denote type 1, type 3, and fluorescence observations, respectively. [c] Data sets were fitted to $ae^{-k_1 t} + c$ or to $ae^{-k_1 t} + be^{-k_2 t}$. [d] $(a \times 100)/(a + b)$. [e] Only the last exponential part of the multiphase reaction was used for fitting. [f] This reaction (actually monophasic) had an artifactually offset base line.

are monophasic following the single reduction–reoxidation cycle at pH 8.5 (Figure 1E). Thus, a single turnover is apparently sufficient to convert laccase to fully active species. Activation is detected in type 1 Cu(II) reduction by an initial turnover phase rather than the initial rapid, net reduction seen with the resting enzyme.

Anaerobic Rereduction at Various Times after Reoxidation. The differences in the reduction kinetics of the native and reoxidized enzymes suggest that the kinetic profiles of the latter will relax to the kinetics of the resting enzyme. The time dependence for this relaxation process at pH 7.4 was determined by reoxidizing reduced laccase with equimolar O_2. At various times thereafter, the enzyme was rereduced with $Ru(NH_3)_6^{2+}$ at a concentration which gives biphasic type 3 reduction (and fluorescence increase) kinetics with the resting enzyme (Hansen et al., 1984). These experiments were performed at 5 °C where the reoxidations of the type 1 and type 3 copper sites were complete within 5 s, but the rate of transformation from activated to resting kinetic profiles was slow enough to monitor.

At early times after reoxidation, a prominent turnover phase in the type 1 Cu(II) reduction profile is detected which gradually disappears with increasing time after reoxidation (Figure 2A). The type 1 Cu(II) rereduction patterns progressively approach that of the anaerobic reduction profile of the resting enzyme (Figure 2A, control). Analogous trends are observed in the reduction patterns of the type 3 Cu(II) (Figure 2B) and the fluorescence enhancement associated with reduction (Figure 2C). At very early times after reoxidation, both exhibit monophasic kinetics (with a small back-decay in type 3 reduction). These patterns gradually transform to the biphasic kinetic characteristics of the resting enzyme (Figure 2B,C, controls). None of the profiles returns completely to the kinetics of the native oxidized enzyme over these time frames. Apparently, an additional very slow change must occur to reach the native state. Qualitatively similar results are obtained with 10 mM hydroquinone.

The rate of the transformation was estimated from the data for type 1 reduction by assuming that a slow first-order conversion from fully active to mixed active plus inactive laccase

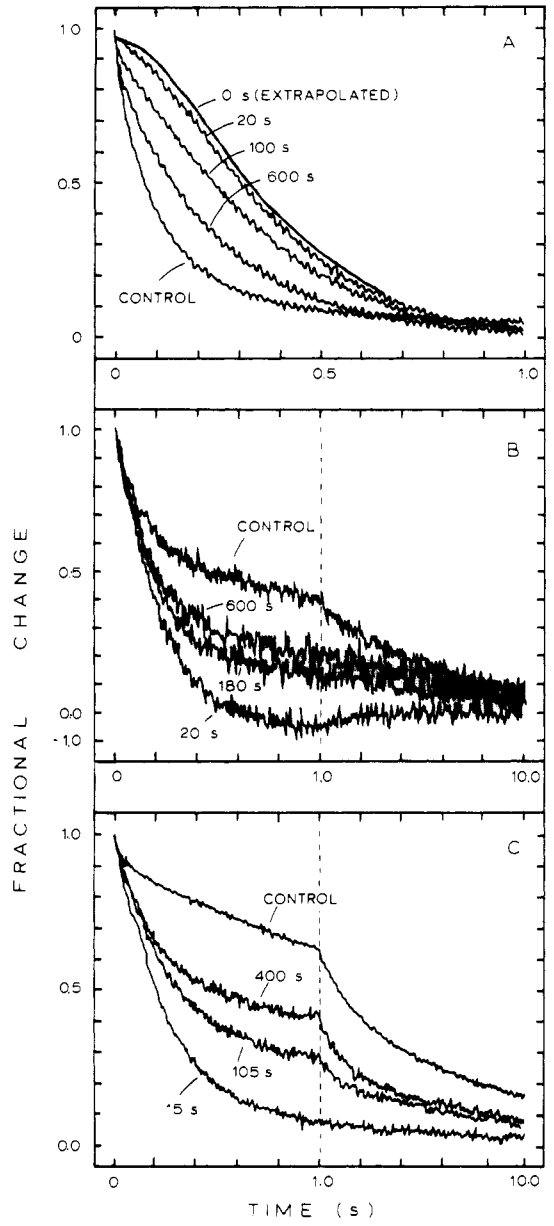


FIGURE 2: Anaerobic rereduction of *Rhus* laccase by 1.0 mM Ru-$(NH_3)_6^{2+}$, pH 7.4, 5 °C, at various times after oxidation of the reduced enzyme. Time-labeled curves: The protein in sodium phosphate buffer was incubated with 4.5 equiv/mol of enzyme of sodium ascorbate for 3 h. The *reduced* protein was mixed with equimolar oxygen (cf. Materials and Methods), and the freshly oxidized protein was then mixed with the reductant at the indicated times after oxidation. The final protein concentration was approximately 10 μM. The type 1 (A), type 3 (B), and fluorescence kinetic reduction profiles (C) all represent one accumulation. Note the change in time scale at 1.0 s in panels B and C. The solid line (panel A) represents the reduction of type 1 Cu(II) extrapolated to zero time after oxidation. "Control" curves: These profiles were generated by mixing anaerobic, native, *oxidized* protein (9 μM) with anaerobic reductant under otherwise identical conditions. All kinetic profiles are averages of six accumulations.

accounts for the transformation (Appendix). The calculated rate of transformation at various times of rereduction is indeed constant, (6.59 ± 0.62) × 10^{-3} s^{-1}. Similar transformation rate constants were calculated from type 3 reduction and fluorescence increase profiles (Table II).

Rate of Reoxidation of Type 2 Cu(I). The rate constant for reoxidation of type 2 Cu(I) at pH 7.4, 25 °C, has been shown to be about 0.05 s^{-1}, which also corresponds to the rate of disappearance of an oxygen radical intermediate (Aasa et al., 1976a; Brändén & Deinum, 1978). Since the calculated

Table II: Rates of Oxygen Radical Disappearance and Type 2 Cu(I) Oxidation and Rates of Transformation from Activated to Resting Kinetics of Type 1, Type 3, and Fluorescence at pH 7.4, 5 °C

rate process	rate (s^{-1} × 10^3)
oxygen radical disappearance[a]	8.3
type 2 Cu(I) oxidation[b]	9.5
transformation of fluorescence reduction profiles[c]	9.5
transformation of type 1 reduction profiles[d]	6.6 ± 0.6
transformation of type 3 reduction profiles[e]	6.5

[a] From Figure 3B. [b] From Figure 3A. [c] Fit of the data in Figure 2C at 1 s after reduction. [d] See the Appendix. [e] Fit of the data in Figure 2B at 1 s after reduction.

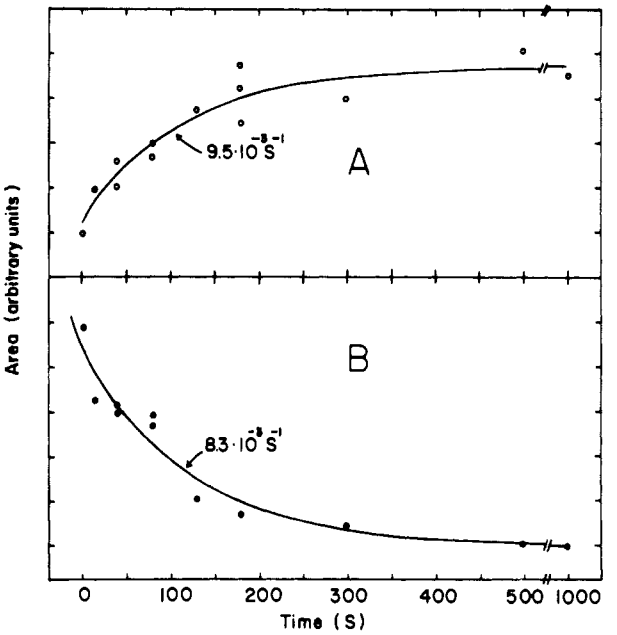
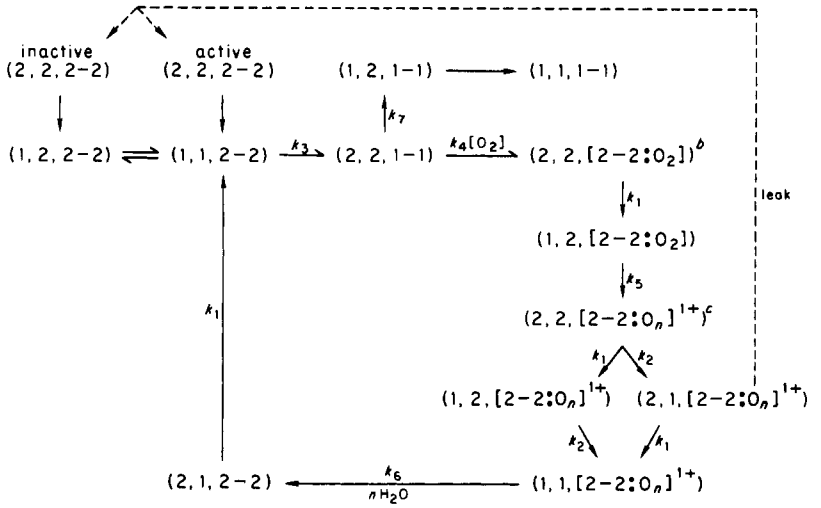


FIGURE 3: Rate of oxidation of type 2 Cu(I) and disappearance of the oxygen radical after mixing reduced *Rhus* laccase (170 μM) with excess O_2 (0.95 mM) at pH 7.4, 5 °C. The anaerobic protein, in 0.1 M sodium phosphate buffer, was incubated with 4 equiv/mol of protein of sodium ascorbate for 2 h and then mixed with O_2 as described under Materials and Methods. ESR spectra were recorded at 13 K, 9.04–9.05 GHz. (A) Type 2 Cu(I) oxidation. The concentration of type 2 Cu(II) was calculated by integrating the low-field type 2 signal (Materials and Methods). The solid line and the rate constant are the best fits of the data points to $ae^{-kt} + b$ (cf. Materials and Methods). (B) Disappearance of the oxygen radical. The radical concentrations were estimated by integrations of the signal at 3400–4500 G. The solid line and the rate constant are the best fits of the corrected areas to ae^{-kt}.

constants for the relaxation to the kinetics of the resting enzyme are (6.5–9.5) × 10^{-3} s^{-1}, the rate constant for type 2 Cu(I) reoxidation at 5 °C could occur at a comparable rate. The rates of type 2 Cu(I) oxidation and oxygen radical disappearance were obtained by ESR (see Materials and Methods) (Figure 3). The rates of type 2 Cu(I) reoxidation and oxygen radical disappearance were comparable to the transformation rates (Table II).

Effect of Reductant Concentration on the Rate of Type 2 Cu(I) Reoxidation. Assuming a catalytic role for the type 2 Cu, (Andréasson & Reinhammar, 1976, 1979; Malmström, 1982; Reinhammar & Oda, 1979; Morpurgo et al., 1980), the type 2 Cu(II) may be expected to reach its steady-state concentration as rapidly as the type 1 copper does when the steady

Scheme I: Proposed Reaction Sequences for Anaerobic, Post-Steady-State, and Aerobic Steady-State Reduction[a]



[a] Active and inactive forms which are not in equilibrium are proposed to account for the anaerobic reduction data (Hansen et al., 1984). The branch following the (2, 2, [2-2:O_n]$^{1+}$) state accounts for the steady-state concentrations of reduced type 1 and type 2 Cu (see text). The indicated leak reactions convert activated to resting enzyme in the experiments represented by Figure 2. [b] Peroxy intermediate. [c] Oxygen radical intermediate.

state is approached beginning with reduced enzyme. This possibility was examined by adding O_2 to anaerobic laccase plus excess hydroquinone in an ESR tube at 5 °C and then freezing the reaction mixture at various times after mixing. Freezing was completed in 5 s, and the steady state lasted about 40 s under these conditions. ESR spectra of mixtures frozen as early as 4–9 s after mixing had a well-developed low-field type 2 signal, and the magnitude of this signal did not change significantly during the steady state. Thus, the type 2 copper attained its steady-state oxidation states within 10 s in the presence of excess reductant and oxygen, in marked contrast to the results in the absence of excess reductant, where it took 80 s to reach half-oxidation (Figure 3). This confirms that the type 2 Cu(I) is only rapidly oxidized during type 3 site reduction. The steady-state concentrations of reduced type 1 and type 2 were both estimated to be 30% under these conditions. The difference between this result at 5 °C and that at 20 °C given above may be due to the temperature difference or oxidation-state changes associated with freezing (Morpurgo et al., 1981).

Discussion

The results reported here clearly demonstrate that immediately following one reduction–reoxidation cycle, laccase does not exhibit the inhibitory, rate-limiting step that is observed with the native oxidized enzyme at pH 7.4. An inhibitory step is detected in the approach to steady-state experiments as an overshoot in type 1 Cu(II) reduction. The inhibitory step is detected during anaerobic reduction as a slow, substrate-independent reduction of type 3 Cu(II) and a slow, concurrent increase in fluorescence. An important characteristic of the reduction–reoxidation effect is the gradual return to the kinetic characteristics of native laccase (Figure 2).

The effects of reduction–reoxidation are apparently related to the oxidation state of the type 2 copper site. Both type 1 and type 3 Cu(I) sites are oxidized very rapidly (<1 s), whereas the half-times for type 2 Cu(I) oxidation and oxygen radical disappearance are both approximately 75 s at pH 7.4, 5 °C (Figure 3). Thus, when reduction is initiated soon after the reoxidation, the initial species being reduced is (2, 1, [2-2:O_n]$^{1+}$), whereas after longer waiting times the initial species is (2, 2, 2-2)[1] (see Scheme I). [While the net charge at the type 3 site is known to be 1+, the distribution of electrons and the state of the oxygen are unknown (Malmström, 1982).] The (2, 1, [2-2:O_n]$^{1+}$) species exhibits activated kinetics while the (2, 2, 2-2) species exhibits the kinetic characteristics of the resting enzyme. Clearly, the initial oxidation state of the type 2 site influences the rate of type 3 site reduction and the turnover rate of the type 1 site. The fact that the rate constant determined for the transformation from uninhibited to inhibited kinetic profiles equals the rate constant for type 2 Cu(I) oxidation quantitatively substantiates this inference.

Biphasic type 3 reduction and fluorescence increase kinetics have been attributed to a slow, substrate-independent step which precedes the reduction of the type 2 Cu(II) site (Andréasson & Reinhammar, 1976, 1979; Hansen et al., 1984). That the type 3 reduction and fluorescence increase profiles are monophasic when the (2, 1, [2-2:O_n]$^{1+}$) species is further reduced is easily rationalized in these terms. The slow step is simply bypassed when the type 2 copper is already reduced [assuming that type 2 is not oxidized by the radical (Reinhammar, 1981)].

The (2, 1, [2-2:O_n]$^{1+}$) species may also be a pivotal reaction intermediate in the normal, steady-state catalytic mechanism. Kinetic schemes which have been proposed for laccase cannot account for the steady-state concentrations of the reduced type 2 site. Since the scheme proposed by Farver et al. (1980, 1981) predicts that each enzymic state which contains reduced type 2 copper is short-lived, the type 2 site is predicted to be 100% oxidized under aerobic steady-state conditions. Similarly, if the rate of type 3 reduction by reduced type 1 and type 2 is greater than 560 s^{-1} at pH 7.4, 25 °C, as suggested by anaerobic reduction and steady-state kinetics (Andréasson & Reinhammar, 1976, 1979; Peterson & Degn, 1978), the type 2 site should also be fully oxidized by the scheme proposed by Andréasson and Reinhammar under conditions in which type 1 reduction is rate limiting. Yet, the steady-state concentration of reduced type 2 Cu was found to be 30% at pH 7.4, 5 °C, with 2.5 mM hydroquinone, in agreement with earlier results. If the (2, 1, [2-2:O_n]$^{1+}$) species were an in-

[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.

termediate, it would contribute significantly to the steady-state concentration of reduced type 2 (see below). Current concepts of laccase kinetics also cannot account for the observed steady-state concentration of reduced type 1 copper since oxidation of type 1 Cu(I) occurs after very rapid substrate-independent steps which must all have rate constants ≥ 560 s^{-1}, which is the substrate-independent turnover rate (Peterson & Degn, 1978).

The above discussion indicates that refinements of current concepts of laccase kinetics at pH 7.4 must account for (a) the effects of one reduction–reoxidation cycle on the kinetics of type 3 reduction, type 1 turnover, and fluorescence increase, (b) the observed concentrations of reduced type 1 and type 2 copper under steady-state conditions, (c) the steady-state concentration of reduced type 1 being substrate independent, (d) the high steady-state concentration of the oxygen radical intermediate (Andréasson et al., 1973a, 1976; Aasa et al., 1976a,b; Brändén & Deinum, 1978), and (e) the post-steady-state reduction profiles of the type 1 Cu(II), the type 3 Cu(II), and the fluorescence increase (Andréasson & Reinhammar, 1976; Holwerda & Gray, 1974).

The tentative scheme (Scheme I)[1] involves the following assumptions: (a) As proposed by Andréasson & Reinhammar (1979), both type 1 Cu(I) and type 2 Cu(I) are involved in the reduction of the type 3 Cu(II) pair. The fact that pre-reduction of the type 2 site has such striking consequences further supports the thesis that type 1 reduction precedes type 2 reduction under anaerobic conditions or during the pre-steady-state period. (b) Biphasic anaerobic reduction and pre-steady-state overshoot phenomena are accounted for by two enzyme forms not in equilibrium as previously proposed (Hansen et al., 1984). (c) As proposed by Pecht (1969) and Malmström (1982), the peroxide intermediate is rapidly reduced by oxidation of type 1 Cu(I). The oxidation state of the type 2 site during this process is unknown. (d) Reduction of the oxygen intermediate is associated with both type 1 and type 2 sites being in their reduced states. This restriction is represented as a bifurcation at (2, 2, $[2\text{-}2{:}O_n]^{1+}$) in Scheme I and is included to account for the steady-state concentrations of the oxygen radical species, reduced type 1 Cu and reduced type 2 Cu. Note that since the rates for formation and disappearance are both substrate dependent, the concentrations of reduced type 1 and type 2 are unaffected by reductant concentration over a wide range of substrate concentrations as observed experimentally. The right side of the branch may be omitted, but this requires that the rates of reduction of (1, 2, $[2\text{-}2{:}O_n]^{1+}$) and (2, 1, 2-2) be exactly identical to account for the identical steady-state concentrations of type 1 and type 2. (e) Following reduction of the oxygen radical intermediate, the type 2 Cu remains reduced; i.e., the (2, 1, 2-2) state is an important intermediate in the normal steady-state catalytic cycle. This restriction is required to account for monophasic type 3 reduction after a single turnover or in the post steady state. Since the fully oxidized enzyme or the enzyme state in which only the type 1 site is reduced is only attained via slow "leak" reactions, and is therefore only exhibited by the resting or pre-steady-state enzyme, the slow steps in type 3 reduction are bypassed in the post steady state. Excluding participation by the fully oxidized enzyme during steady-state turnover would appear to be a necessary requirement in any model to account for the experimental facts noted above.

Transformation from biphasic to monophasic kinetics was addressed earlier to rationalize monophasic type 3 reduction profiles observed under post-steady-state conditions. Andréasson & Reinhammar (1976, 1979) proposed that the rate of converting active to inactive oxidized laccase was slow; and therefore, monophasic type 3 reduction was expected in the post steady state. However, recent pH-jump experiments showed that the slow-equilibrium assumption cannot be valid (Hansen et al., 1984). Pecht and co-workers noted that there were pronounced spectral differences between native, oxidized and postturnover, oxidized laccase (Farver et al., 1980; Goldberg et al., 1980; Goldberg & Pecht, 1978). A catalytically active C state was thus proposed which could exhibit monophasic kinetics. The C state is relatively long-lived (at least 24 h) by spectral criteria. However, as can be seen in Figure 2, the return to biphasic kinetics is relatively rapid. Thus, the reoxidized protein exhibits inhibited kinetics even while in the C state (Figure 2).

If the postulated kinetic loop occupied during steady-state conditions is considered first, a rate law is derived by assuming that each of the type 1 Cu(II) reduction steps has the same rate constant [the same assumption was made for each type 2 Cu(II) reduction step]. The expression is further simplified when the rate of type 1 reduction is much less than the rates of all intramolecular electron-transfer steps, as pertained here. The form of the rate law is identical with that derived by Peterson & Degn (1978), which they showed fits the steady-state kinetic data. The rate law also correctly predicts that the steady-state concentrations of reduced type 1 and 2 Cu are independent of the substrate concentration at the reductant concentrations used here. Profiles from computer simulations of the approach to steady state from the oxidized enzyme have the correct form, and the observed pre-steady-state undershoot when beginning with reduced enzyme is also predicted from Scheme I. The validity of the rate law was further tested by calculating the predicted steady-state concentrations of reduced type 1 and type 2 Cu when their concentrations are equal as observed. A value of 31% was obtained which is in excellent agreement with the experimentally determined value. Under these conditions, the concentration of the oxygen radical intermediate is predicted to be 54%, which also agrees with the observed concentration and reported value (Andréasson & Reinhammar, 1979).

Computer simulations of the rereduction kinetics following the single reduction–reoxidation cycle (Figure 1) were also attempted as previously described (Hansen et al., 1984). This focuses on the following steps involved in the overall reaction sequence shown in Scheme I:

$$(1,1,1\text{-}1) \xrightarrow{O_2} (2,1,[2\text{-}2{:}O_n]^{1+}) \xrightarrow{\text{ascorbate}} (1,1,1\text{-}1)$$

The observed Cu(II) reduction and fluorescence increase patterns at pH 6.0, 7.4, and 8.5 are approximated well by Scheme I (Figure 1). While the actual rate constants for the k_1 steps in Scheme I are unknown, the predicted values of k_7 are in good agreement with the experimental values for the final type 1 reduction step given in Table I [pH 6.0, 0.085 s^{-1} (predicted), 0.072 s^{-1} (determined); pH 7.4, 0.22 s^{-1} (predicted), 0.11 s^{-1} (determined)]. The experimental value at pH 8.5 appears to be influenced by a relatively slow substrate-independent type 3 reduction at this pH. In summary, while other schemes are undoubtedly possible, Scheme I represents a reaction sequence which reasonably approximates the rereduction results and the steady-state concentrations of intermediates detected thus far.

A questionable aspect of the proposed scheme is the mandatory requirement for both type 1 and type 2 reduction after O_2 binding, but prior to reducing the oxygen radical intermediate. Since the oxygen radical is consumed by type 2

depleted laccase (Reinhammar & Oda, 1979; Reinhammar, 1981), reduced type 2 would not appear to be required. However, the exact mechanism and products under type 2 depleted conditions are not well characterized, and water product formation or release has not been demonstrated. It also is quite possible that when the type 2 copper is present, it must be in its reduced state to provide a protein state which is required for reduction of the radical. In any event, if the proposed substrate-dependent steps which lead to the (1, 1, $[2\text{-}2{:}O_n]^{1+}$) state are not involved, the steady-state concentrations of reduced type 1 and type 2 would need to be accounted for by other reduction steps which lead to relatively long lifetimes of reduced type 1 and type 2 Cu. These steps apparently must occur between O_2 binding and H_2O release.

The observed effects of a single reduction–reoxidation cycle on the fluorescence kinetics help clarify the significance of the fluorescence changes and their origin. The fluorescence kinetic profiles are as characteristic of the active and inactive states as the Cu reduction profiles (Figure 1). This substantiates their potential value as probes of kinetically relevant protein conformation changes (Hansen et al., 1984). Under activated conditions, the fluorescence increases always follow the same form as type 3 reduction and always proceed at a slower overall rate than type 3 reduction, but faster than type 1 Cu(II) reduction. During postturnover rereduction, the fluorescence increases are apparently associated with two separate reduction steps as was also deduced for anaerobic reduction (Hansen et al., 1984). Moreover, the same relative fluorescence intensities were assumed in the simulations shown in Figure 1 as were previously assumed to account for anaerobic reduction data (Hansen et al., 1984).

Malmström (1982) has suggested possible parallels between laccase and cytochrome *c* oxidase kinetics. In a similar vein, the similarity of the scheme proposed here for laccase to the proposal for the catalytic scheme of cytochrome *c* oxidase by Antonini et al. (1977) can be noted. Since anaerobic reduction of fully oxidized, "resting" cytochrome *c* oxidase was found to be slow relative to the rate of reduction of the reduced–reoxidized enzyme, the fully oxidized state was suggested not to participate in the catalytic cycle. However, in contrast to what has been proposed for laccase, the fully reduced state was proposed as a transient intermediate, and the heme *a* ESR undetectable Cu(II) pair analogue of the laccase type 3 Cu(II) pair could remain reduced even in the presence of oxygen intermediates according to their model (Antonini et al., 1977).

Appendix

Consider the following reaction sequence:

$$A_1 \xrightarrow{k_1} A_2 \xrightarrow{k_2} \cdots \xrightarrow{k_{i-1}} A_i \xrightarrow{k_i} \cdots \xrightarrow{k_{n-1}} A_n$$

The initial conditions are $A_1(0) = A_1^0$ and $A_i(0) = 0$ ($2 \le i \le n$). The time dependence for $A_i(t)$ is given by the familiar $A_i(t) = (A_1^0)^{-k_1 t}$. The $A_i(t)$ value ($2 \le i \le n$) may be derived by solving the linear first-order differential expression:

$$\frac{dA_i}{dt} = k_{i-1}A_{i-1} - k_iA_i$$

This is straightforward once $A_{i-1}(t)$ is known. We find

$$A_2(t) = \frac{k_1A_1^0}{k_2 - k_1}(e^{-k_1t} - e^{-k_2t})$$

Then

$$A_3(t) = \frac{k_1k_2A_i^0}{k_2 - k_1}\left[\frac{1}{k_3 - k_1}(e^{-k_1t} - e^{-k_3t}) - \frac{1}{k_3 - k_2}(e^{-k_2t} - e^{-k_3t})\right]$$

Table III: Rate of Transformation from Activated to Resting Kinetics of Type 1 Reduction Profiles at Various Times after Initiation of Reduction at pH 7.4, 5 °C[a]

time after start of rereduction ($s \times 10^1$)	% oxidation: type 1 at t_∞ after oxidation[b]	% oxidation: type 1 at t_0 after oxidation[c]	rate of transformation[d] ($s^{-1} \times 10^3$)
0.25	79.94	94.38	6.066
0.50	71.57	93.79	7.697
0.75	62.97	90.91	6.774
1.00	59.85	88.92	7.601
1.25	51.55	84.21	6.800
1.50	47.15	79.96	6.748
2.00	37.98	72.01	6.500
2.50	31.02	62.45	6.300
3.00	25.30	53.56	6.166
3.50	20.88	44.97	5.815
4.00	16.81	38.23	6.020
			6.59 ± 0.62[e]

[a] Reactions were performed as described in Figure 2 and under Materials and Methods. Five reduction curves initiated at 20, 60, 100, 250, and 600 s after oxidation were used. The normalized absorbancies at various fixed times after the start of reduction were fitted to $ae^{-k_1t} + b$ (t signifies the time after oxidation). See the Appendix for treatment. [b] Corresponds to b in the equation given in footnote *a*. [c] Corresponds to $a + b$ in the equation shown in footnote *a*. [d] Corresponds to k_1. [e] Average. The average percent error of the fit was ≤1%.

Any $A_i(t)$ value ($3 \le i \le n-1$) may be conveniently obtained by multiplying $A_{i-1}(t)$ by k_{i-1} and substituting all exponential terms, e^{-k_it} ($1 \le j \le i-1$), of this product by $(e^{-k_it} - e^{-k_jt})/(k_j - k_i)$. Thus, the expression for $A_i(t)$ or any linear combination of $A_i(t)$ values will be strictly proportional to A_1^0, the initial concentration of A_1.

Consider now the reaction

$$\xleftarrow{k_{leak}} (2, 1, [2\text{-}2{:}O_n]^{1+}) \xrightarrow{k_1} (1, 1, [2\text{-}2{:}O_n]^{1+}) \xrightarrow{k_2}$$
$$(2, 1, 2\text{-}2) \xrightarrow{k_3} (1, 1, 2\text{-}2) \xrightarrow{k_4} (2, 2, 1\text{-}1) \xrightarrow{k_5} (1, 2, 1\text{-}1)$$

Under the conditions of the experiment shown in Figure 2, k_{leak} is 2 orders of magnitude lower than k_1, and its effect on the reduction profiles may be neglected. The reduction profile for type 1 Cu(II) is obtained as a linear combination of the equations for (2, 1, $[2\text{-}2{:}O_n]^{1+}$), (2, 1, 2-2), and (2, 2, 1-1) as described above. The reduction profile for type 1 absorption may be expressed as $I_i(t) = \epsilon^{615}A_1f_1(t^R)$ in which t^R is the time after the initiation of reduction, A_1 is the concentration of (2, 1, $[2\text{-}2{:}O_n]^{1+}$) at $t^R = 0$, $f_1(t^R)$ is the sum of exponentials such as $A_2(t)$ and $A_3(t)$ given above, and ϵ^{615} is the extinction coefficient for type 1 Cu(II). The time-dependent expression for the reduction of type 1 Cu(II) in the two-form model may be derived similarly and may be expressed as $I_2(t^R) = \epsilon^{615}A_2[\alpha f_{2,1}(t^R) + (1 - \alpha)f_{2,2}(t^R)]$. α is the fraction of A_2 of the total concentration of (2, 2, 2-2) at $t^R = 0$, committed to the inactive path.

However, A_1 and A_2 change during the waiting period since $A_1 \xrightarrow{k_{leak}} A_2$ (cf. Discussion); thus, we expect $A_1(t^o) = A_{tot}e^{-k_{leak}t^o}$ and $A_2(t^o) = A_{tot}(1 - e^{-k_{leak}t^o})$ in which t^o is the time after the start of oxidation. If t^R is fixed, we get $I_1(t^o) = A_1(t^o)C_1 = C_1'e^{-k_{leak}t^o}$ and $I_2(t^o) = A_2(t^o)C_2 = C_2'(1 - e^{-k_{leak}t^o})$; the "$C$'s" denote constants. Since the observed type 1 Cu(II) absorption is a sum of I_1 and I_2, k_{leak} can be estimated by fitting the observed absorbancies at a fixed t^R to $Ce^{-k_{leak}t^o} + C_2$. It follows that k_{leak} should be independent of the value for the fixed t^R. Table III shows the rates of transformation from activated to resting kinetics of type 1 reduction profiles at various times after initiation of reduction.

Registry No. Cu, 7440-50-8; L-ascorbic acid, 50-81-7; laccase, 80498-15-3.

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Effect of Trypsin Binding on the Hydrogen Exchange Kinetics of Bovine Pancreatic Trypsin Inhibitor β-Sheet NH's†

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ABSTRACT: The hydrogen–deuterium exchange rates of the slowest exchanging β-sheet NH's in the bovine pancreatic trypsin inhibitor (BPTI) have been determined in free BPTI and in the trypsin–BPTI complex at pH 9–10, 25–40 °C. Rate constants for individual protons have been measured from their assigned resonances in the ^{1}H NMR spectrum. Trypsin binding has a highly localized effect; the Tyr-35 NH exchange rate is slowed by a factor of $>10^3$ in the complex, while the other NH's measured are slowed by a factor of 3–15. In free BPTI, under conditions where the exchange rate constants have activation energies in the range 11–32 kcal/mol, the NH's of Tyr-21, Phe-22, and Tyr-23 are several orders of magnitude slower than the other β-sheet NH's.

The exchange of isotope between water hydrogens and labile protein hydrogens is a measure of the accessibility of solvent to the protein's internal sites. The largest group of exchangeable protons in proteins are peptide NH's. Exchange rate constants of NH's in extended polypeptides at pH 3 and 25 °C are around 10^{-1} min^{-1}, while exchange rate constants of NH's in folded proteins under the same conditions vary from about 10^{-1} to 10^{-10} min^{-1}. Although labile protons in folded proteins are clearly shielded from solvent, the fact that buried peptide NH's exchange with finite rates indicates that proteins undergo internal fluctuations that expose buried regions of the protein to solvent (Hvidt & Nielsen, 1966; Englander et al., 1972; Woodward & Hilton, 1979; Woodward et al., 1982; Barksdale & Rosenberg, 1982).

Traditionally, measurements of protein hydrogen exchange kinetics average over all exchangeable protons (Englander & Englander, 1978). More recently, assignments of average NH exchange rates to segments of the protein sequence have been made by rapid proteolysis methods (Rosa & Richards, 1981; Englander et al., 1980). Now, with the development of procedures for assigning NMR NH resonances (Dubs et al., 1979; Delpierre et al., 1982; Kuwajima & Baldwin, 1983), it is

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