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KINETIC STUDIES OF *RHUS VERNICIFERA* LACCASE

ROLE OF THE METAL CENTERS IN ELECTRON TRANSFER

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Summary

The reactions of *Rhus vernicifera* (monophenol,dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) with the reducing substrates hydroquinone and ascorbic acid have been investigated with the stopped-flow technique.

Rhus laccase appears to be present in two molecular forms with a pH-sensitive equilibrium constant regulating the relative concentrations of each species.

A model for the reaction of *Rhus* laccase with reducing substrates has been formulated. The model is similar to one formulated earlier for the anaerobic reduction of laccase from *Polyporus versicolor* (Andréasson, L.-E., Malmström, B.G., Strömberg, C. and Vänngård, T. (1973) Eur. J. Biochem. 34, 434—439) and accounts for the reduction also of this enzyme.

The essentials of the model are as follows: Electrons are taken up from reductants one at a time. The type 1 Cu^{2+} has a central role in mediating the transfer of at least one of the electrons needed for the reduction of the cooperative two-electron acceptor. Intramolecular reactions determine the concentrations of two molecular forms of the enzyme and influence the rate of reduction of the two-electron acceptor. The model, which has been used for successful simulations of the anaerobic reduction of *Rhus* laccase, is capable of explaining the reduction of laccases also in the presence of the inhibitor F^-. In addition, the model gives an explanation of the behaviour of the laccases when reducing substrates and O_2 are simultaneously present and is consistent with earlier observations of the post-steady-state reduction of the type 1 Cu^{2+} and the two-electron acceptor (Holwerda, R.A. and Gray, H.B. (1974) J. Am. Chem. Soc. 96, 6008—6022).

Introduction

The copper in (monophenol,dihydrophenylalanine:oxygen oxidoreductase, EC 1.14.18.1) is present in three different forms. The oxidation state of the type 1 and 2 copper can be followed by EPR spectroscopy. In addition, the type 1 Cu^{2+} strongly absorbs light at around 610 nm. A cooperative two-electron acceptor associated with a 330-nm absorbance band is believed to be formed by the two remaining, non-paramagnetic copper ions. A detailed account of the properties of the three different forms of copper in "blue" oxidases can be found in a recent review [1].

In laccase from the fungus *Polyporus versicolor* the type 1 Cu^{2+} has been shown to be the first electron acceptor to be reduced by substrates [2—4]. In this enzyme the two-electron acceptor (the type 3 copper) appears to be reduced via the type 1 copper, i.e. at least one of the required electrons enters by way of the type 1 copper [3]. The inhibitor F^- which binds to the type 2 Cu^{2+}, inhibits the electron transfer between the type 1 copper and the two-electron acceptor [3], but the exact mechanism of inhibition is not known. Recently, in anaerobic reduction experiments with fungal laccase, observations have been made which imply participation also of the type 2 copper in the electron transfer [4].

Holwerda and Gray have recently published results from kinetic experiments with laccase from the lacquer tree *Rhus vernicifera* [5] which they interpret as showing that electron transfer through the type 1 copper is not necessary for the reduction of the two-electron acceptor in the catalytic mechanism of this enzyme. Supported by the results in the present study, we suggest that this conclusion is incorrect and maintain that the model for the anaerobic reduction originally given for fungal laccase [3], with the incorporation of a minor modification, is valid also for *Rhus* laccase. In addition, the model is able to give an explanation of the behaviour of the laccases when reducing substrate and O_2 are simultaneously present or when inhibitors, such as F^-, are bound to the enzymes.

Materials and Methods

Rhus vernicifera laccase was prepared from lacquer acetone powder (Saito and Co., Ltd., Tokyo) by the method of Reinhammar [6]. The concentration of enzyme was determined from the absorbance difference, oxidized minus reduced, at 615 nm (molar absorbance 5500 $M^{-1} \cdot cm^{-1}$) [7].

Analytical grade ascorbic acid (British Drug Houses Ltd., Poole, England) and hydroquinone (Schuchart Chemical Company, Munich, G.F.R.), recrystallized, were used. Analytical grade chemicals and deionized water were used for the preparation of solutions. The pH of the solutions was adjusted to the proper value before degassing. Phosphate buffer of 0.25 M ionic strength was used in all experiments.

The technique for the preparation of anaerobic solutions as well as the stopped-flow apparatus were the same as described earlier [3,8]. Under the conditions employed for the anaerobic reduction experiments the O_2 concentration is below 0.3 μM.

All kinetic experiments were performed at 25°C. An IBM 360/65 computer was used for the simulation of reaction courses.

Results

Anaerobic reduction of Rhus laccase by hydroquinone and ascorbate

The reaction of *Rhus* laccase was studied at concentrations of hydroquinone or ascorbate ranging from 50 μM to 50 mM at enzyme concentrations of 10—20 μM. Most of the experiments were carried out close to the optimum pH for catalytic activity, 7.4 [9]. The results obtained with hydroquinone have been chosen to illustrate the general behaviour of the enzyme towards reducing substrates. The 615-nm absorbance of the type 1 Cu^{2+} generally decreases in a rather complicated manner (Fig. 1E). Initially, there is a relatively rapid loss of

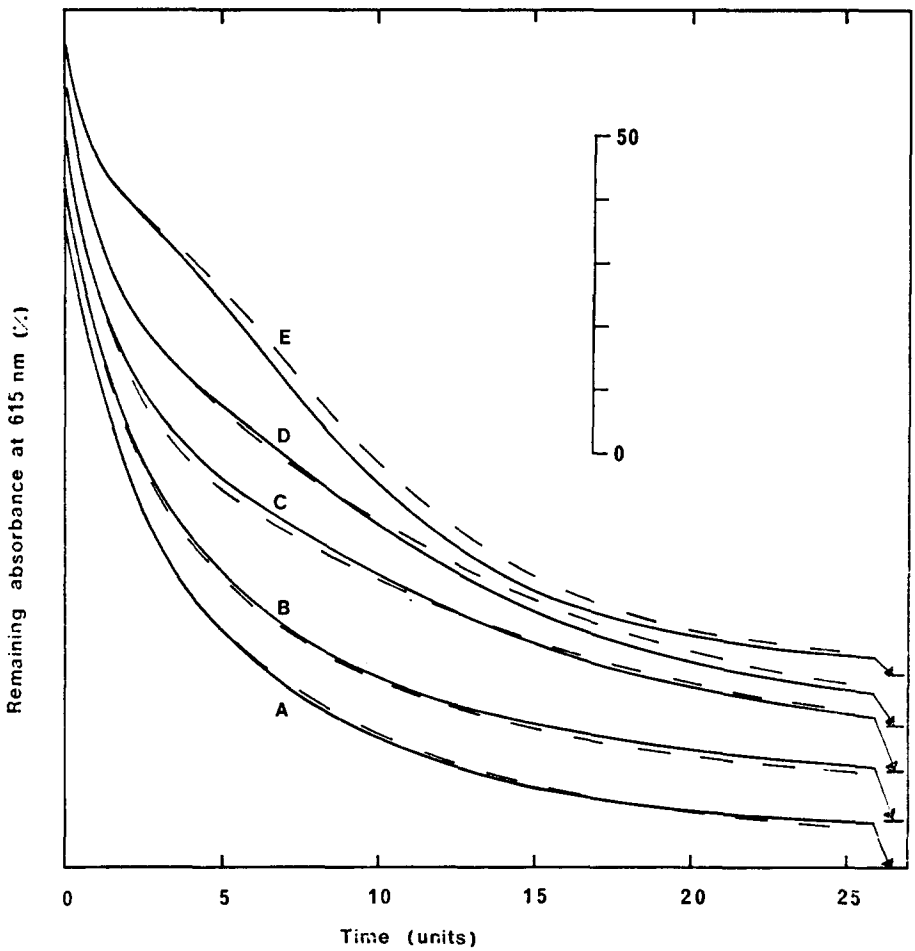


Fig. 1. Time courses of the anaerobic reduction of type 1 copper at pH 7.4. Hydroquinone concentrations were: 10 mM (A), 5 mM (B), 1 mM (C), 0.5 mM (D) and 0.1 mM (E). Full lines show the experimental results. Dashed lines represent simulations according to Scheme 1 with rate constants shown in Table III. Enzyme concentration was 15 μM. Curves are displaced vertically for clarity. The time scale is 0.02, 0.04, 0.2, 0.4 and 2 s per unit for curves A—E, respectively.

blue colour, the rate of which is proportional to both enzyme and substrate concentrations. The experimentally determined rate constants are shown in Table I. The amount of the 615-nm absorbance reduced in this phase appears to increase with increasing substrate concentration. The initial phase is succeeded by a reaction phase where the rate of the type 1 reduction levels off. Finally, the 615-nm absorbance is lost completely, in a reaction which is slower than the initial one. The reaction is first order in enzyme and substrate concentrations. With increasing amounts of substrate the initial and final reduction phases become increasingly dominant at the expense of the 'plateau' phase (Fig. 1A—C).

The absorbance changes at 340 nm, representing changes in the redox state of the two-electron acceptor, are also rather complicated. Initially there is, in fact, a very rapid small increase in absorbance, the amplitude of which is more prominent at low concentrations of substrate and accounting for at most about 10% of the total absorbance decrease at this wavelength. The rate of the initial increase appears to depend on the concentration of substrate. The absorbance decrease at 340 nm appears monophasic at low concentrations of substrate with the rate proportional to both enzyme and substrate concentrations (Fig. 2D,E, Table I. The figure shows the change in absorbance from the maximum to the final value). However, as the substrate concentration is increased, the reduction becomes distinctly biphasic (Fig. 2A,B, Fig. 3). The rate of the initial portion of the 340-nm absorbance loss (following the very rapid small increase described above) is still dependent on both the enzyme and substrate concentrations but the half-time of the succeeding slower reaction becomes constant. In experiments with a constant, high concentration of substrate and varying concentrations of enzyme, the time courses of the slow reaction, normalized to the same amplitude, fall along the same curve (Fig. 4). The rate constant for

TABLE I

RATE CONSTANTS FOR THE REDUCITON OF *RHUS* LACCASE WITH REDUCING SUBSTRATES ($10^{-4} - 10^{-2}$ M)

The rate constants for the initial reduction at 615 nm were calculated from the initial slope of the progress curves and are related to the total enzyme concentration. Other rate constants were determined as the slope of the linear log $(A_t - A_\infty)$ vs. time plots. At 340 nm, pH 6.0, the reduction was biphasic. The rate constant is calculated for the initial dominant phase.

Substrate	pH	Reduction phase			
		615 nm		340 nm	
		Initial ($M^{-1} \cdot s^{-1}$)	Final ($M^{-1} \cdot s^{-1}$)	Initial ($M^{-1} \cdot s^{-1}$)	Final (s^{-1})
Hydroquinone	6.0	—	25	300	—
Hydroquinone	6.5	360	160	400	—
Hydroquinone	7.4	1 580	800	1450	0.40
Hydroquinone	8.5	18 000	4000	8000	0.25, 0.06
Ascorbate	7.4	250	80	125	0.32
Hydroquinone + 0.1 M F^-	7.4	1 690	—	—	0.005
Ascorbate + 0.1 M F^-	7.4	250	—	—	0.002—0.004

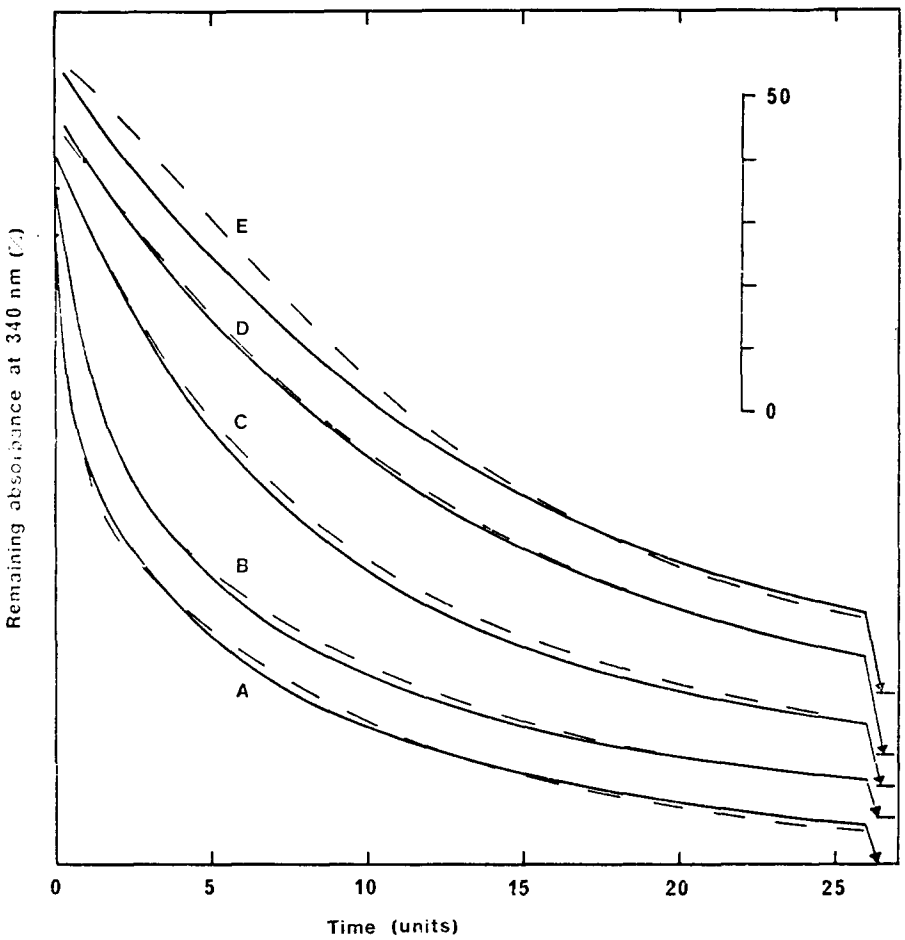


Fig. 2. Time courses of the anaerobic reduction of the two-electron acceptor. Conditions are identical to those in Fig. 1. The time scale is 0.2 s per unit for curves A—D and 0.4 s per unit for curve E.

this phase is not very much affected by the choice of substrate, hydroquinone or ascorbate (Table I). Neither is the percentage of the absorbance at 340 nm disappearing in this reaction affected by the concentrations of enzyme and substrate nor by the type of substrate and amounts to about 50% of the total decrease at pH 7.4.

Anaerobic reduction in the presence of F^- at pH 7.4

The second-order rate constant for the initial reduction of the 615-nm chromophore by hydroquinone or ascorbate is not significantly affected by the presence of F^- (Table I). There is no evidence of a 'plateau' phase even at low concentrations of substrate (Fig. 5B). More than one reduction phase can be observed but the analysis is complicated by the presence of some uninhibited enzyme (see below and Discussion).

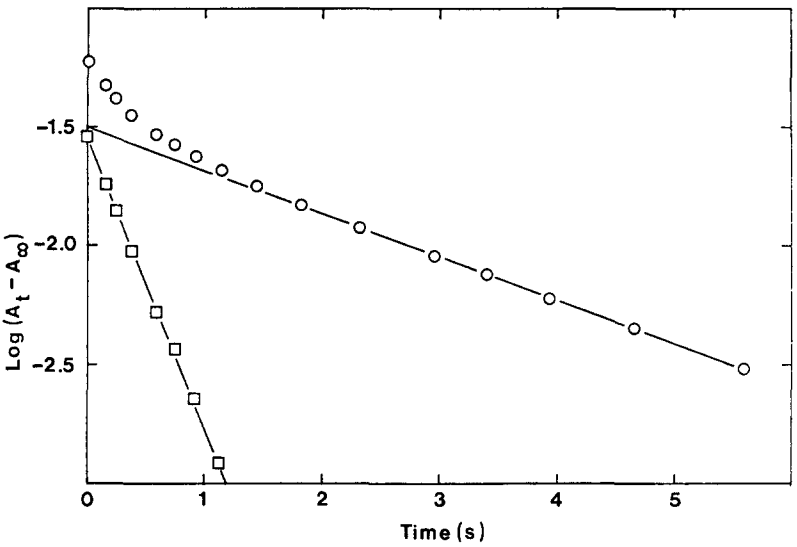


Fig. 3. First-order plots of the disappearance of the 340-nm absorbance to show the biphasic reduction of the two-electron acceptor. Circles represent the plot of log ($A_t - A_\infty$) for the time course shown in Fig. 2B. Squares represent the log plot of the difference between the circles and the extrapolated line.

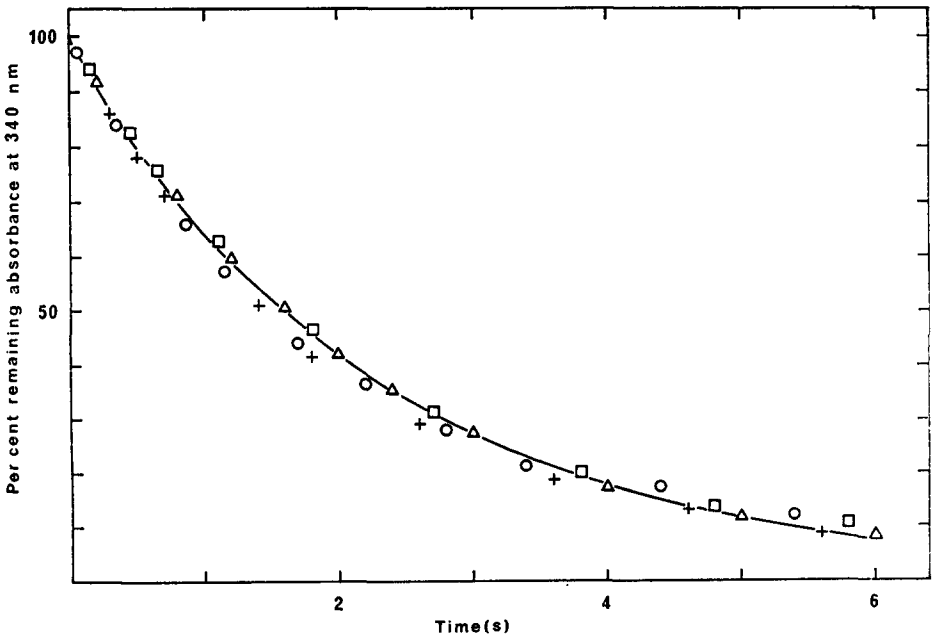


Fig. 4. Disappearance of the substrate concentration-independent part of the 340-nm absorbance. The amplitude of this part of the reaction was obtained by extrapolation as shown in Fig. 3 and the absorption changes ($A_0 - A_\infty$) normalized to 100% for different enzyme concentrations. Hydroquinone concentration was 5 mM. Protein concentrations were: 2.5 μM (○), 5 μM (□), 10 μM (+) and 20 μM (△), respectively. Enzyme and substrate were dissolved in anaerobic phosphate buffer, pH 7.4. The line represents a first-order reaction with a half-time of 1.6 s.

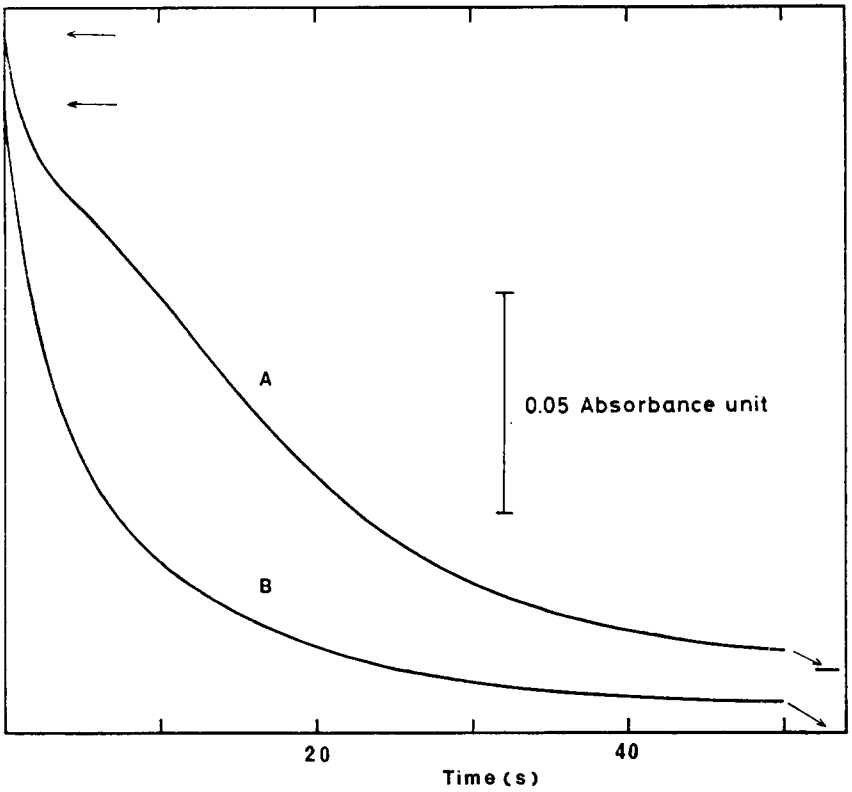


Fig. 5. Effect of F^- on the anaerobic reduction by hydroquinone of type 1 copper as studied at 615 nm. Conditions after mixing were: A; 10 μM enzyme, 0.1 mM hydroquinone, phosphate buffer, pH 7.4. B; as in A plus 0.1 M NaF. The enzyme was equilibrated with 0.1 M NaF for 24 h before mixing with an equal volume of NaF-containing substrate solution. Arrows indicate the starting and final absorbances.

The rate of reduction at 340 nm is dramatically altered by the interaction of the enzyme with F^-. The two-electron acceptor of the inhibited enzyme is reduced in a process, the half-time of which is independent of the enzyme and substrate concentrations. The rate constant calculated for this reaction is 0.005 s^{-1} when hydroquinone is used or about 1% of the value for the substrate concentration independent reduction of the two-electron acceptor in the native enzyme (Fig. 6). When ascorbate is used as the reducing substrate a similar value is found for the rate constant of the inhibited reduction at this wavelength (Table I). The presence of uninhibited enzyme is also evident at 340 nm since part of the reaction is characterized by the same rate constants as are found in the absence of F^-. The dissociation constant for the enzyme · F^- complex can be estimated from the relative concentrations of inhibited and uninhibited enzyme as measured at 340 nm. At 0.1 M F^- about 70% of the laccase is initially inhibited which corresponds to a dissociation constant of 40 mM. At this wavelength the small initial rapid increase in absorbance can still be detected.

The effect of pH on the anaerobic reduction of Rhus laccase

Changes in pH bring about modifications in the anaerobic reduction behaviour of *Rhus* laccase. The rate constants of the different substrate concen-

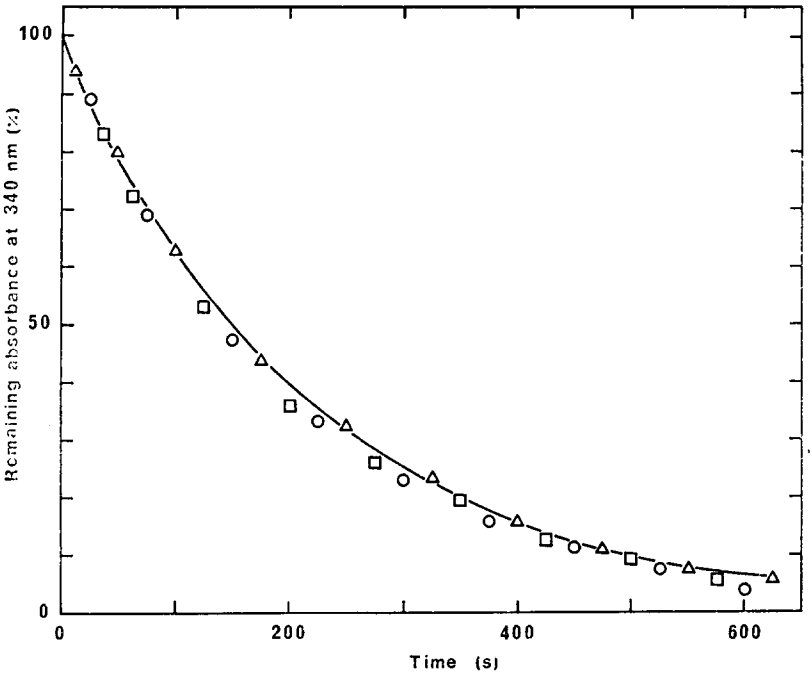


Fig. 6. Kinetics of disappearance of the 340 nm absorbance band in F^--inhibited enzyme. The contribution to the decrease originating from uninhibited enzyme has been subtracted from the total change and the remaining part normalized to 100%. Hydroquinone concentrations were: 5 mM (□), 0.5 mM (○) and 0.1 mM (△), respectively. Other conditions as in Fig. 5B. The line represents a first-order reaction with a half-time of 150 s.

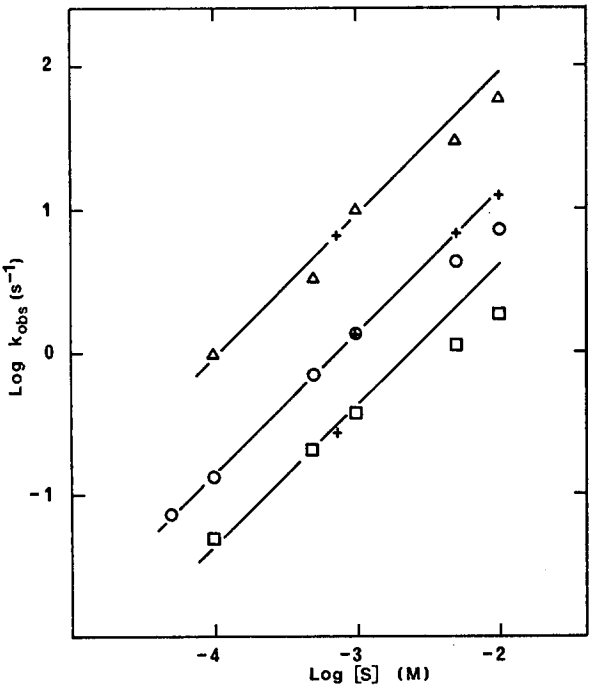


Fig. 7. Observed rate constants vs. hydroquinone concentration for the substrate concentration-dependent reduction of the two-electron acceptor. The anaerobic reduction experiments are represented by the following symbols: □; pH 6.5, ○; 7.4 and △; pH 8.5. Crosses represent values obtained from post-steady-state reduction in separate aerobic experiments. The lines are drawn with a slope of unity. Enzyme concentration was about 10 μM.

tration dependent reaction phases increase with an increase in pH as exemplified in Figs. 7 and 8. At lower pH a striking effect is the decrease in the amplitude of the reaction phase at 340 nm with the rate independent of the substrate concentration. This phase is practically absent at pH 6.5 and 6.0 so that the reduction of the two-electron acceptor is monophasic. At these pH values the reduction behaviour of the type 1 Cu^{2+} is generally the same as that found at pH 7.4 with an initial decrease in absorbance followed by a short 'plateau' and a final decrease. At concentrations of hydroquinone below 1 mM, however, the initial reaction is practically absent and the reaction can be described as reduction preceeded by a lag (Fig. 9). Rate constants can be obtained for the initial as well as the final decrease and are listed in Table I.

At pH 8.5 the 340-nm reduction can be analyzed in terms of three reaction phases. Initially there is a reduction of the absorbance which is first order in both enzyme and substrate concentrations amounting to about 25% of the total change. The rate of the remainder of the reaction at this wavelength is independent of the concentration of substrate but is characterized by two first-order rate constants. The slowest reaction corresponds to about 50% of the total change in absorbance. The rate constants obtained at this wavelength are listed in Table I. The rate of the initial rapid absorbance increase at 340 nm seems to decrease with decreasing pH. The reduction of the type 1 Cu^{2+} can be

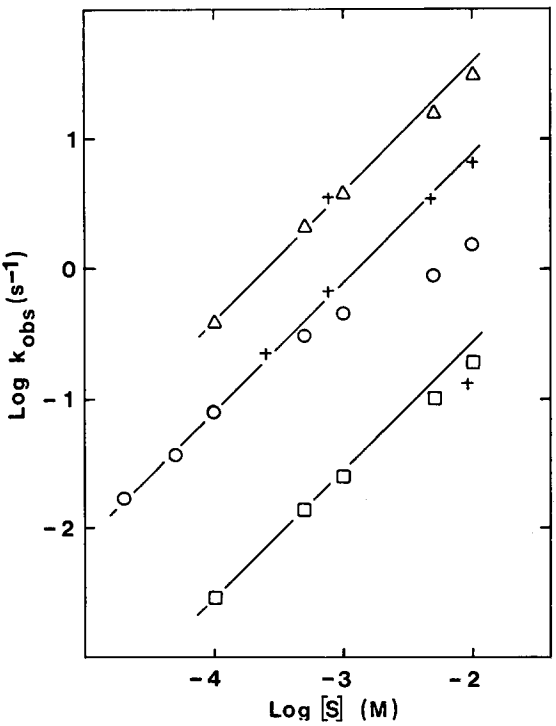


Fig. 8. Observed rate constants vs. hydroquinone concentration for the final reduction of type 1 copper. The anaerobic reduction experiments are represented by the following symbols: □; pH 6.0, ○; pH 7.4 and △; pH 8.5. Crosses represent values obtained from post-steady-state reduction in separate aerobic experiments. The lines are drawn with a slope of unity. Enzyme concentration was about 10 μM.

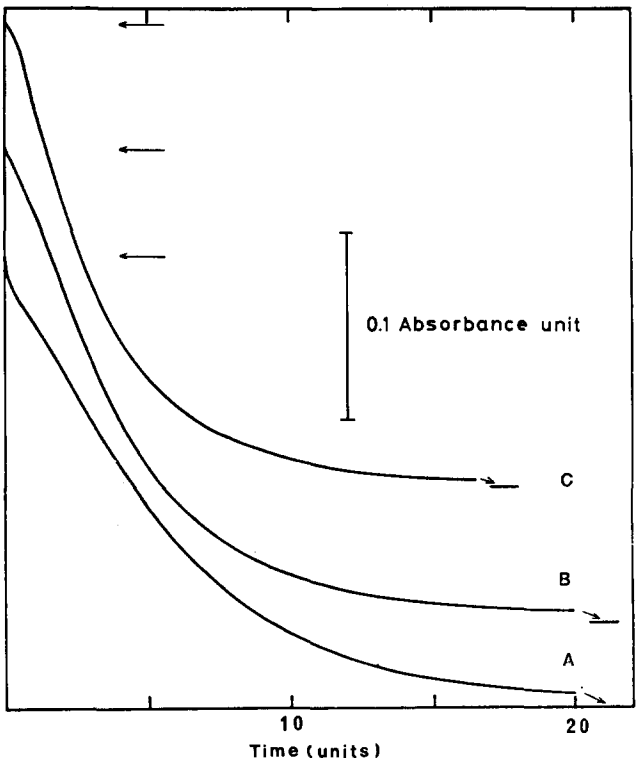


Fig. 9. Time courses of the anaerobic reduction of type 1 copper at pH 6.0. Enzyme concentration was 21 μM. Hydroquinone concentrations were: 10 mM (A), 1 mM (B) and 0.1 mM (C). Arrows mark the absorbances at the start and the end of the reactions. The time scale is 1, 10 and 100 s per unit for curves A, B and C, respectively.

resolved into two second-order reactions. The two phases are not separated by a 'plateau' in this case. The rate constants for the type 1 copper reduction are listed in Table I.

Reduction of Rhus laccase in the presence of O_2 at pH 7.4

In the presence of O_2 the reduction with excess substrate results in a rapid initial decrease in absorbance at 615 nm followed by a slow return of colour towards a steady-state level (Fig. 10 A), the duration of which is dependent on the substrate concentration. The magnitude of the initial decrease is related to the amount of reducing substrate present but even with very high concentrations of reductant (10 mM) it does not amount to more than about 50% of the possible total decrease at this wavelength. The rate constant based on the initial reduction rate is identical with that found in the absence of O_2 (Tables I and II).

Following the initial decrease the 615-nm absorbance increases and approaches the steady-state level exponentially (Fig. 10A). The extracted rate constant, 0.3 s^{-1}, is independent of the substrate concentration and the extent of the initial reduction and is unaffected by the type of reductant, hydroquinone or ascorbate. Following the exhaustion of O_2 the 615-nm absorbance

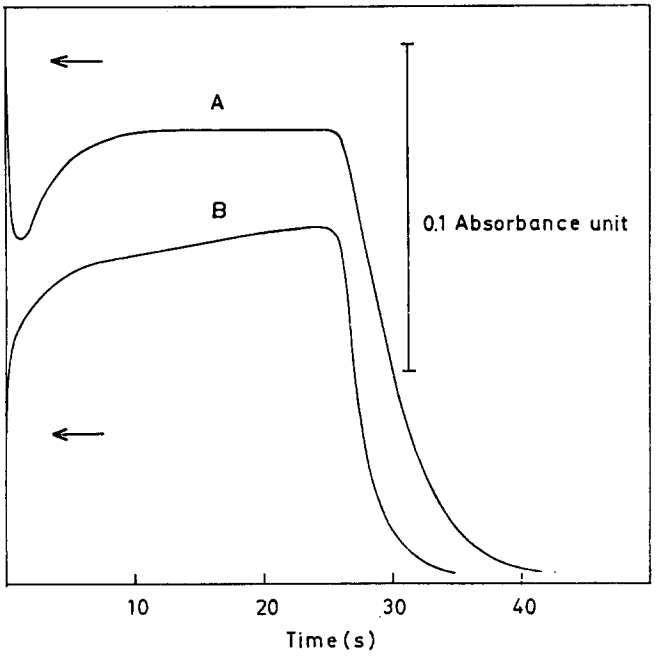


Fig. 10. Reduction of *Rhus* laccase by hydroquinone in the presence of oxygen. Conditions after mixing were: 15 μM enzyme, 125 μM O_2 and 0.5 mM hydroquinone at pH 7.4. Curves A and B represent the absorbance changes at 615 and 340 nm, respectively. Arrows mark starting absorbance values.

drops to a value characterizing full reduction of this chromophore. This phase in the reaction appears to be governed by a second-order mechanism. The rate constant is indistinguishable from the one obtained for the final reduction phase at 615 nm in the anaerobic reduction experiments.

At 340 nm the absorbance actually shows an initial biphasic increase to the steady-state level and drops to the level of fully reduced enzyme upon the exhaustion of O_2 (Fig. 10B). The rapid part of the increase is similar to that found in the absence of O_2 and may have a similar explanation (see discussion).

TABLE II

RATE CONSTANTS FOR THE REDUCTION OF *RHUS* LACCASE BY HYDROQUINONE (10^{-4} — 10^{-2} M) IN THE PRESENCE OF O_2 (125 μM)

The rate constants for the initial reduction at 615 nm were calculated from the initial slope of the progress curves and are related to the total enzyme concentration. The post-steady-state rate constants were determined as the slope of the linear log $(A_t - A_\infty)$ vs. time plots.

pH	Reduction phase			
	Initial 615 nm ($M^{-1} \cdot s^{-1}$)	Post-steady-state		
		615 nm ($M^{-1} \cdot s^{-1}$)	340 nm	
			($M^{-1} \cdot s^{-1}$)	(s^{-1})
6.0	—	15	320	—
6.5	—	160	400	—
7.4	1 580	800	1400	0.3
8.5	18 000	4000	8000	0.07

Due to the absorbance of the quinone product the steady-state absorbance level shows a slight increase when hydroquinone is used as the reductant. This is seen at all pH values. When ascorbate is used the steady-state level is constant. The rate constant for the post-steady-state decay of the 340-nm absorbance is the same as that found for the initial, substrate concentration dependent reduction in the anaerobic experiments. At the highest concentrations of reductant used (about 10 mM hydroquinone or ascorbate) a minor part of the decrease at 340 nm (less than 15%) is reduced at a rate zero-order in substrate concentration. The first-order rate constant is similar to that found under anaerobic conditions at this wavelength for the substrate concentration-independent reduction phase (Table II).

The tendency for decrease in second-order rate constants with increasing substrate concentration found under anaerobic conditions at high concentrations of substrate (>1 mM) is eliminated in the presence of O_2 (see Figs. 7 and 8, pH 7.4).

The effect of pH on the kinetics in the presence of O_2

The extent of the pre-steady-state absorption decrease at 615 nm is greatly influenced by the hydrogen ion concentration. At 1 mM hydroquinone this reaction phase at pH 6.5 only corresponds to a few per cent of the total possible decrease while it is about 60% at pH 8.5. At pH 6.0 there is no return of colour following the initial reduction phase (cf. pH 7.4).

The second-order rate constant obtained from the post-steady-state decay of the 615-nm absorbance shows the same pH dependence as the rate constants calculated for the anaerobic reduction. It has a value similar to that found for the final reduction phase under anaerobic conditions at least at low substrate concentrations (Fig. 8 and Table II).

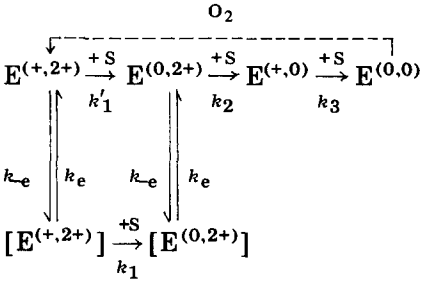
At pH 6.5 the 340-nm absorbance shows the same initial biphasic increase as was seen at pH 7.4. The post-steady-state decrease at 340 nm is monophasic with no evidence of a reduction phase independent of substrate concentration.

At 340 nm, pH 8.5, the same initial absorbance increases can be seen as at the other pH values. At the time of exhaustion of O_2 the decrease in absorbance at this wavelength is biphasic. The rate of one of these reaction phases is substrate concentration-independent with a rate constant similar to the one found for one of the reduction phases under anaerobic conditions (0.07 s^{-1}).

Discussion

Considering the straightforward rate dependence which can be derived for the initial reduction of the 'blue' chromophore, a bimolecular mechanism for this reaction seems likely. Also, the late reduction of this site appears to be governed by a reaction mechanism of this sort, but with a smaller rate constant than in the former case. The existence of two forms of the enzyme, with different rates of reduction, could account for this observation. This explanation alone, however, fails to account for the 'plateau' phase at 615 nm seen at low concentrations of substrate. The 'plateau' would, on the other hand, be expected in a sequential scheme if electrons are transferred from the reduced type 1 copper to an electron-accepting site at a rate comparable to the reduc-

tion of type 1 Cu^{2+}. The likely candidate is the two-electron acceptor which has been shown to be able to undergo turnover in ceruloplasmin, another blue oxidase [10]. The biphasic reduction of the two-electron acceptor again supports the idea of two forms of the enzyme under the present experimental conditions. The rate of reduction of one of the forms appears to be limited by an intramolecular reaction step. Support for this hypothesis is derived from the observation that the half-time for reduction is independent of the enzyme and substrate concentrations (Figs. 2 and 4) and the same with either of the substrates hydroquinone or ascorbate. The reduction of the two-electron acceptor of the other form, on the other hand, is dependent on a bimolecular reaction. Two observations support the hypothesis that the two enzyme forms are in equilibrium with each other. First, the distribution between the forms seems to be sensitive to changes in pH (see below), and second, in the post-steady-state reduction of the enzyme at pH 7.4, the rate of the dominant part of the decrease at 340 nm is dependent on the substrate concentration compared to only 50% in anaerobic reduction. A model of the reactions of tree laccase must therefore incorporate the following features: sequential electron transfer, substrate concentration independent reactions and an equilibrium between different forms of the enzyme. The following scheme has been formulated to describe the anaerobic reduction of *Rhus* laccase:



Scheme 1

$E^{(+,2+)}$ denotes laccase in the fully oxidized state, the first superscript (+) representing the oxidized type 1 copper. The second superscript (2+) represents the oxidized two-electron acceptor. The superscript "0" indicates that the particular oxidation-reduction centre is in its reduced state. The enzyme molecules are supposed to exist in two forms, one, the "inactive", being indicated by brackets. The equilibrium between the two states is determined by the equilibrium constant K_e which under the conditions used in most of this study (phosphate buffer, pH 7.4, 25°C) is close to 1 (calculated from the amount of "inactive" form).

A rough estimation of the accuracy of the model can be obtained from the computer simulations in Figs. 1 and 2. Since the experimental curves at 340 nm represent absorbance decrease from the maximum value which includes the small initial absorbance increase of unknown origin (see results and below) and the simulated curves only represent true reduction, some smaller differences between the two sets of curves should not be surprising. With hydroquinone the relative magnitudes of the rate constants are $k_1 \approx 3k'_1 \approx 3k_3$ with k_1 about 2500 $M^{-1} \cdot s^{-1}$ (see Table III). For good fit k_2 has been estimated to be

TABLE III

RATE CONSTANTS USED FOR COMPUTER SIMULATIONS, ACCORDING TO SCHEME 1, SHOWN IN FIGS. 1 AND 2

[Hydroquinone] (M)	k_1 ($M^{-1} \cdot s^{-1}$)	k_1' ($M^{-1} \cdot s^{-1}$)	k_2 ($M^{-1} \cdot s^{-1}$)	k_3 ($M^{-1} \cdot s^{-1}$)	k_e (s^{-1})	$K_e = \frac{k_e}{k_{-e}}$
10^{-2}	2400	900	10^5	800	0.5	0.7
$5 \cdot 10^{-3}$	2400	1000	10^5	800	0.5	0.7
10^{-3}	3000	1100	10^5	850	0.4	0.9
$5 \cdot 10^{-4}$	2600	1300	10^5	900	0.4	1.0
10^{-4}	2500	1300	10^5	1100	0.4	0.8

about $30k_1$. The value of k_e has been determined from the rate of the substrate concentration independent reduction phase at 340 nm (Fig. 4). An equally good fit to experimental results using ascorbate as a substrate was obtained with the rate constants listed in Table IV. The second-order rate constants, used in the simulations for the reduction of the type 1 Cu^{2+}, have been given somewhat lower values at the highest substrate concentrations in order to accomodate for the rate saturation observed experimentally (see Figs. 7,8). The rate constant k_e is much smaller than $k_1 \cdot [S]$ at the highest substrate concentrations used and of comparable magnitude at the lowest concentrations (see Figs. 1 and 2). At high substrate concentrations the rate of reduction of the two-electron acceptor in about half of the molecules at pH 7.4 should be determined by k_e, thus explaining the zero-order substrate concentration dependence seen at 340 nm for about 50% of the reaction. In the other half of the enzyme molecules the rate of reduction of the two-electron acceptor is determined by k_1' ($k_1' << k_2$) in a reaction dependent on the substrate concentration. This corresponds to the initial rapid reduction of the 340-nm absorbance. The observed initial reduction of the 615-nm absorbance is largely determined by k_1 since in half of the molecules the rapid reoxidation of this site, accompanying the reduction of the two-electron acceptor, is hampered by the slow intramolecular step. At later stages k_3 contributes to the observed rate constant for the reduction of the 615-nm band. At low substrate concentrations, the intramolecular reaction determined by k_e is no longer rate-limiting for the overall reduction of the two-electron acceptor resulting in a largely monophasic disappearance of absorbance at 340 nm. At 615 nm the initial drop in absorbance should be less pronounced for the same reason. For most of the reaction the rate of reduction will be determined by a rate expression also containing k_3.

TABLE IV

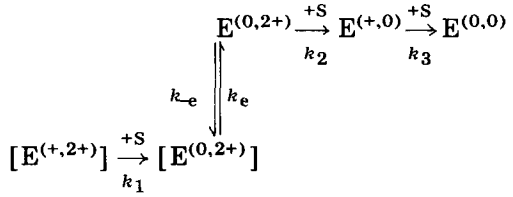
RATE CONSTANTS USED FOR COMPUTER SIMULATIONS OF THE ANAEROBIC REDUCTION OF *RHUS* LACCASE BY ASCORBATE AT PH 7.4

[Ascorbate] (M)	k_1 ($M^{-1} \cdot s^{-1}$)	k_1' ($M^{-1} \cdot s^{-1}$)	k_2 ($M^{-1} \cdot s^{-1}$)	k_3 ($M^{-1} \cdot s^{-1}$)	k_e (s^{-1})	$K_e = \frac{k_e}{k_{-e}}$
$5 \cdot 10^{-2}$	300	80	5000	80	0.4	0.8
10^{-2}	380	130	5000	85	0.35	0.8
$5 \cdot 10^{-3}$	420	120	5000	100	0.30	0.9
10^{-3}	300	100	5000	130	0.32	0.8

The effect of F^- on the anaerobic reduction of *Rhus* laccase can be fairly well understood in this model if it is assumed that the reaction step leading to the reduction of the two-electron acceptor (determined by k_2) is influenced. An analysis shows that if this reaction is determined by the rate constant for the F^--inhibited 340-nm reduction (0.005 s^{-1}) and leaving all other reactions unaffected, a good fit to the experimental results is obtained. The inhibition of the electron transfer between the type 1 copper and the two-electron acceptor explains why the 'plateau' in the 615-nm reaction curves, seen at low concentrations of substrate, is abolished in the presence of F^- (see Fig. 5). F^- is known to bind to the type 2 Cu^{2+} in the blue oxidases as are several other inhibitory anions [11—14]. For fungal laccase it has been shown that, in assays of F^--inhibited enzyme, the catalytic activity increases with time and at the same rate as free F^- appears in the reaction medium [14]. This rate is similar to that determined for the F^--inhibited anaerobic reduction of the two-electron acceptor in this enzyme [3]. One obvious interpretation is that the F^--induced inhibition of the catalytic turnover and reduction of the two-electron acceptor is abolished as F^- dissociates from the type 2 copper. If the situation is the same in the tree enzyme it provides an explanation for the substrate concentration independent rate of the 340-nm reduction in the presence of F^-. Assuming that the affinity for F^- is independent of the redox state of sites other than the type 2 copper, the on-constant for F^- at pH 7.4 can be estimated to be about 0.1 $M^{-1} \cdot s^{-1}$ from the equilibrium constant and the off-constant characterizing the F^--complex with *Rhus* laccase.

A mechanism similar to that just discussed for the F^- inhibition could conceivably be responsible for the substrate concentration independence of part of the 340-nm reduction in the native enzyme. We find that this reduction phase is sensitive to changes in pH. It is absent at pH 6.0 and 6.5 and amounts to about 50% and 75% at pH 7.4 and 8.5, respectively. A similar pH dependence is observed for the corresponding reaction in the post-steady-state reduction in agreement with observations by Holwerda and Gray [5]. It is therefore likely that the elimination of an OH^- ion from the type 2 Cu^{2+} influences the rate of reduction of the two-electron acceptor at high pH as suggested by these authors, but matters are probably a good deal more complicated. This is indicated by the unusual distribution of the different reduction phases at 340 nm at different pH values and the pH dependence of the rate constants of the substrate concentration insensitive phase. For example, conformational changes, affecting the reactivities of the redox sites, could be influenced by changes in pH.

At this stage it is useful to make a comparison with the model proposed for the anerobic reduction of fungal laccase [3]. This can be rewritten as follows:



Scheme 2

The position of the equilibrium is shifted far to one side ($k_{-e} = 100\ k_e$, ref. 3). Scheme 1, deduced from the anaerobic reduction of the tree laccase, should give as good a representation of the kinetics of the fungal enzyme as Scheme 2 with the use of the proper rate constants (see ref. 3) and K_e (k_e/k_{-e}) for the equilibrium reactions equal to 0.01. Fungal laccase could, therefore, very well react according to Scheme 1. A close mechanistic similarity between the two enzymes would hardly be surprising in view of the great similarities in other features such as distribution of different forms of copper, visible absorbance, magnetic properties, oxidation-reduction behaviour and affinity towards anions etc. (see ref. 1). For the fungal laccase the binding of F^- was originally suggested to affect the rate constant for the rate-limiting reaction step, k_e, in the above scheme [3]. An alternative explanation also consistent with the experiments would be an inhibitory effect on the reduction of the two-electron acceptor according to the scheme proposed for the tree enzyme. In conclusion, the essential characteristics in the anaerobic reduction of the two laccases with several substrates can be accounted for by the same reaction model.

The scheme proposed above also offers an explanation to the kinetic behaviour when reducing substrate and O_2 are both present. Under these conditions a rapid initial drop in 615-nm absorbance is seen. This result is consistent with Scheme 1 if O_2 reacts as indicated since the reduced type 1 copper of the 'inactive' form is prevented from rapid reoxidation because of the slow intramolecular reaction step. The reaction of the reduced enzyme with O_2 indicates three-electron transfer with the formation of an oxygen intermediate. Support for this hypothesis comes from recent reoxidation experiments with the laccases (see below).

Experimentally the 615-nm absorbance is gradually shifted to a new, higher, level after the initial decrease (Fig. 10). A time-dependent increase in the amount of the 'active' enzyme should give rise to this effect. Such an increase could be caused by a bound oxygen intermediate shifting the equilibrium towards the 'active' state by preventing the return to the 'inactive' state (decreasing k_{-e}). The rate of shift of the 615-nm absorbance level should be equal to the rate of the 'inactive' to 'active' conversion which is determined by the rate constant, k_e, of the intramolecular reaction step.

At the exhaustion of O_2, the model predicts that the 340-nm absorbance of the 'active' molecules should decrease at a rate determined by the rate constant k_1' ($k_2 >> k_1'$). The post steady-state reduction of the 615-nm absorbance is expected always to be slower than this in a sequential scheme with the proposed properties. This is also seen experimentally (Fig. 10). Usually a small substrate concentration-independent reaction phase is detected in the post-steady-state reduction at 340 nm (see also ref. 5). The amplitude of this phase should be determined by the extent of the 'inactive' to 'active' transition before the exhaustion of O_2.

For the fungal enzyme it has been found that the path leading to the reduction of the type 3 copper site under anaerobic conditions is too slow to be part of the catalytic mechanism, and that the introduction of O_2 into the system must somehow change the electron transfer pathway [8]. With the present results on the tree laccase at hand it is possible to suggest how this might be accomplished. With the tree enzyme the rate constants for the reductive steps

in the 'active' form are large enough to explain the turnover rate at steady state at all concentrations of reducing substrate. For example, with 0.5 mM hydroquinone the turnover number, calculated from the time required for exhaustion of O_2 (0.125 mM), is 0.32 s^{-1} (from Fig. 10) while the limiting pseudo first-order rate constants in the catalytic reduction sequence are about 0.4 s^{-1} (k_3 multiplied by the substrate concentration). The rate constant for the interconversion between the two forms of the enzyme (about 0.4 s^{-1}) is, on the other hand, too small for this reaction to be involved in the turnover at higher concentrations of substrate in analogy with the same reaction in the fungal enzyme. This enzyme is initially present only as the 'inactive' form because of the position of the equilibrium but if the model is correct an increasing amount of 'active' enzyme will be available for rapid turnover of substrate. The turnover number determined from the amount of product formed during steady state should, therefore, be calculated from the amount of 'active' enzyme integrated over the whole steady-state period. When the length of the steady state is not much larger than the half-time for the formation of the 'active' enzyme, too small a turnover number will be found when the amount of product formed is related to the total enzyme concentration. This is a probable explanation of the low figure found at high concentrations of fungal laccase [8] compared to that found with catalytic amounts of enzyme [2]. A successive increase in the amount of 'active' enzyme during turnover should also account for the lag phase in the product formation observed earlier for fungal laccase (see ref. 2, Fig. 5). For the tree enzyme a rough estimation of the turnover number can be obtained by multiplying the rate constant k_1' or k_3 by the substrate concentration, keeping in mind the limitations outlined above.

Holwerda and Gray [5] have investigated the reaction of tree laccase with hydroquinone under conditions somewhat similar to our aerobic experiments. This is apparent from the rather long steady-state period in their experiments (see for example Fig. 1 in ref. 5). The interpretation of their results is therefore derived from the study of the post-steady-state reduction of the enzyme. They claim that this reduction phase gives an equally good representation of the anaerobic reduction as the initial reaction between fully oxidized enzyme and substrate. It is obvious from the present study that this cannot be the general situation. It is, however, possible to understand why, under certain conditions, the same rate constants can be extracted from truly anaerobic reduction experiments and post-steady-state reduction. As noted earlier the initial distribution between the 'active' and 'inactive' forms of the enzyme is sensitive to the hydrogen ion concentration of the medium. At pH 6.0 and 6.5 for example, practically all of the enzyme is initially present as the 'active' form. Therefore, at these pH values the present model predicts that nearly identical results will be obtained from anaerobic reduction and post-steady-state reduction at low substrate concentrations, since in neither case is the electron transfer hampered by the slow intramolecular transition determined by k_e. A control experiment at pH 6.5 (1 mM hydroquinone) verified predictions completely, i.e. the rate constants obtained at 615 and 340 nm were the same as those calculated from the post-steady-state reaction at the corresponding wavelengths.

Under experimental conditions where only the 'active' form is initially pres-

ent the addition of reducing substrate in the presence of O_2 is expected to cause the 615-nm absorbance to drop directly to the steady-state level without the return of colour seen at higher pH, since the very basis for this recovery, the 'inactive' to 'active' transition, has been removed. Accordingly, there is no return of 615-nm absorbance at pH 6.0, and similar observations were made by Holwerda and Gray at pH 6.0 [5].

Inhibitors such as F^- are expected to quench the steady state at 615 nm and cause the type 1 Cu^{2+} of the inhibited enzyme to be reduced by substrates as in the absence of O_2. The electron transfer reaction leading to the reduction of the two-electron acceptor and the simultaneous reoxidation of the type 1 copper is now rate-limited by the slow dissociation of the enzyme-inhibitor complex. Therefore, the observation, made by Holwerda and Gray [5], that the reduction rate of type 1 copper was about five times greater in the presence of F^-, at pH 7.0, finds an explanation. In the experiments with F^- they measured the reduction of this electron acceptor in the pre-steady-state whereas rate parameters for the reduction of native enzyme were measured after the consumption of oxygen present.

The presence of inhibitor at low pH or very high concentrations of inhibitor complicates the reduction of laccase, possibly because of interactions of the inhibitor directly with the type 1 copper [5]. Interaction of this sort is known from the effect of N_3^- on the visible absorption of the type 1 copper [5,13,15]. No attempt has been made to expand the model to account for these effects.

No definite explanation exists for the initial rapid absorbance increase at 340 nm which is observed in both anaerobic and aerobic experiments. Since the rate of this phase is greater than the absorbance loss at 615 or 340 nm, it could possibly represent the binding of substrate to the oxidized enzyme. Obviously, this reaction should be examined in greater detail.

The part of the initial rise in absorbance at 340 nm only seen at reduction in the presence of O_2 and the larger than 100% steady-state level at this wavelength have recently found a possible explanation. During the steady state a reaction intermediate is present with an absorbance extending from about 500 nm to below 330 nm [16]. Moreover, EPR measurements have indicated a possible relation between the optical intermediate and a new EPR signal [17] associated with oxygen [18]. Optical intermediates with similar properties have been detected during the reoxidation of other blue oxidases [19,20]. This again stresses the mechanistic similarities between the enzymes of this kind.

The presented model for the reaction of the laccases with reducing substrates and O_2 rather successfully accounts for many of the observations made in connection with these enzymes. It should, of course, be remembered that the model necessarily represents a simplified picture of the actual case. Thus, the role of the type 2 copper has not been specified although it is known that it is capable of undergoing cyclic reduction-reoxidation in the fungal enzyme [4]. This site could very well participate in the transfer of one of the electrons required for the two-electron accepting type 3 site (cf. ref. 5). It is also evident that hydrogen ions are mechanistically involved in the reduction reactions of tree laccase although the nature of the involvement remains obscure. Holwerda and Gray have proposed the phenolate ion as the main substrate when hydroquinone is used [5]. This certainly could explain part of the pH dependence of

the reactions involving substrate.

A reasonably good fit to the experimental results is obtained without the incorporation of enzyme-substrate complexes into the model although such are probably present. Such complexes could be the cause of the rate saturation seen under anaerobic conditions in the substrate concentration dependent reactions. Why the saturation is absent in the post-steady-state reduction remains unclear.

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