Biochimica et Biophysica Acta, 568 (1979) 145—156

BBA 68731

THE MECHANISM OF ELECTRON TRANSFER IN LACCASE-CATALYSED REACTIONS

LARS-ERIK ANDRÉASSON and BENGT REINHAMMAR

Department of Biochemistry and Biophysics, University of Göteborg and Chalmers Institute of Technology, Fack, S-402 20 Göteborg (Sweden)

(Received September 11th, 1978)

Key words: Laccase; Electron transfer; Oxygen reduction; (Mechanism)

Summary

1. The reaction of the electron acceptors in *Rhus vernicifera* laccase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) have been studied with stopped-flow and rapid-freeze EPR techniques. The studies have been directed mainly towards elucidation of the role of the type 2 Cu^{2+} as a possible pH-sensitive regulator of electron transfer.

2. Anaerobic reduction experiments with *Rhus* laccase indicate that the type 1 and 2 sites contribute one electron each to the reduction of the two-electron-accepting type 3 site. There is also evidence that the reduction of the type 1 Cu^{2+} triggers the reduction of the type 2 Cu^{2+}.

3. Only at pH values at which the reduction of the two-electron acceptor is limited by a slow intramolecular reaction can an OH^- be displaced from the type 2 Cu^{2+} by the inhibitor F^-.

4. A model describing the role of the electron-accepting sites in catalysis is formulated.

Introduction

A model for the reaction of *Rhus vernicifera* laccase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) with reducing substrates has been presented earlier [1]. It was based on studies of the behaviour of the optical absorption bands of the enzyme in its reactions with the substrates. The type 1 Cu^{2+}, monitored at 615 nm, was found to act as a primary electron acceptor, mediating electron transfer between the reducing substrate and the two-electron-accepting type 3 site, measured at 340 nm. The role of the type 2 Cu^{2+} in the electron transfer reaction was not specified in

detail, although it was suggested to act as a regulator of the electron flow to the two electron acceptor, permitting reduction of this site at low pH. The sluggish reduction of the type 3 site at high pH values was suggested to originate from the slow elimination of an inhibiting OH^- bound to the type 2 Cu^{2+}. Binding of OH^- to the type 3 site has also been suggested to be responsible for the slow reduction of this site [2]. The strong affinity of the type 2 Cu^{2+} for F^- has here been utilized to probe the state of the type 2 site.

The studies of the transient kinetic behavior of *Rhus* laccase have been expanded to include the paramagnetic properties. EPR permits observations of the type 1 and 2 sites in their oxidized states. By the combination of rapid-freeze quenching and EPR, the oxidation states of the two sites can be studied at different times after the initiation of the reaction of laccase with reducing substrates. We have used this approach in attempts to determine the mechanistic role of the type 2 Cu^{2+}. In particular, it is interesting to know if this copper ion acts as electron acceptor together with the type 1 Cu^{2+} and contributes one of the electrons necessary for the reduction of the type 3 site.

Materials and Methods

R. vernicifera laccase was prepared from lacquer aceton powder (Saito and Co., Ltd., Tokyo) according to the method by Reinhammar [3]. In the reduction experiments the enzyme was dissolved in potassium phosphate buffers with ionic strength of 0.25. Fungal laccase B from *Polyporus versicolor* was prepared by a method described earlier [4]. The fungus was grown in a medium containing isotopically pure ^{63}Cu. Both enzymes were dialyzed against sodium ascorbate for removal of any contaminating fluoride [5]. Analytical grade hydroquinone was obtained from Schuchart Chemical Company, Munich, F.R.G., and was recrystallized before use. All solutions were made with analytical grade chemicals and deionized distilled water.

Stopped-flow studies were performed at 25° C. The technique for preparation of anaerobic solutions, the apparatus used and the method for analysing the stopped-flow data have been described elsewhere [6,7].

Rapid-freeze experiments were made according to a technique previously described [7]. The temperature at mixing was 25° C. EPR measurements at about 9.2 GHz were made at 77 K in a Varian E-3 spectrometer or at 12 K in a Varian E-9 instrument. The amount of F^- bound to type 2 Cu^{2+} was measured by analysis of the change into a doublet of the low-field line when F^- binds to this copper ion [8]. The analysis was performed with the use of a computer program which allowed a predetermined fraction of the native type 2 Cu^{2+} low-field line to be subtracted from the spectra of F^--treated enzyme until only the pure F^--Cu^{2+} spectra remained.

The rates of anaerobic reduction of the electron acceptors in *Rhus* laccase were obtained by the following procedure. Type 1 Cu^{2+} and the two-electron-acceptor concentration changes were determined from stopped-flow experiments by recording the optical absorption changes at 615 and 340 nm, respectively (cf. Ref. 1). The concentration of enzyme was about 20 μM in these studies. The amount of type 2 Cu^{2+} as well as type 1 Cu^{2+} was determined by double integration of the EPR spectra obtained from anaerobic rapid-freeze

experiments with about 300—400 μM enzyme. Thus, the amount of residual tupe 2 Cu^{2+} was determined by integrating the low-field line of this copper [9] and the amount of type 1 Cu^{2+} was obtained by subtracting the type 2 Cu^{2+} intesity from the total spectrum. Proper corrections for the different g factors were made in the integrations of spectra [10].

Measurements of pH were made with a Radiometer (Copenhagen, Denmark) glass electrode GK 2322 C coupled to a Radiometer 26 pH meter. In order to prevent CO_2 to disturb in the pH measurements, when F^- was added to the laccases, nitrogen gas was constantly flushed over the reaction mixture.

Results

Anaerobic reduction of Rhus laccase at different pH values

Anaerobic reduction studies were made at pH 6.0, 6.5, 7.4 and 8.5 in rapid-freeze EPR and stopped-flow experiments. The time courses for the reduction of types 1 and 2 Cu^{2+} and the two-electron acceptor are summarized in Figs. 1—4. In general, the reduction behaviour of type 1 copper and the two-electron acceptor, according to the stopped-flow data, is identical to what has been reported earlier [1]. There is very good agreement between the rate of reduction of type 1 Cu^{2+} as measured in stopped-flow and rapid-freeze EPR experiments.

At pH 6.5 (Fig. 1) the integrated EPR intensity of type 1 Cu^{2+} is within about 5% the same as the amount of type 2 Cu^{2+}. Also with 5 or 20 mM hydroquinone the EPR intensities of both types of paramagnetic copper ions decrease with identical rates. Note that there is a lag phase in the reduction of

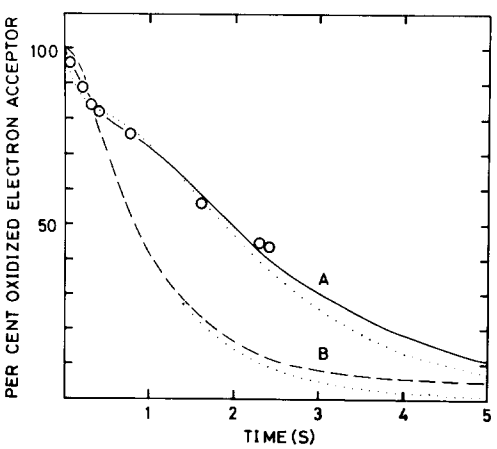


Fig. 1. Time courses for the anaerobic reduction of the electron acceptors in *Rhus* laccase at pH 6.5. Enzyme and hydroquinone (final concentrations 0.3 and 10 mM, respectively) were mixed in the rapid-freeze apparatus at 25°C. The reaction was quenched at different times after mixing by freezing at about 150 K. The circles represent the integrated EPR intensity of the type 2 Cu^{2+}. The intensity of the type 1 Cu^{2+} signal was within 5% the same as that of the type 2 Cu^{2+} and is for clarity not included in the figure. The lines A (615 nm) and B (340 nm) show absorbance changes in separate stopped-flow experiments with lower enzyme concentration (0.01 mM). · · · · · ·, computer simulations according to Scheme 1 and the rate constants shown in Table I. EPR spectra were recorded at 77 K at a microwave frequence of 9.15 GHz with a microwave power of 20 mW and a modulation amplitude of 2 mT.

the two-electron acceptor and that types 1 and 2 copper are reduced faster than the two-electron acceptor at the beginning of the reaction. The rates of reduction of type 1 copper and the two-electron acceptor at pH 6.5 with the preparation of enzyme used in this experiment are about a factor of three slower than found with an earlier preparation [1]. However, a rapid-freeze reduction experiment showed that both types 1 and 2 copper are reduced simultaneously also in the earlier preparation. At the moment we have no explanation of the difference in reduction rates of the two preparations of enzyme studied. The changes in concentration of the three electron acceptors during anaerobic reduction can be stimulated to fair agreement with the experiments using Scheme 1 (see Discussion) and the rate constants shown in Table I.

Experiments at pH 6.0 with 5 or 10 mM hydroquinone reveal that also that this pH the types 1 and 2 copper are reduced with similar rates but with a lower rate constant (see Ref. 1).

Although the reduction of type 2 Cu^{2+} appears to be simultaneous with that of the type 1 Cu^{2+} at pH 6.0 and 6.5 it is reduced much more slowly at higher pH. The EPR spectra shown in Fig. 2 illustrates the reduction of types 1 and 2 Cu^{2+} at pH 7.4. After 0.5 s almost only type 2 Cu^{2+} contributes to the EPR spectrum. There is a clear lag phase in the reduction of type 2 Cu^{2+} (see Fig. 3). Also at this pH the reduction of all three electron acceptors can be simulated according to Scheme 1 and the rate constants shown in Table I.

Except for a smaller amount (about 20%) the type 2 Cu^{2+} at pH 8.5 (Fig. 4) is reduced in a slow reaction with an estimated half-time of about 2 min. Earlier kinetic studies show that the reduction of the two-electron acceptor at this pH is complicated [1]. Therefore, the reaction scheme must be expanded in order to account for the complete kinetic behaviour at this pH. However, no attempt has yet been made to include more steps in the reaction scheme.

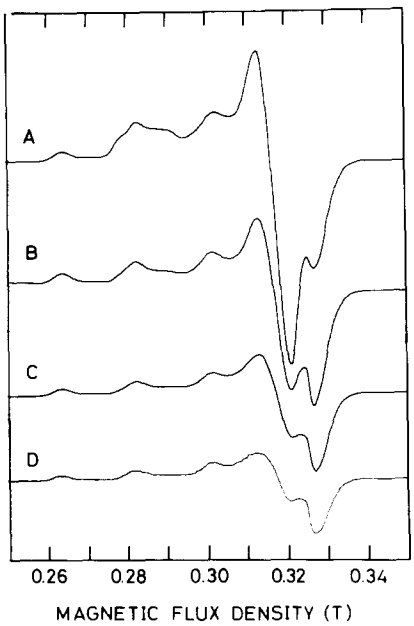
Reduction of Rhus laccase in the presence of O_2 at pH 6.5

In order to determine the steady-state level of the three electron acceptors during turnover, reduction was carried out in the presence of O_2. Stopped-flow experiments at pH 6.5 demonstrate that the type 1 Cu^{2+} is almost fully oxidized during steady-state in a reaction mixture containing 0.01 mM enzyme, 1 mM hydroquinone and 0.125 mM O_2. The absorption at 340—400 nm indicates that the two-electron acceptor is virtually fully oxidized and that an oxygen intermediate is present (cf. [1]).

TABLE I

Rate constants used in computer simulations of the anaerobic reduction of *Rhus* laccase by 10 mM hydroquinone at pH 6.5 and 7.4. Rate constants k_1, k_2, k_4, k_5, and k_9 in $M^{-1} \cdot s^{-1}$. Other rate constants in s^{-1}. The initial concentrations of the two forms of the enzyme can be calculated from the rate constants k_{10} and k_{-10}.

pH	k_1	k_2	k_3	k_4	k_5	k_9	k_{10}, k_{11}	k_{-10}, k_{-11}
6.5	110	$\geqslant 10^4$	5	80	$\geqslant 10^4$	300	3.6	0.4
7.4	900	$\geqslant 10^4$	$\geqslant 300$	900	200	2400	0.5	0.75



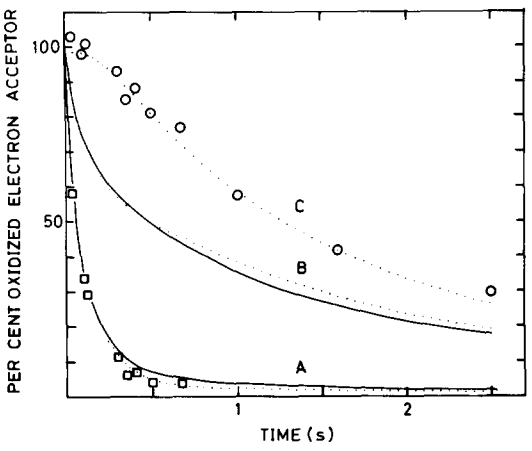


Fig. 2. EPR spectral changes during anaerobic reduction of *Rhus* laccase at pH 7.4. The reaction of laccase with hydroquinone was quenched by the rapid-freeze technique 100 ms (B), 500 ms (C) and 1 s (D) after mixing. The sample in (A) was obtained 30 ms after mixing the enzyme with anaerobic buffer. All experimental conditions, except for the pH, were the same as in the legend to Fig. 1.

Fig. 3. Time courses for the anaerobic reduction of *Rhus* laccase at pH 7.4. The full lines A (615 nm) and B (340 nm) represent changes in stopped-flow studies. The amounts of type 1 Cu^{2+} (squares) and type 2 Cu^{2+} (circles) were obtained by double integration of EPR spectra from rapid-freeze experiments. The dotted lines were obtained by computer simulations according to Scheme 1 and the rate constants shown in Table I. The experimental conditions (except for pH) were identical to those in the legend to Fig. 1.

The steady-state level of the type 2 Cu^{2+} was measured by EPR. In these experiments a hydroquinone solution was placed on top of the enzyme solution in EPR tubes and then mixed with a plunger. The reaction mixture was rapidly frozen in cold iso-pentane (160 K) at different times after mixing. Fig. 5B shows the EPR spectrum obtained after a reaction time of 60 s at 20°C. Identical spectra were obtained after 30 or 45 s. The steady-state lasted for about 70 s under these conditions, Double integration of the spectrum in Fig. 5B shows that the total integral is about 75% of that of the untreated enzyme. This decrease in EPR intensity is found to be due to about equal reduction of both types of paramagnetic copper ions. The EPR spectrum of type 2 Cu^{2+} is changed during turnover. The low-field line is shifted up-field about 1—1.5 mT and the linewidth is increased by about 1.5 mT at 12 K in all samples. No line broadening was detected at 77 K. Similar results were also found if the enzyme (0.075 mM) was treated with 2.4 mM hydroquinone for about 1 min, to obtain complete reduction, and then reoxidized by mixing with air and allowed to react for 20 s before freezing. This indicates that the same steady-state is reached in the two kinds of experiments. The lower steady-state level of type 1

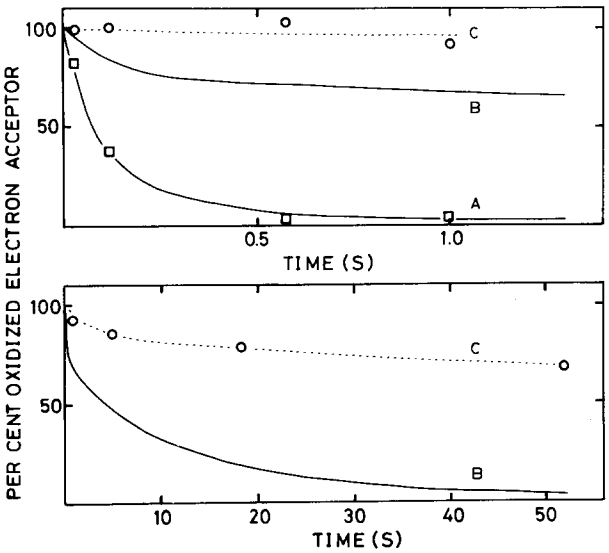


Fig. 4. Time courses for the anaerobic reduction of the electron acceptors in *Rhus* laccase at pH 8.5. The results are shown in two time scales. For explanation of lines and symbols see the legend to Fig. 3. Hydroquinone concentration was 1 mM. Other conditions were the same as in the legend to Fig. 1.

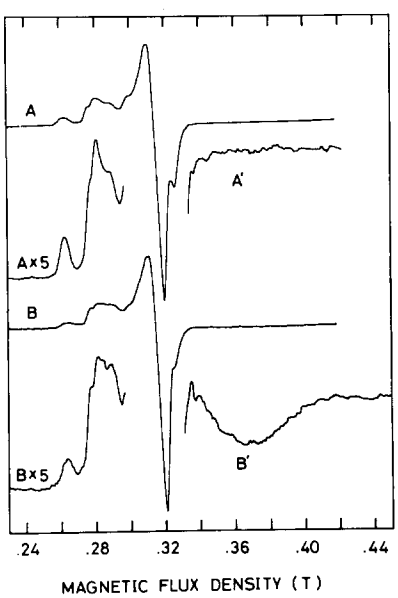


Fig. 5. EPR spectral changes of *Rhus* laccase during turnover. (A) The spectrum of native enzyme (0.1 mM) in potassium phosphate buffer, pH 6.5 $I = 0.25$. (B) The spectrum of laccase reduced in the presence of O_2. The mixture contained 0.083 mM enzyme, 1.7 mM hydroquinone and about 0.25 mM O_2 at the beginning of the reaction. Spectra were recorded at 12 K and 9.2 GHz with a microwave power of 30 dB and a modulation amplitude of 2 mT. Gain settings: (A) $1.25 \cdot 10^3$; (B) $2 \cdot 10^3$. Part of the spectra A' and B', were recorded with a microwave power of 10 dB and with a gain setting of $8 \cdot 10^3$. Details of the experimental technique are described in Results.

Cu^{2+} observed in the EPR experiments as compared to what is found in stopped-flow studies is probably due to the difference in concentrations of reactants in the two types of experiments.

All samples frozen during turnover show the presence of an EPR-detectable oxygen intermediate (Fig. 5) with the same signal shape as found in reoxidation experiments [11]. The amount of the oxygen intermediate, present during turnover, was estimated by comparison of the EPR signal amplitudes at about 0.36 T in the steady-state experiments and the maximal signal obtained in reoxidation experiments (cf. [11]). It was found that about 50% of the enzyme molecules contain the oxygen intermediate during turnover.

Displacement of OH^- from type 2 Cu^{2+} by F^- in Polyporus laccase

As reported earlier [1], and also shown here (Figs. 3 and 4), the reduction of the two electron acceptor becomes inhibited in a characteristic way with increasing pH. A similar type of inhibition is caused by F^- [6] which is known to bind strongly to type 2 Cu^{2+} [8]. It was therefore suggested that the inhibition at increasing pH is due to the formation of a type 2 Cu^{2+}-OH^- complex [1]. This copper ion would not permit the reduction of the two electron acceptor until the OH^- has been dissociated or converted to a bound water molecule. Since about 50% of the *Rhus* enzyme is inhibited at pH 7.4 about 50% of the type 2 Cu^{2+}-OH^- complex would exist at this pH. The binding of F^- to the type 2 Cu^{2+} should liberate any bound OH^- and result in a pH change, Unfortunately, F^- is only relatively weakly bound to *Rhus* laccase at pH 7.4 [1]. The binding of F^- to the same copper ion in *Polyporus* laccase at pH 5.5 is much stronger. Addition of one equivalent of F^- to the enzyme resulted in the formation of at least 90% of the type 2 Cu^{2+}-F^- complex [12]. At this pH the reduction of the two electron acceptor [6] indicates that there is close to 100% of the type 2 Cu^{2+}-OH^- complex according to the hypothesis presented above. *Polyporus* laccase should therefore be ideally suited to test for release of OH^-.

Fig. 6 shows the EPR spectral changes at low-field upon addition of 1.2 equivalents of NaF to *Polyporus* laccase B at pH 5.60. The first hyperfine line of type 2 Cu^{2+} (at about 0.263 T in the untreated enzyme) is gradually changing into two lines due to the formation of the F^- complex [8]. Under these conditions about 85% of the 1 : 1 complex was formed. Analysis of the spectra show that the linewidth of the type 2 Cu^{2+}-F^- complex is only about 70% compared to that of the uncomplexed ion.

The increase in pH of unbuffered enzyme after addition of 1.2 equivalents of NaF is shown in Fig. 7. Both the amount of the type 2 Cu^{2+}-F^- complex formed and the kinetics of its formation parallel the changes in pH. The change in pH represented by 100% was obtained by measuring the increase in pH after addition of 1.0 equivalent of NaOH to the native enzyme. Due to the strong buffering capacity of the enzyme this increase was only 0.23 pH unit.

Upon addition of 10.6 equivalents of NaF to the enzyme the rate of increase and the total change in pH were the same as with 1.2 equivalents. Therefore, the rate of binding of F^- to type 2 Cu^{2+}, as reflected by the changes in pH, appears to be limited by an intramolecular step with a rate constant of about $6 \cdot 10^{-3}\ s^{-1}$. This value is about seven times greater than found earlier [12] which might be due to the differences in conditions in the two experiments

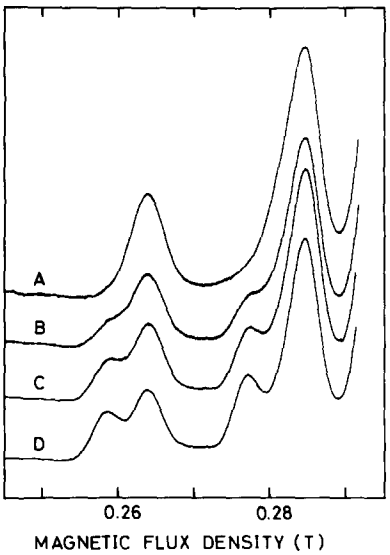


Fig. 6. EPR spectral changes at low-field of *Polyporus* laccase B induced by NaF. Enzyme and NaF in 0.1 M Na_2SO_4 were titrated to pH 5.60 before mixing. Concentrations of enzyme and NaF after mixing were 0.30 mM and 0.35 mM, respectively. The temperature was 293 K. Samples for EPR analysis were withdrawn and frozen at 77 K after 1.6 min (B), 5.3 min (C) and 25 min (D). Untreated enzyme (A). EPR spectra were recorded under the conditions described in the legend to Fig. 1.

(e.g. buffer, ionic strength and enzyme concentration). With 10.6 equivalents of NaF about 75% of the type 2 Cu^{2+}-$2F^-$ complex was formed at the end of the experiment (after 40 min).

Addition of NaF to Rhus laccase

According to earlier kinetic results there is not inhibition of the reduction of the two electron acceptor in *Rhus* laccase at pH values below 6.5 [1]. In order to test if OH^- can be liberated by F^- treatment of a laccase under such condi-

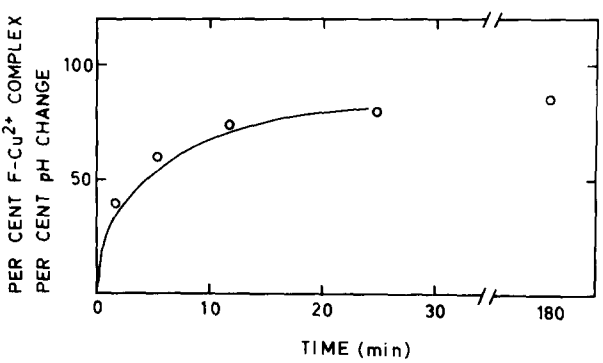


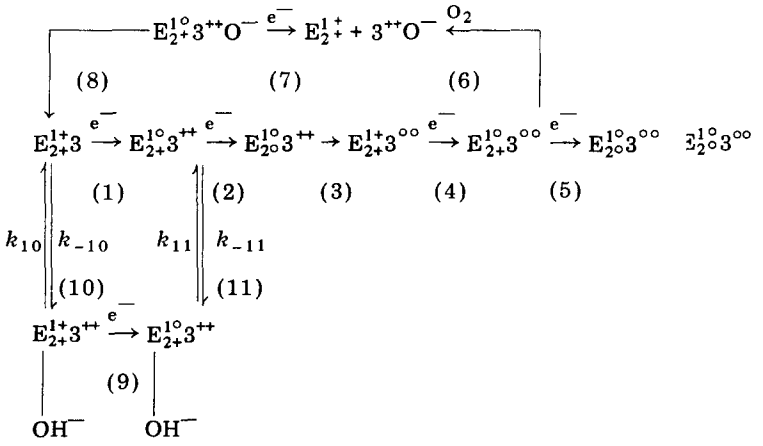
Fig. 7. Formation of type 2 Cu^{2+}-F^- complex and pH change on mixing of *Polyporus* laccase and NaF. Reaction conditions according to the legend to Fig. 6. The change in pH (line) was continually recorded and aliquots for EPR measurements (see Fig. 6) were withdrawn and frozen at the times indicated by the circles. The pH change induced by addition of 1.0 equiv. of NaOH was taken as 100%.

tions unbuffered *Rhus* enzyme, dissolved in 0.1 M Na_2SO_4 at pH 5.50, was treated with 1.1 equivalents of NaF. The final concentration of enzyme was 0.3 mM. The changes in pH were recorded as in the experiments with the *Polyporus* enzyme. Samples were withdrawn for analysis of EPR spectra up to 380 min after addition of NaF. At the end of the experiment at least 50% of the tupe 2 Cu^{2+} was changed into the F^- complex. No changes in pH were, however, observed. The increase in pH upon addition of 1.0 equivalent of NaOH to the native enzyme was 0.28 unit.

Discussion

The roles of the type 1 copper and the two-electron accepting type 3 site in the mechanism of anaerobic reduction of laccases were delineated in an earlier study [1]. The type 1 copper functions as a primary electron acceptor and delivers an electron from the reducing substrate to the two-electron acceptor. The type 2 copper was suggested as a possible donor of the second electron required by the cooperative two-electron accepting unit [1,13].

Biphasic reduction of the type 1 Cu^{2+} of the kind exemplified in Fig. 1 was earlier shown to be the result of its reduction by substrate and subsequent reoxidation, as the electron is passed along to the two electron acceptor, followed by final reduction [1]. At pH 6.0 and 6.5 the type 2 Cu^{2+} EPR intensity closely follows that of the type 1 Cu^{2+} which strongly supports the hypothesis that the type 2 copper also functions as an electron shuttle between reducing substrate and the type 3 site. This behaviour, which was observed over a range of substrate concentrations (5—20 mM), is consistent with a sequential mechanism in which the reduction of one site must preceed the reduction of the other. We suggest that the first site to be reduced is the type 1 Cu^{2+}. This is supported by the fact that this site can be reduced even if the reduction of the type 2 Cu^{2+} is blocked by F^- [14] or OH^- (see below). The role of the type 2 Cu^{2+} is rationalized in Scheme 1 which is an expanded version of the original model [1].



Scheme 1.

In the scheme the oxidized states are denoted by '+', the reduced by 'o'. The type 3 site is represented by two symbols to emphasize its function as a two-electron acceptor. At pH 6.0 and 6.5 the experimental results are consistent with the reaction sequence 1—5, with the reduction of the type 1 Cu^{2+} limiting the rate of electron transfer (see above and [1]). As the pH is increase, however, the reduction of the two-electron acceptor in a growing fraction of the molecules is rate-limited by a slow intramolecular pH-dependent reaction, while the type 1 copper is readily reduced [1]. These results imply that the laccases can exist in at least two interconvertible states with relative concentrations regulated by the pH of the medium and the pK of some titrable group in the enzyme. The additional reactions of the 'inactive' high-pH form are represented by reactions 9—11 (lower pathway). The relative abundancies of the high- and low-pH forms permit an estimation of the pK of the group involved to about 7.4 for *Rhus* laccase (1 : 1 at pH 7.4) and below 3.5 for *Polyporus* laccase (about 100 : 1 at pH 5.5) [1,6]. The regulating titrable group has been suggested to be a water molecule bound to the type 2 Cu^{2+} [1]. Several lines of evidence indicate that water (or OH^-) is in fact bound at this site, such as proton relaxation rate measurements [15] and the observation that the type 2 Cu^{2+} EPR spectrum is broadened in $H_2{}^{17}O$ medium [16]. One of the water molecules formed on reoxidation of reduced enzyme also appears to bind at the type 2 Cu^{2+} site [17]. Accordingly, in the 'inactive', high-pH state of the enzymes OH^- is likely to be bound to the type 2 Cu^{2+} while water might be bound in the 'active', low-pH state (Scheme 1). The demonstration that the binding of F^- to the type 2 Cu^{2+} in *Polyporus* laccase at pH 5.5 (high-pH form) results in the net release of one OH^- (Figs. 6 and 7) is fully consistent with this hypothesis as is the observation that F^- binding to this site in *Rhus* laccase at the same pH (low-pH form) has no effect on solution pH.

The elimination of OH^- represents the rate-limiting step in the reduction of the two-electron acceptor (k_{10}, k_{11}). Further support for an inhibiting OH^- is obtained from the observed pH effect on the rate of reduction of the two-electron acceptor. In *Polyporus* laccase the rate was found to increase by a factor of 3 when the pH was changed from 5.5 to 5.0 [13] which is consistent with a pK of 3.5 for the type 2 Cu^{2+}-bound water. Expected and observed rates agree fairly well also for *Rhus* laccase although other effects are seen at pH values around 8.5 [1].

Only the type 1 site is rapidly reduced by $Fe(CN)_6^{4-}$ in *Polyporus* laccase [6,14] which is consistent with the idea that a bound OH^- makes the type 2 site inaccessible to reductants. Under some conditions the type 2 Cu^{2+} appears to be reduced despite the OH^- [13]. In this case the reduction of the two electron acceptor could be the inhibited step.

With *Polyporus* laccase it has been observed that the rate of reduction of the type 2 Cu^{2+} appears to be influenced by the redox state of the type 3 site, being higher when this site is oxidized [13]. Such an effect might be responsible for the slow reduction of the type 2 Cu^{2+} EPR signal in *Rhus* laccase at higher pH values when the two-electron acceptor is reduced (Figs. 3 and 4). The disappearance of the EPR signal should then represent the final reduction of type 2 Cu^{2+} (step 5).

The present model has been used for computer simulations of the

anaerobic reduction of *Rhus* laccase at a wide range of hydroquinone concentrations ($1 \cdot 10^{-4}$—$1 \cdot 10^{-2}$ M). A list of rate constants used for simulations at two pH values with hydroquinone as reductant appears in Table I. The pH dependence of the measurable rate constants in reactions where the substrate is involved is explained by the assumption that the hydroquinone anion is the true substrate [1,2]. To obtain reasonable agreement with the experiments the rate constant k_3 had to be given a marked pH dependence.

The slow final reduction at some pH values (Figs. 3 and 4) of the type 2 Cu^{2+} (Reaction 5) excludes such a reaction from the catalytic pathway, keeping in mind that the reduction of the type 1 Cu^{2+} is rate-limiting for the overall reaction [1]. Reoxidation by O_2 should therefore take place before the final reduction of the type 2 copper. Furthermore, reoxidation studies at pH 4.4 [17] and 7.4 [18] have shown that the electron transfer between reduced type 2 copper and the oxygen intermediate, which occurs as a consequence of reoxidation of the fully reduced enzyme, is too slow to account for the catalytic rate.

The presence during turnover of an optical species with increased near-ultraviolet absorption [18] and an EPR signal (this study, Fig. 5), which has earlier been proposed to result from an O^- radical [11], indicates that a three-electron transfer takes place as a normal step in catalysis. Electron transfer to the oxygen intermediate likely occurs via the type 1 copper site ($k_7 \approx k_1$, $k_8 \gg k_7$) since the decay of the optical intermediate and the reaction of the two electron acceptor, as observed at 340 nm in post-steady-state reduction, are both observed to be limited by the rate of electron transfer to the type 1 copper site (see Ref. 1, Figs. 7 and 10). A tentative reoxidation pathway is included in the reaction scheme (Reactions 6—8).

The model predicts a high steady-state level of the type 2 Cu^{2+} during turnover. This is confirmed by the rapid-freeze studies. The type 2 Cu^{2+} low-field line is broadened and shifted towards higher field in the samples frozen during turnover when the oxygen intermediate is present (Fig. 5) (cf. [9]). The temperature-dependent line broadening of type 2 Cu^{2+} (Fig. 5) is consistent with a magnetic interaction between the oxygen intermediate and this Cu^{2+} (between 25 and 50% of the enzyme molecules contain both the oxygen intermediate and type 2 Cu^{2+}). No calculations of the distance between these paramagnetic centers, based on the observed line broadening, have been made. However, the small increase in linewidth excludes a close proximity.

Acknowledgements

We are indebted to Professors Bo G. Malmström and Tore Vänngård and to Dr. Roland Aasa for critical reading of the manuscript and helpful discussions. This work has been supported by grants from Statens Naturvetenskapliga Forskningsråd.

References

1 Andréasson, L.-E. and Reinhammar, B. (1976) Biochim. Biophys. Acta 445, 579—597
2 Holwerda, R.A. and Gray, H.B. (1974) J. Am. Chem. Soc. 96, 6008—6022

3 Reinhammar, B. (1970) Biochim. Biophys. Acta 205, 35—47
4 Fåhraeus, G. and Reinhammar, B. (1967) Acta Chem. Scand. 21, 2367—2378
5 Brändén, R., Malmström, B.G. and Vänngård, T. (1971) Eur. J. Biochem. 18, 238—241
6 Andréasson, L.-E., Malmström, B.G., Strömberg, C. and Vänngård, T. (1973) Eur. J. Biochem. 34, 434—439
7 Andréasson, L.-E., Brändén, R., Malmström, B.G., Strömberg, C, and Vänngård, T. (1973) in Oxidases and Related Redox Systems. Proceedings of the 2nd International Symposium (King, T.E., Mason, H.S. and Morrison, M., eds.), pp. 87—95, University Park Press, Baltimore
8 Malkin, R., Malmström, B.G. and Vänngård, T. (1968) FEBS Lett. 1, 50—54
9 Vänngård, T. (1967) in Magnetic Resonance in Biological Systems (Ehrenberg, A., Malmström, B.G. and Vänngård, T., eds.), p. 213, Pergamon Press, Oxford
10 Aasa, R. and Vänngård, T. (1975) J. Magn. Res. 19, 308—315
11 Aasa, R., Brändén, R., Deinum, J., Malmström, B.G., Reinhammar, B. and Vänngård, T. (1976) Biochem. Biophys. Res. Commun. 70, 1204—1209
12 Brändén, R., Malmström, B.G. and Vänngård, T. (1973) Eur. J. Biochem. 36, 195—200
13 Brändén, R. and Reinhammar, B. (1975) Biochim. Biophys. Acta 405, 236—242
14 Malmström, B.G., Finazzi Agrò, A. and Antonini, E. (1969) Eur. J. Biochem. 9, 383—391
15 Malmström, B.G., Reinhammar, B. and Vänngård, T. (1968) Biochim. Biophys. Acta 156, 67—76
16 Deinum, J. and Vänngård, T. (1975) FEBS Lett. 58, 62—65
17 Brändén, R. and Deinum, J. (1977) FEBS Lett. 73, 144—146
18 Andréasson, L.-E., Brändén, R. and Reinhammar, B. (1976) Biochim. Biophys. Acta 438, 370—379
19 Andréasson, L.-E., Brändén, R., Malmström, B.G. and Vänngård, T. (1973) FEBS Lett. 32, 187—189