Biochimica et Biophysica Acta, 438 (1976) 370—379
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

BBA 67845

KINETIC STUDIES OF *RHUS VERNICIFERA* LACCASE

EVIDENCE FOR MULTI-ELECTRON TRANSFER AND AN OXYGEN INTERMEDIATE IN THE REOXIDATION REACTION

LARS-ERIK ANDRÉASSON, ROLF BRÄNDÉN and BENGT REINHAMMAR

Institutionen för Biokemi, Göteborgs Universitet och Chalmers Tekniska Högskola, Fack, S-402 20 Göteborg 5 (Sweden)

(Received December 8th, 1975)

Summary

1. The reoxidation of reduced *Rhus vernicifera* laccase (monophenol,dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) by molecular oxygen has been studied by optical absorption and EPR methods.

2. The reoxidation by oxygen of the type 1 Cu^+ and the two-electron acceptor is characterized by a second-order rate constant of about $5 \cdot 10^6\ M^{-1} \cdot s^{-1}$.

3. The appearance of an optical intermediate (with an absorbance maximum around 360 nm) parallels the reoxidation of type 1 Cu^+ and the two-electron acceptor. It disappears in a first-order reaction with a half-time of 20 s. A similar intermediate is formed during normal turnover.

4. The type 2 Cu^+ appears to be reoxidized in an intramolecular reaction with a half-time of about 20 s, suggesting a correlation between the reoxidation of this site and the disappearance of the optical intermediate.

5. The results suggest that three electrons are rapidly transferred to oxygen leading to the formation of an enzyme-bound oxygen intermediate.

Introduction

Rhus vernicifera laccase (monophenol,dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1), like the related enzyme from the fungus *Polyporus versicolor*, contains four copper ions, present in three different forms, all of which seem to take part in the catalytic mechanism. In their oxidized states types 1 and 2 copper each exhibit specific EPR signals. In addition, type 1 Cu^{2+} is associated with an intense optical absorption band around 610 nm. The two non-paramagnetic copper ions (called type 3 in this report) are supposed to be associated with a cooperative two-electron acceptor, which shows a strong op-

tical absorption at about 340 nm in the oxidized state (for a recent review, see ref. 1).

To reduce fully the electron-accepting sites, four electrons are taken up from a substrate [2]. An important question is whether the enzyme transfers electrons to oxygen one at a time or if oxygen is reduced in multi-electron steps. A reduction involving one-electron transfers to oxygen would lead to intermediates such as O_2^- and OH. These would have to be strongly bound to the enzyme for two reasons. First, the final product of oxygen reduction is water, a reaction which requires additional electron transfer. Second, the free energy change for the formation of free O_2^- is substantial because of the large difference in oxidation-reduction potential (>0.6 V) between the electron acceptors in the enzyme and the O_2/O_2^- couple [3,4] so that a mechanism involving this reaction would be extremely slow.

The presence of a cooperative two-electron acceptor [4] in blue oxidases has led to the suggestion that reduction of oxygen occurs in consecutive two-electron steps [5]. Earlier results from the reoxidation of partly reduced fungal laccase also give some support to this idea [6].

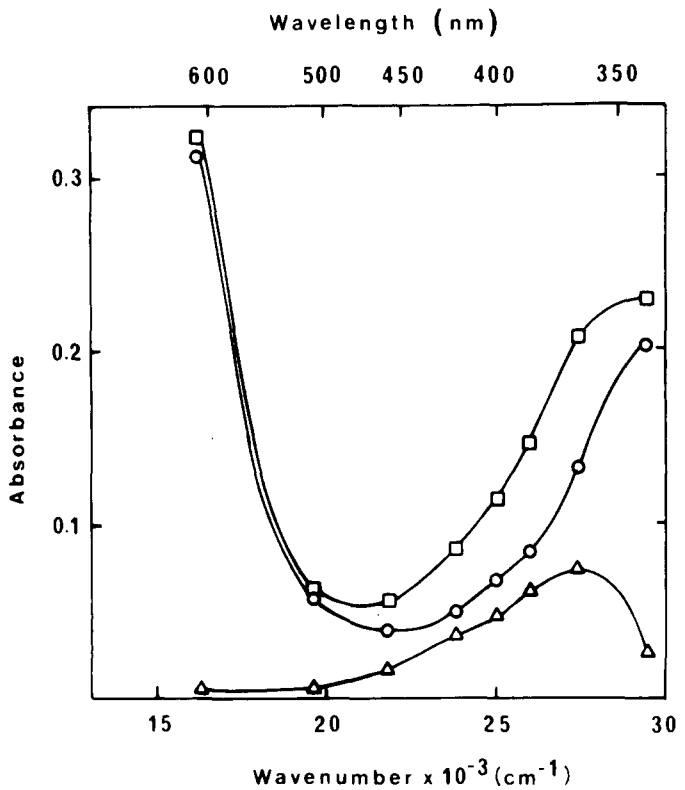

Fig. 1. Optical absorption spectral changes observed in the reaction between fully reduced laccase and O_2. The experiment was performed in a stopped-flow apparatus (2 cm optical path, 4 ms dead-time) at 25°C and in 0.1 M phosphate buffer, pH 7.4. The concentrations of enzyme and O_2 were 28 and 125 μM, respectively. Ascorbate was used for the reduction of the enzyme. The figure shows the difference spectral changes at 50 ms (□) and 3 min (○) after mixing of reduced enzyme and oxygen-containing buffer. The difference between these two curves is also shown (△).

The results in this communication suggest a more complex reoxidation mechanism, as three electrons seem to be involved in a rapid reaction between reduced *Rhus* or *Polyporus* laccase and oxygen. This finding indicates that oxygen receives three electrons from the reduced enzyme in a rapid reaction and that an oxygen intermediate, for example an OH radical, is formed. Although this interpretation appears to be the most likely one, other mechanisms cannot be excluded at the present.

Materials and Methods

R. vernicifera laccase was prepared as described earlier [7]. Protein concentration was determined spectrophotometrically at 615 nm on the basis of an absorbance coefficient of 5.7 $mM^{-1} \cdot cm^{-1}$ [8].

Analytical grade ascorbic acid and hydroquinone, used to produce reduced enzyme, were obtained from Merck AG (Darmstadt, Germany) and Schuchart Chemical Co. (Munich, Germany), respectively. Other chemical used in the experiments were also of analytical grade. Deionized distilled water was used to prepare aqueous solutions. Protein, reducing agents and oxygen were dissolved in 0.1 M potassium phosphate buffer, pH 7.4.

Stopped-flow experiments were performed at 25°C. The equipment and the technique adopted for preparation of anaerobic enzyme and substrate solutions were essentially the same as those described earlier [9,10].

Rapid-freeze studies were carried out according to a previously described method [9].

Electron paramagnetic resonance (EPR) measurements were made at about 9.2 GHz and 77°K in a Varian E-3 spectrometer. The concentrations of types 1 and 2 Cu^{2+} were determined by integration [11] of spectra using oxidized native enzyme as a reference.

Results

The reoxidation of *Rhus* laccase, which had previously been anaerobically reduced with four electron equivalents of reductant and then rapidly mixed with oxygen-containing buffer at 25°C, was studied in a stopped-flow apparatus and by the rapid-freeze EPR technique. Some results from stopped-flow experiments are shown in Figs. 1 and 2, the wavelengths used being indicated in Fig. 1. It is seen that when reduced laccase and O_2 are allowed to react, strong absorption bands at 615 and 340 nm appear rapidly. These are similar to those seen in fully oxidized enzyme. However, the rapidly formed intermediate spectrum exhibits higher absorption between 500 and 340 nm than the oxidized minus reduced difference spectrum (see Fig. 1). The maximum absorption difference between these two states is around 360 nm. The absorption at the different wavelengths reaches a maximum after about 15 ms and then slowly decreases to values characterizing the fully oxidized enzyme (see Fig. 2). The pseudo first-order rate constant estimated from the observable part of the absorption increase at 615, 385 and 340 nm is about 350 s^{-1}. The relaxation towards the fully oxidized state is a first-order reaction at all wavelengths indicated in Fig. 1. The reaction as studied at 340 nm is shown in Fig. 3. The half-

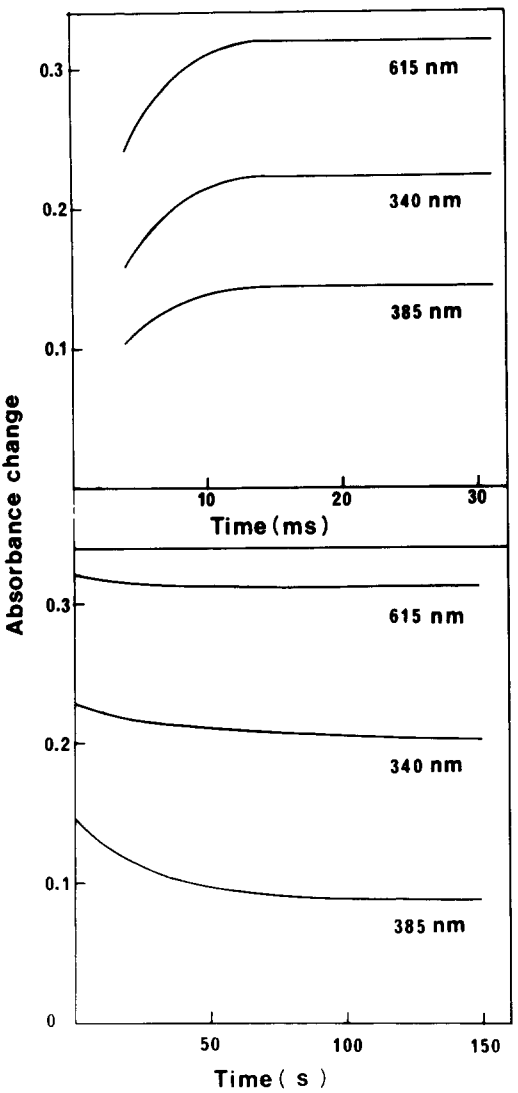

Fig. 2. Time course of the reaction between fully reduced laccase and O_2 recorded at 615, 385 and 340 nm on two different time scales. Experimental conditions were identical to those in Fig. 1.

time of the reaction, about 20 s, is independent of the concentration of enzyme. The absorption changes accompanying this decay were smaller when enzyme reduced with less than four equivalents of reductant was reoxidized.

Aerobic reduction experiments with an excess of ascorbate over oxygen concentration reveal that the steady-state absorption spectrum differs from the spectrum of the oxidized enzyme in a way similar to that of the intermediate spectrum obtained in reoxidation experiments (cf. Figs. 1 and 4). In the aerobic reduction experiments the enzyme is fully oxidized at the beginning of the reaction. The optical absorption changes in one such experiment are summarized in Fig. 4. The values for the difference between the steady-state absorbance (after 10 s) and the absorbance of the reduced enzyme have been cor-

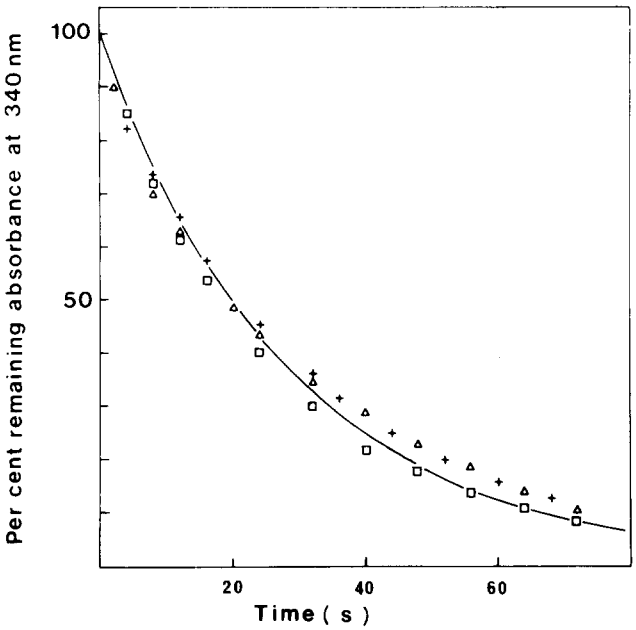

Fig. 3. Kinetics of the disappearance of the intermediate spectrum formed during reoxidation of fully reduced enzyme. Absorption decreases were measured at 340 nm. The maximum changes at the different enzyme concentrations were normalized to 100%. The protein concentrations were: 14 μM (□), 8 μM (+) and 4 μM (△). The oxygen concentration was 30 μM. Other conditions were as in Fig. 1. The line represents a first-order reaction with a half-time of 20 s.

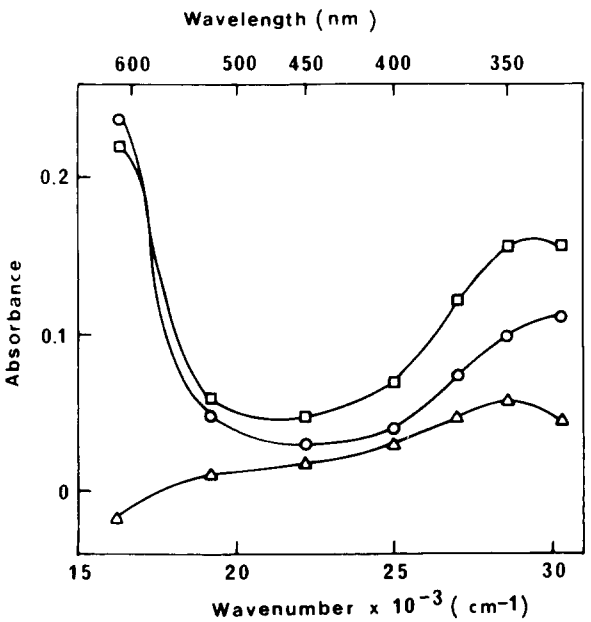

Fig. 4. Optical spectral changes observed during aerobic reduction of laccase. The symbols indicate: □, difference between the absorption recorded at the steady state and that of reduced enzyme; ○, difference absorption between native oxidized and reduced enzyme; △, the difference between these two spectra. (The value at 615 nm falls below zero because the steady-state level for type 1 Cu^{2+} is less than 100%). Concentrations of enzyme, ascorbate and oxygen were 22 μM, 5 mM and 250 μM, respectively. Other conditions as in Fig. 1.

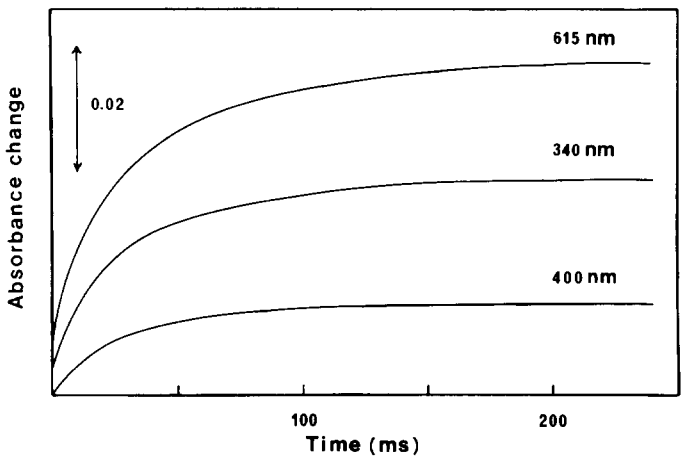

Fig. 5. Time course for the reaction between fully reduced laccase and O_2. The figure shows the absorption increases at 615, 400 and 340 nm versus time when reduced enzyme was mixed with oxygen-containing buffer. Concentrations of enzyme and O_2 were 4.5 and 5 μM, respectively. The traces have been vertically displaced for clarity.

rected for an absorption increase (less than 15% of the total change) occurring between 330 and 400 nm. This increase was much faster than the reduction of type 1 Cu^{2+} and might arise from the binding of reducing substrate, since it was also seen in anaerobic reduction experiments. It was also observed that the intermediate spectrum disappears much faster when the oxygen has been consumed in steady-state experiments than it does after reoxidation of previously reduced enzyme. In fact, its decay in the post-ready-state reaction was at least as fast as the reduction of the type 3 copper (0.44 s^{-1}).

Under strictly anaerobic reduction conditions the laccase absorption spectrum can be described in terms of the absorption properties of the known electron acceptors. The intermediate spectrum described above must, therefore, represent some phenomenon connected with the reoxidation of the enzyme by oxygen.

Reoxidation of fully reduced laccase was also studied at constant enzyme concentration (2 or 4.5 μM) and with an oxygen concentration varying between 0 and 30 μM. The rapid absorption increase at 615, 400 and 340 nm are shown in Fig. 5. An analysis of the absorbance changes at these three wavelengths reveals that they represent reactions proceeding with the same rate. The rapid absorption increase at the different wavelengths depends on the proportion between oxygen and enzyme concentrations. With a ratio of one or higher, maximum absorbance increase is observed. However, only about 50% of this change is found if the oxygen concentration is one half of the enzyme concentration. A logarithmic plot of the absorbance changes versus time yields a straight line. At concentrations of oxygen close to that of the enzyme the expected deviations from first-order behaviour are observed at later stages of the reaction. In these cases only the initial part of the first-order plot was used for the calculation of the rate constant. The absorption changes at 615 and 340 nm at low concentrations of oxygen were also analyzed according to a second-order reaction. In the second-order plots straight lines were obtained for the greater

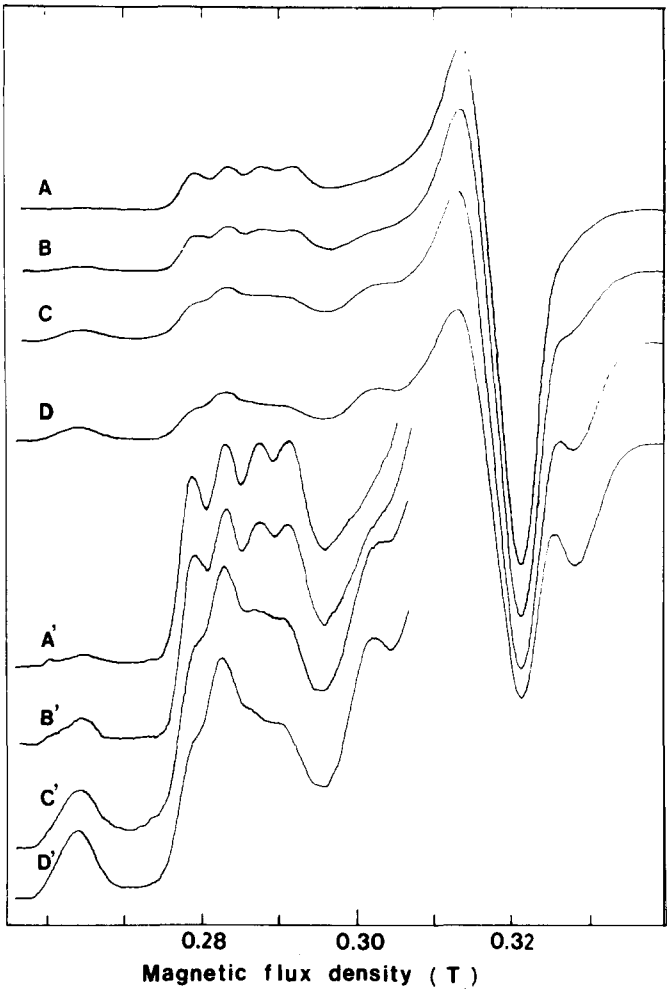

Fig. 6. EPR spectral changes observed during the reaction between fully reduced enzyme and oxygen. Enzyme (500 μM) was reduced with four electron equivalents of ascorbate and then rapidly mixed with oxygen-saturated buffer in the rapid-freeze apparatus. The reaction was quenched 25 ms (A), 200 ms (B), 25 s (C) and 10 min (D) after mixing by freezing at about 150°K. Parts of the spectra are also shown with 10 times higher grain (primed letters). Spectra were recorded at about 77°K with a microwave power of 10 mW. Modulation amplitude was 2.5 mT. Frequency was 9.15 GHz.

TABLE I

OBSERVED RATE CONSTANTS FOR THE REOXIDATION OF TYPE 1 COPPER AND THE TWO-ELECTRON ACCEPTOR (30 μM O_2)

Enzyme concentration (μM)	k_{obs} (s^{-1})	
	Type 1 copper	Two-electron acceptor
2	144	153
4	139	137
8	142	144
14	130	122

TABLE II

PERCENT REOXIDIZED TYPES 1 AND 2 COPPER VERSUS TIME OBSERVED IN RAPID-FREEZE EXPERIMENTS

The differences between the type 1 Cu^{2+} values are within experimental error. The values at 10 min represent full reoxidation. Values have been corrected for differences in EPR tube diameters.

Time after mixing	Type 1 Cu^{2+} (%)	Type 2 Cu^{2+} (%)
25 ms	105	7
200 ms	106	16
25 s	104	58
10 min	100	100

part of the reaction. Apparently, reoxidation at these wavelengths can be described in terms of a second-order reaction with a rate constnat of $5 \cdot 10^6 - 7 \cdot 10^6$ $M^{-1} \cdot s^{-1}$. At 125 μM oxygen this reaction shows saturation (see above).

Reoxidation of fully reduced enzyme was also performed with varying enzyme concentrations at constant oxygen concentration. The pseudo first-order rate constant so obtained (Table I) corresponds to a second-order rate constant of about $5 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ in agreement with the data presented above.

Results from the rapid-freeze reoxidations of enzyme, reduced with four electron equivalents of reductant, are shown in Fig. 6. Type 1 copper is completely reoxidized even at the shortest observation time (25 ms) in agreement with the stopped-flow data. The reappearance of type 2 copper EPR signal is, however, a much slower reaction with an estimated half-time of about 20 s (see Table II). When the type 2 copper is still reduced and the optical intermediate is present the EPR spectrum of the type 1 Cu^{2+} differs from that of the fully oxidized enzyme. This is most clearly seen from the difference in linewidth between the two states (Fig. 6 (A′ and D′), cf. Fig. 2 in ref. 8). Also spectrum A in Fig. 6 is characterized by $g_{\parallel}$ and $A_{\parallel}$ of 2.291 and $4.6 \cdot 10^{-3}$ cm^{-1}, respectively, whereas these parameters in the oxidized enzyme are 2.298 and $4.3 \cdot 10^{-3}$ cm^{-1}, respectively [8]. The differences are well beyond the errors of measurement.

Discussion

When reduced laccase and oxygen are allowed to react the optical absorption increase at 615 nm, due to type 1 Cu^{2+}, and the reappearance of the type 1 Cu^{2+} EPR spectrum, show that this site is rapidly reoxidized. In contrast, type 2 Cu^{2+} is not formed as quickly (see Fig. 6). Simultaneously with the reappearance of the 615 nm band, an absorption band at about 340 nm develops. This band is similar in shape and position to that representing the oxidized two-electron acceptor in the native enzyme. This band, therefore, probably originates from oxidation of the two-electron acceptor. However, the enzyme during reoxidation shows higher absorption than the native enzyme between 500 and 340 nm. This difference might be due to an interaction between the oxidized two-electron acceptor and a bound oxygen intermediate, which would be formed as a result of transfer of electrons from the rapidly reoxidized sites in the protein. An alternative interpretation would be that the intermediate spec-

trum is a superposition of the normal spectrum of the oxidized two-electron acceptor and the spectrum of a protein-bound oxygen intermediate. One cannot, however, definitely rule out that the rapid absorption increase at 340 nm does not at all represent reoxidation of the two-electron acceptor, but is the result of an interaction between hyperoxide, formed through the reoxidation of type 1 copper, and the partially reduced enzyme. This possibility seems, on the other hand, rather remote considering the similarity in shape and position of the rapidly appearing 340 nm band and the normal spectrum of the oxidized two-electron acceptor. Also, the very presence of a cooperative two-electron redox site in the enzyme is strong circumstantial evidence that two-electron transfer is involved in the reaction mechanism, maybe to bypass energetically unfavourable reactions.

The arguments given indicate that both type 1 copper and the two-electron acceptor are oxidized in reactions which are not resolved on the time scale of the rapid kinetic experiments and which are first-order in protein concentration. This would exclude a dependence of the reoxidation of these sites on electron transfer between enzyme molecules (which under certain conditions seems to limit the oxidation of type 1 copper in the analogous enzyme from the fungus *P. versicolor* [12]). Data from the reoxidation of these electron acceptors also show that this reaction is first order in oxygen concentration and that the amounts of type 1 copper and the two-electron acceptor which are reoxidized depend on the ratio between oxygen and enzyme concentrations. Taken together these facts suggest that each reduced enzyme molecule interacts with only one oxygen molecule in the rapid phase of oxidation. As type 2 copper is oxidized much more slowly than the other two acceptors, the most straightforward explanation is that oxygen rapidly receives three electrons, this leading to the formation a protein-bound oxygen intermediate. This intermediate then slowly disappears in an intramolecular reaction. This is in agreement with the finding that the half-times for the oxidation of type 2 Cu^+ and the decay of the optical intermediate appear to be the same. The decay of the oxygen intermediate would thus be coupled to electron transfer from type 2 Cu^+ with the formation of water and fully oxidized enzyme. However, the oxidation of type 2 Cu^+ and the disappearance of the optical intermediate are very slow reactions (half-time 20 s). If they represent reactions in the catalytic mechanism, they must proceed with higher rates during turnover as the turnover number for oxygen reduction with 5 mM ascorbate and 20 μM enzyme is about 1 s^{-1} (Andréasson, L.-E. and Reinhammar, B., to be published). It was also observed in steady-state experiments that an optical intermediate, very similar to the one just discussed, quickly disappears at the exhaustion of oxygen. The slow electron transfer between type 2 Cu^+ and the intermediate, observed in reoxidation experiments, might then represent a reaction not taking place in the normal catalytic reaction. Therefore, in the presence of excess reducing substrate, the apparent decay of the optical intermediate could be due to its further reduction, possibly mediated by type 1 copper.

Optical intermediates of similar appearance, as the one presented in this communication, have also been observed in the reoxidation by oxygen of reduced *Polyporus* laccase [6] and ceruloplasmin [13]. These common features, and the fact that the intermediate is also formed under steady-state conditions

with the *Rhus* enzyme, suggest that it represents a true intermediate in the oxidation mechanism of blue copper-containing oxidases in general.

Reoxidation of type 2 Cu^+, much slower than that of type 1, has also been observed in studies with *Polyporus* laccase which was reduced with four electron equivalents of ascorbate before reoxidation with oxygen [14]. This similarity between two different laccases further suggests that the mechanisms for oxidation of these enzymes are in principle the same.

Under the conditions for the EPR experiments no new signals seem to appear during the reoxidation of the *Rhus* or *Polyporus* laccase. This appears to be inconsistant with the present results, which indicate that three electrons are transferred to oxygen in the rapid reaction phase. Recent studies [14] at lower temperatures have, however, shown that a new EPR signal, corresponding to about one unpaired spin, is formed during reoxidation of both laccases. It can only be detected below 20° K, which explains the 77° K data. Furthermore, the new signal seems to be associated with the optical intermediate.

The parameters of type 1 Cu^{2+} in the *Rhus* enzyme changes, as the enzyme becomes fully oxidized. These effects are probably due to conformational changes accompanying reoxidation of type 2 Cu^+ or to the disappearance of the optical intermediate.

The present results indicate that both laccases, and perhaps all blue oxidases, utilize the same pathway for the reduction of oxygen. Currently, experiments are being performed to investigate further the nature of the intermediate and the reactions leading to its formation and decay.

Acknowledgements

This study has been supported by grants from the Knut and Alice Wallenberg Foundation and the Swedish Natural Science Research Council. The authors wish to thank professors Bo G. Malmström and Tore Vänngård for their critical examination of the manuscript.

References

1 Malmström, B.G., Andréasson, L.-E. and Reinhammar, B. (1975) The Enzymes (Boyer, P.D., ed.), Vol. 12, pp. 507—579, Academic Press, Inc., New York
2 Reinhammar, B. and Vänngård, T. (1971) Eur. J. Biochem. 18, 463—468
3 Wood, P. (1974) FEBS Lett. 44, 22—24
4 Reinhammar, B. (1972) Biochim. Biophys. Acta 275, 245—259
5 Malmström, B.G. (1970) Biochem. J. 117, 15P—16P
6 Andréasson, L.-E., Brändén, R., Malmström, B.G. and Vänngård, T. (1973) FEBS Lett. 32, 187—189
7 Reinhammar, B. (1970) Biochim. Biophys. Acta 205, 35—47
8 Malmström, B.G., Reinhammar, B. and Vänngård, T. (1970) Biochim. Biophys. Acta 205, 48—57
9 Andréasson, L.-E., Brändén, R., Malmström, B.G., Strömberg, C. and Vänngård, T. (1973) Oxidases and Related Redox Systems, Proc. 2nd Int. Symp. (King, T.E., Mason, H.S. and Morrison, M., eds.), pp. 87—95, University Park Press, Baltimore
10 Andréasson, L.-E., Malmström, B.G. and Vänngård, T. (1973) Eur. J. Biochem. 34, 434—439
11 Aasa, R. and Vänngård, T. (1975) J. Magn. Res. 19, 308—315
12 Malmström, B.G., Finazzi Agró, A. and Antonini, E. (1969) Eur. J. Biochem. 9, 383—391
13 Manabe, T., Manabe, N., Kiromi, H. and Hatano, H. (1972) FEBS Lett. 23, 268—270
14 Aasa, R., Brändén, R., Deinum, J., Malmström, B.G., Reinhammar, B. and Vänngård, T. (1976) FEBS Lett. 61, 115—119