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THE EFFECT OF pH ON THE OXYGEN INTERMEDIATE AND THE DIOXYGEN REDUCING SITE IN BLUE OXIDASES

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Summary

A new method for the preparation of laccase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) from acetone powder from the latex of the Vietnamese lacquer tree *Rhus succedanea* is described. The spectral and catalytic properties resemble more those of the fungal laccase from *Polyporus versicolor* than those of the lacquer tree enzyme from *Rhus vernicifera*.

Laccase from *R. succedanea* and human ceruloplasmin form similar types of oxygen intermediates, shown by EPR, as the laccases from *R. vernicifera* and *P. versicolor*.

The oxygen intermediate formed in the reoxidation of anaerobically-reduced *R. vernicifera* laccase shows pH-dependent characteristics, suggesting the presence of at least one protonable group in the dioxygen reducing site. At pH 7.4 a broad line is formed which at pH 4.0 becomes much more narrow and rather similar to that obtained from *P. versicolor*.

Protons can exchange rapidly with the oxygen intermediate or its near surroundings as shown by the fast change in line shape of the intermediate EPR signal, when pH is rapidly dropped from 7.4 to 4.0 or increased from 4.0 to 7.4.

The decay of the oxygen intermediate in *R. vernicifera* laccase is pH dependent with a $t_{1/2}$ = 13 s at pH 7.4 and a $t_{1/2}$ = 1 s at pH 4.0. With *R. succedanea* and *P. versicolor* laccase the decay of the oxygen intermediates has a $t_{1/2}$ = 1 s at pH 5.5.

There is a linear correlation between the decrease of the oxygen intermediate and the reoxidation of the Type 2 copper. This suggests that the oxygen intermediate can accept only one electron and water is formed.

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Introduction

When fully reduced *Rhus vernicifera* and *Polyporus versicolor* laccase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) react with O_2 the Type 1 copper and the Type 3 copper pair are rapidly reoxidized, while the Type 2 copper remains EPR undetectable [1]. The EPR spectrum at 77 K shows only the presence of Type 1 Cu^{2+}, but at lower temperatures a paramagnetic intermediate becomes EPR detectable [2]. Reoxidation of *P. versicolor* laccase with $^{17}O_2$ gave conclusive evidence that this paramagnetic species is a true oxygen intermediate [3].

The ultimate product, water, formed in the reduction of O_2 by the fully reduced *R. vernicifera* laccase was found to be coordinated to the Type 2 Cu^{2+} and it was proposed that the Type 2 copper ion, the Type 3 copper pair and the oxygen intermediate are situated in the dioxygen reducing site [4]. It was further suggested that this site is a cavity which can be open or closed, a property that would be important for the catalytic mechanism of the enzyme in stabilizing reactive oxygen intermediates.

In this study we give more information about the dioxygen reducing site and the nature of the oxygen intermediate.

Materials and Methods

Chemicals. The ^{17}O-enriched O_2 (91.8%) was purchased from Miles Laboratories Ltd., Slough SL2 4LY, U.K. All other chemicals were of analytic grade. Buffers were prepared from deionized, distilled water.

Proteins. R. vernicifera laccase was prepared as described earlier [5]. Protein concentrations were determined on the basis of an extinction coefficient of 5.7 $mM^{-1} \cdot cm^{-1}$ at 615 nm. The enzyme from some preparations shows heterogeneity with respect to its EPR characteristics. The low-field line of the Type 2 Cu^{2+} sometimes contained as much as 20% of a copper ion different from the "normal" Type 2 Cu^{2+}. Furthermore, the distinct hyperfine structure around $g_\perp$, which can be observed at low pH [4], is in such preparations less resolved. Since most of the results in the present study deal with changes in EPR signals we have only used an enzyme which shows an EPR spectrum with a clean low-field line of the Type 2 Cu^{2+} and distinct well-resolved hyperfine structure around $g_\perp$ at pH 4.0.

Ceruloplasmin was prepared from Cohn fraction III (a gift from AB Kabi, Stockholm). A modification of the procedure of Deutsch was used for the preparation of this enzyme [6].

Acetone powder, prepared from the natural lacquer of *Rhus succedanea* of Vietnamese origin, was used for the preparation of *R. succedanea* laccase. The acetone powder was obtained from Saito and Co. Ltd., Tokyo, Japan. The enzyme was prepared according to the following procedure. 100 g of acetone powder was suspended in 2 l of 100 mM sodium acetate buffer at pH 5.5. The suspension was homogenized at full speed in a Waring blendor for 2 min, stirred overnight at 4°C and after that centrifuged for 10 min at 28 000 $\times g$. The supernatant was concentrated to a volume of about 400 ml using an Amicon ultrafiltration cell supplied with a PM-30 filter. Solid ammonium sulfate was

added until the solution turned turbid. After 2 h at 4°C a white floatage was carefully removed from the top of the solution. A precipitate was discarded after centrifugation at 28 000 $\times g$ for 10 min and solid ammonium sulfate was added to the green supernatant until saturation. The saturated solution was stirred for 2 h at 4°C before it was centrifuged for 10 min at 28 000 $\times g$. The blue-green precipitate was resuspended in 50 mM sodium acetate buffer at pH 5.5 and applied to a column of concanavalin A-Sepharose 4B (4 × 15 cm) equilibrated with the same buffer. The column was washed with 500 ml of the same buffer. A blue protein was eluted from the column with 50 mM sodium acetate buffer at pH 5.5, containing 1 M glucose and 1 M NaCl, and the eluate was concentrated to a volume of 10—20 ml. The protein was desalted by passing it through a Sephadex G-25 column, equilibrated with 20 mM sodium phosphate buffer at pH 5.5 and directly applied to a DEAE A-50 Sephadex column (4 × 20 cm) equilibrated with the same buffer. A green protein fraction was eluted with 20 mM sodium phosphate buffer at pH 5.5, directly after the void volume, while a blue protein fraction was retarded and nicely separated from the green fraction. Still another blue protein fraction was formed at the top of the column. This last blue fraction could be eluted with 200 mM sodium phosphate buffer at pH 5.5.

Analytical methods and anaerobic technique. The total amount of copper was determined with 2,2'-biquinoline [7]. The anaerobic technique used has been described elsewhere [8].

Electron paramagnetic resonance (EPR) measurements. EPR spectra were run at 9 GHz at 77 K and 15 K with a Varian E-3 and a E-9 spectrometer, respectively. EPR-detectable copper was determined by double integration of the 9 GHz spectra and corrections for the different g-factors were applied [9].

Rapid freeze technique. The rapid freeze technique has been described before [10].

Results

Some properties of R. succedanea laccase

Fig. 1 shows the optical absorbance spectrum of the first blue fraction (see Materials and Methods). The second blue fraction, with the same extinction coefficient at 610 nm as the first one, absorbs about twice as much in the near ultraviolet region from 400 to 300 nm. However, both blue fractions have identical oxidized-reduced difference spectra.

The EPR spectra for the two blue fractions are also identical (see Fig. 2A). The EPR-detectable copper content was 47% of the total amount of copper and Type 2 Cu^{2+} corresponds to about 50% of the total EPR-detectable copper ions. The estimated EPR parameters are the following $g_{\|} = 2.204$, $A_{\|} = 0.0076$ cm^{-1} and $g_{\perp} = 2.045$ for Type 1 Cu^{2+} and $g_{\|} = 2.217$, $A_{\|} = 0.0184$ cm^{-1} for Type 2 Cu^{2+}.

In 20 mM sodium phosphate buffer at pH 5.5 the extinction coefficient for the optical absorbance at 610 nm is 5.8 $mM^{-1} \cdot cm^{-1}$/Type 1 Cu^{2+}. The band at 330 nm, in the oxidized-reduced difference spectrum, has an extinction coefficient of 3.2 $mM^{-1} \cdot cm^{-1}$/2 EPR non-detectable copper ions.

R. succedanea laccase has a turnover of 40 s^{-1} in 20 mM sodium phosphate

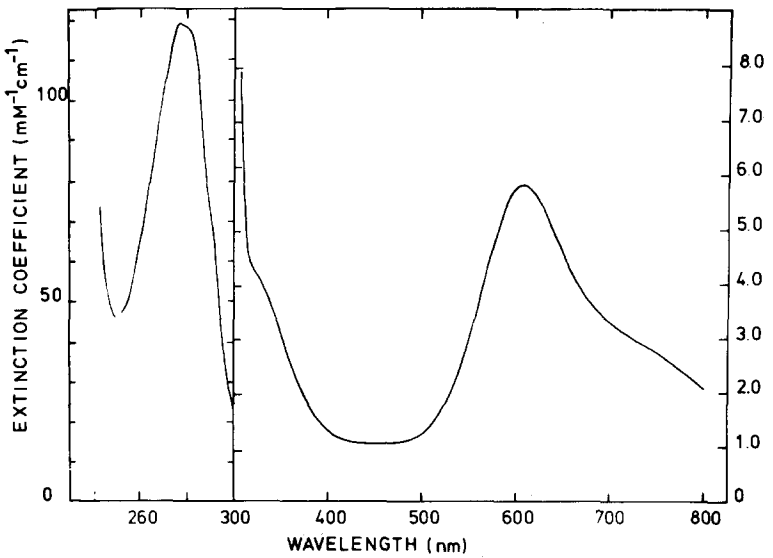


Fig. 1. Optical absorbance spectrum of *R. succedanea* laccase in 20 mM sodium phosphate buffer at pH 5.5.

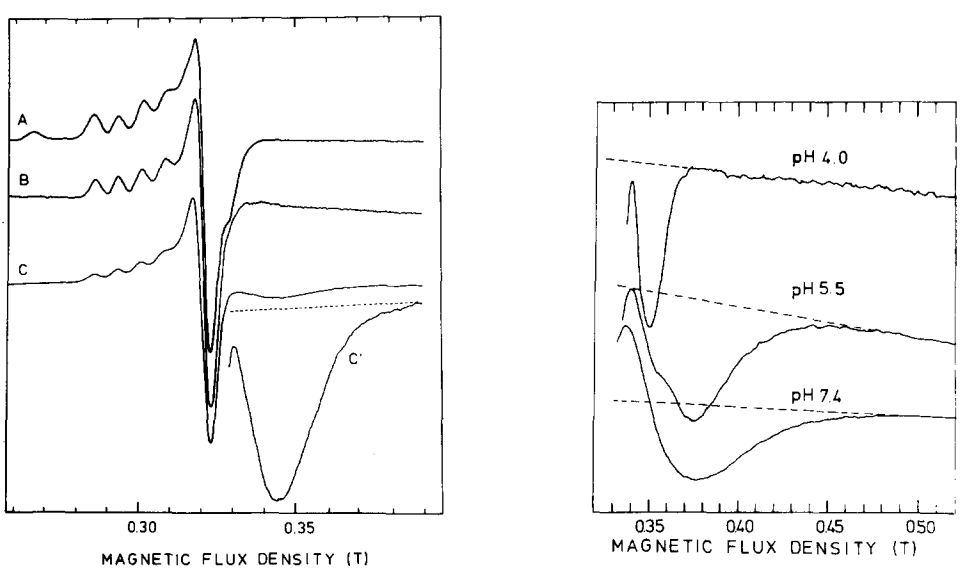


Fig. 2. EPR spectra of *R. succedanea* laccase in 20 mM sodium phosphate buffer at pH 5.5. (A) Aerobic solution of the laccase. (B and C) An anaerobic solution of the laccase, reduced with 1.0 electron equivalent of ascorbic acid/copper ion, was mixed with O_2-saturated buffer. The reaction was quenched after 30 ms. Spectra A and B were recorded at 77 K and 9.2 GHz with a microwave power of 10 mW. Spectra C and C' were recorded at 15 K and 9.15 GHz. The microwave power was 20 and 170 mW, respectively, and the gain was $10 \cdot 10^3$ and $3.2 \cdot 10^3$, respectively.

Fig. 3. Rapid freeze EPR spectra of *Rhus vernicifera* laccase at pH 4.0, 5.5 and 7.4. An anaerobic solution of the laccase, reduced with 4.2 electron equivalents of ascorbic acid was mixed with O_2-saturated buffer and the reaction was quenched after 5 s. The final protein concentration was 0.6 mM. 100 mM sodium acetate buffers were used at pH 4.0 and 5.5 and 20 mM sodium phosphate buffer was used at pH 7.4. The spectra were recorded at 15 K and 9.13 GHz. The microwave power was 170 mW. The gain was 10^3, $6.3 \cdot 10^2$ and $2.5 \cdot 10^2$ for pH 4.0, 5.5 and 7.4, respectively.

buffer at pH 5.5. This is the same as that for *P. versicolor* laccase under the same conditions.

An intermediate EPR signal appears upon reoxidation of fully reduced *R. succedanea* laccase (Fig. 2c). Both the decay ($t_{1/2}$ = 1 s) and the line shape of the signal at pH 5.5, are similar to that found for *P. versicolor* laccase [2].

Another method for the preparation of *R. succedanea* laccase has been described [11]. When we prepared the enzyme according to this method we lost large amounts of enzyme.

The oxygen intermediate and its pH dependence

When fully reduced *R. vernicifera* laccase is reoxidized at pH 4.0 an intermediate EPR signal is formed (Fig. 3A) with a line shape, temperature dependence and power saturation similar to that found for *P. versicolor* laccase [2]. Substituting $^{17}O_2$ for $^{16}O_2$ at pH 4.4 gives a line broadening of this signal which is consistent with the same effect for *P. versicolor* laccase [3].

To test if the oxygen intermediate was protonated or not at pH 4.0 we made reoxidation experiments in ^2H_2O instead of H_2O. We have not been able to detect any effect of ^2H_2O on the line shape of the intermediate signal.

The narrow EPR intermediate at pH 4.0 becomes much broader if the pH is increased (Fig. 3). The linewidth at half the maximal amplitude increases from 15 mT at pH 4.0 to 55 mT at pH 7.4, simultaneously as the line form of the signal changes. Between pH 4.4 and 6.6 the line shape is very sensitive to pH changes and a more complex line shape is seen (Fig. 3B at pH 5.5), which cannot be made up to a simple addition of the low and high pH forms of the EPR spectra of the oxygen intermediate. This suggests the presence of several species.

The decay of the oxygen intermediate is also pH dependent. At pH 4.0 and 7.4 the $t_{1/2}$ for the decays are 1 and 13 s, respectively.

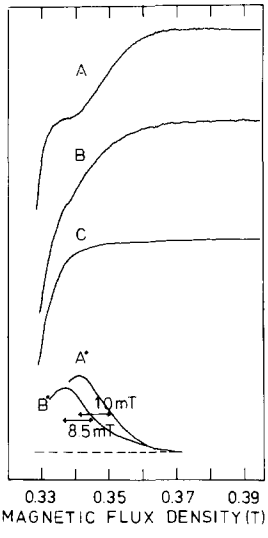
Ceruloplasmin was anaerobically reduced with 1.0 electron equivalent of ascorbic acid/copper ion. One of the Type 1 Cu^{2+} is rapidly reoxidized and an EPR intermediate signal is detectable which is similar to those formed by the laccases [2]. The signal is pH dependent, which can be observed by the shift of its peak (see Fig. 4).

Correlation between the intermediate EPR signal and Type 2 Cu^{2+}

The position and line shape of the intermediate EPR signal formed by *R. vernicifera* laccase is unchanged during its decay. We used the amplitude of the signal as a direct measure for the amount of intermediate formed. In Fig. 4 the amount of intermediate at the shortest quenching time used (30 ms) is taken as 100%. The amplitude for the low field line of Type 2 Cu^{2+}, of the EPR spectrum run at 77 K, was taken as a measure for the amount of Type 2 Cu^{2+}. Fig. 5 shows the linear correlation between the amount of Type 2 Cu^{2+} and the amount of intermediate EPR signal for pH 4.0, 5.8 and 7.4.

Proton exchange

If *R. vernicifera* laccase is reoxidized at pH 4.0 and the pH is rapidly changed to 7.4 and quenched, the total process taking about 50 ms, a broad intermediate EPR signal is formed. On the other hand, if reoxidation is made at pH 7.4



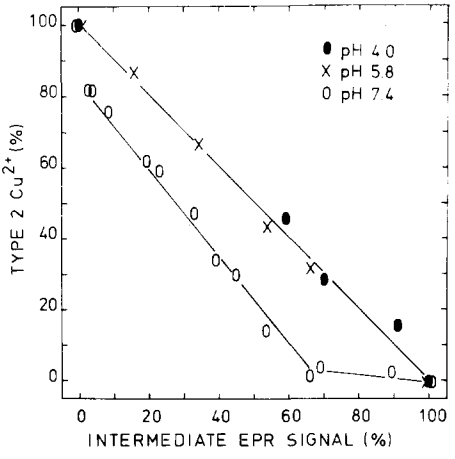


Fig. 4. The high field part of rapid freeze spectra of ceruloplasmin. An anaerobic solution of ceruloplasmin, reduced with one electron equivalent of ascorbic acid per copper ion was mixed with O_2-saturated buffer at pH 5.2 (A) and 7.0 (B). The reaction was quenched after 30 ms. The final protein solution contained 1.8 mM in total copper. 200 mM sodium acetate buffer was used at pH 5.2 and 200 mM sodium phosphate buffer was used at pH 7.0. C represents the spectrum of the native enzyme. A' and B' represents the difference spectra A—C and B—C, respectively. The spectra were recorded at 15 K and 9.13 GHz. The microwave power was 20 mW.

Fig. 5. The correlation between the amount of Type 2 Cu^{2+} and the amount of intermediate EPR signal at pH 4.0, 5.8 and 7.4, when anaerobically-reduced *R. vernicifera* laccase is reoxidized (for the conditions see Fig. 3) and quenched after different reaction times, from 30 ms to 100 s. The amount of the intermediate EPR signals was determined from the amplitude of the high field line of the spectra run at 15 K and 170 mW. The amplitude of the spectrum quenched after 30 ms was taken as 100%. The amount of Type 2 Cu^{2+} was determined from the amplitude of the low field line of the spectra run at 77 K and 10 mW.

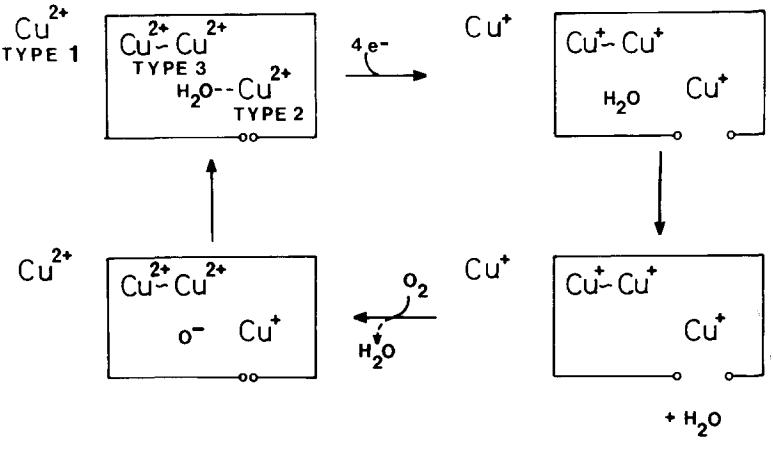


Fig. 6. A model of the dioxygen reducing site.

and the pH is dropped to 4.0 before quenching, the narrow intermediate EPR signal is formed. This means that protons are rapidly exchangeable with the oxygen intermediate or its near surroundings.

Discussion

If the characteristics of the lacquer tree *R. succedanea* laccase are compared to those of the fungal *P. vernicifera* laccase we have found that the EPR parameters for Type 1 Cu^{2+} and Type 2 Cu^{2+} are rather similar (see Fig. 2 and ref. 12). Both enzymes also form intermediate EPR signals, at pH 5.5, with about the same line shape and with the same half time for the decay of the signal [2]. Furthermore, the activity of *R. succedanea* laccase at pH 5.5 is comparable to that found for *P. versicolor* laccase. On the contrary, these properties are distinctly different for the two lacquer tree laccases, *R. vernicifera* laccase and *R. succedanea* laccase.

So far four blue oxidases (*P. versicolor* laccase, *R. vernicifera* laccase, *R. succedanea* laccase and ceruloplasmin) have been examined for their abilities to form EPR-detectable intermediates in the reaction of fully reduced enzyme with O_2. For two of the enzymes (*P. versicolor* laccase and *R. vernicifera* laccase) it has now been demonstrated that the intermediate EPR signals are true oxygen intermediates.

Figs. 2, 3 and 4 show that the line shape of the signal seems to be comparable for three of the blue oxidases at low pH, and under such conditions the $t_{1/2}$ for the decay of the signal is 1 s for *R. vernicifera*, *R. succedanea* and *P. versicolor* laccase. These facts strongly suggest that the nature of the oxygen intermediate is the same for all blue oxidases investigated.

Reoxidation in ^2H_2O instead of H_2O did not effect the linewidth of the intermediate EPR signal. However, this does not exclusively prove that the oxygen intermediate is not protonated at lower pH since the effect on the linewidth of the signal can be too small to be recognized. The fact that both line shape and the rate of decay is pH dependent (see Fig. 3) suggest the presence of at least one protonable group in the dioxygen reducing site. This protonable group can be the oxygen intermediate itself, or there exist protonable groups in the vicinity of the intermediate which influence the line shape of the EPR signal. Since obviously more than two forms of the intermediate EPR signal exist, which can be seen in Fig. 3 at pH 5.5, the nature of the factors influencing the pH dependence is rather complex.

We also wanted to know the quantitation of the intermediate EPR signal and its relation to other electron donors and acceptors. However, an absolute quantitation of the signal, of which only the high field part is separated from Type 1 Cu^{2+} spectrum, is difficult. Fig. 5 shows that the linear correlation between the amount of Type 2 Cu^{2+} and the amount of intermediate EPR signal is consistent throughout the reoxidation reaction at pH 4.0, 5.8 and 7.4. At pH 7.4 the amount of the intermediate EPR signal is 20% less than the amount of reduced Type 2 copper (Fig. 5). This deviation depends on the excess of electron equivalents of ascorbic acid (0.2) used in the anaerobic reduction of the enzyme. At pH 7.4 the reduction of the Type 1 Cu^{2+} is much faster than at pH 5.8 and 4.0. Consequently, some of the reoxidized Type 1

copper is rapidly reduced by the excess of ascorbate. The EPR spectra at pH 7.4, however, show 100% Type 1 Cu^{2+}. This means that 20% of the oxygen intermediate rapidly accepts one electron from the reduced Type 1 copper while the rest of the radical accepts an electron from the reduced Type 2 copper in a much slower process. Thus, it is most probable that the intermediate EPR signal reflects an O^- and this supports earlier attempts to estimate the amount of the intermediate EPR signal by integration [2].

For the final product, water, to be formed in the reduction of O_2, protons must also be available. It has been found that one of the water molecules produced is coordinated to Type 2 Cu^{2+} and exchanges very slowly with the bulk water [4]. Here we show, by a rapid change in pH, that protons in contrast to water rapidly can pass or enter the proposed activity in which the dioxygen reducing site is located. The same conclusion can be drawn from the observed effect on the intermediate EPR signal between pH 5 and 6. Here the line form of the total signal is not changed during its decay and consequently a rapid rate of exchange must exist between protonated and unprotonated species. This rate has been found to be greater than 50 s^{-1} which means that proton exchange is not a rate-limiting step in the catalytic turnover of the enzyme.

We suggest that reactive oxygen intermediates are stabilized by a "closed" cavity in which the dioxygen reducing site is located (Fig. 6). In order to form water protonable groups in vicinity of this cavity are able to rapidly exchange protons from the bulk water to the inside of this cavity.

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