AN INTERMEDIATE IN THE REACTION OF REDUCED LACCASE WITH OXYGEN

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1. Introduction

Fungal laccase (*p*-diphenol: O_2 oxidoreductase, EC 1.10.3.2), like other blue copper-containing oxidases, contains a co-operative two-electron acceptor [1, 2]. This, together with kinetic evidence [3] for multi-electron steps in the catalytic mechanism, has led to the suggestion [4, 5] that the reduction of oxygen with this enzyme involves consecutive two-electron steps. Such a reaction path could provide an efficient way to overcome the kinetic inertness of oxygen [6, 7].

If the proposed laccase mechanism is correct, enzyme-bound H_2O_2 must be formed as an intermediate. We here present kinetic and spectroscopic evidence for the existence of an intermediate in the reaction of reduced laccase with oxygen. The optical and EPR spectra of this new species, recorded by stopped-flow and rapid-freeze techniques, are similar to those of the H_2O_2 complex with Type 2 Cu^{2+} in the oxidized enzyme [8].

A preliminary account of some of the optical results has been given at a recent conference [9]. An intermediate with similar optical properties has been described for ceruloplasmin by Manabe et al. [10].

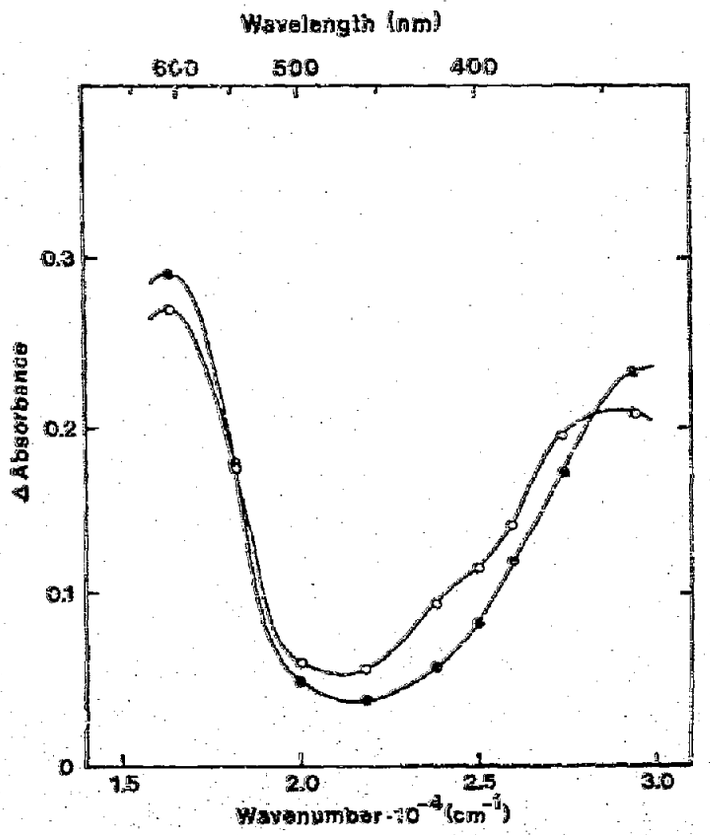

Fig. 1. Optical spectral changes observed in the reaction between partially reduced laccase and O_2. The experiment was carried out in the stopped-flow apparatus (2-cm optical path, 2.5 msec dead-time) at 25°C in 0.1 M phosphate, pH 5.5. Laccase B was reduced anaerobically with hydroquinone corresponding to 2.3 electron equivalents per mole of laccase and was mixed with air-saturated buffer. The concentrations of laccase and O_2 after mixing were 66 and 125 μM, respectively. The figure shows the spectral changes at the indicated wavelengths at 50 msec (o–o–o) and 7 sec (•–•–•) after mixing.

2. Materials and methods

Fungal laccase A and B were prepared by the method of Fåhraeus and Reinhammar [11] and were freed from contaminating F^- as described earlier [8]. The protein concentration was determined spectrophotometrically at 610 nm on the basis of an absor-

bance coefficient of 4.9 mM^{-1} cm^{-1}.

Hydroquinone was obtained from Schuchart Chemical Company, Munich. Deionized distilled water was used, and all solutions were adjusted to pH 5.5 before degassing.

The anaerobic, stopped-flow and rapid-freeze techniques used have been described earlier [12, 13]. EPR measurements were made at 77°K in a Varian E-3 spectrometer at 9.2 GHz.

3. Results

The reoxidation of laccase, which had been reduced anaerobically with about 2.3 electron equivalents of hydroquinone and then mixed with O_2-containing buffer, was followed in a stopped-flow apparatus and by the rapid-freeze technique. The stopped-flow results displayed spectral changes which cannot be described in terms of the absorption properties of the known chromophores, Type 1 Cu^{2+} and the two-electron acceptor, as shown in figs. 1 and 2 (cf. fig. 9 in [9]). Eventually the new spectral species disappears, and the fully reoxidized enzyme is formed (fig. 2). This process has, however, not been studied in detail as, under the conditions used, it would be expected to be very complicated, involving both intra- and inter-molecular electron transfer steps (cf. [3, 13] and Discussion).

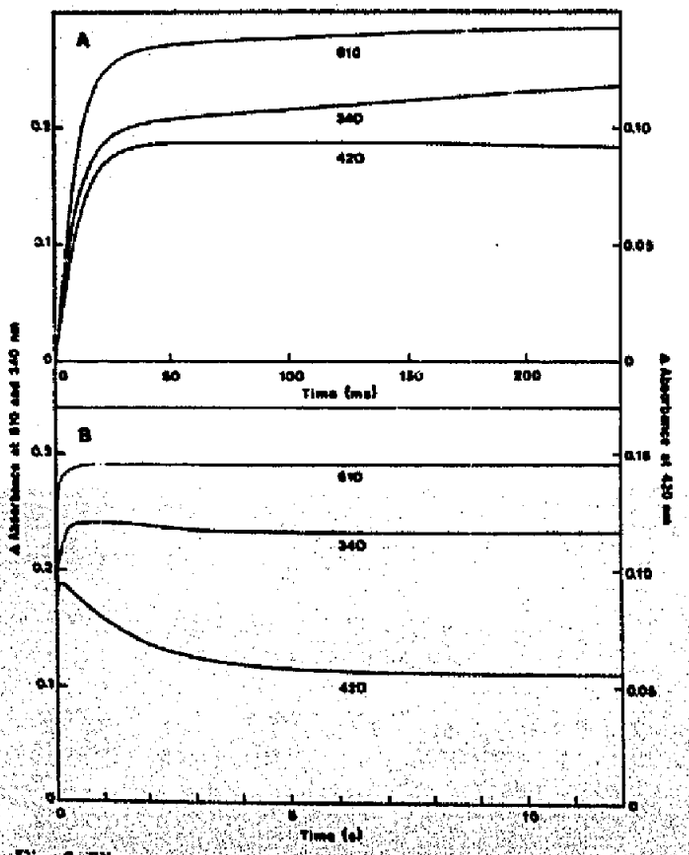

Fig. 2. Time course of the reaction between partially reduced laccase and O_2. A and B show the absorbance changes at 610 and 340 nm (the two main chromophores) and 420 nm on two different time scales. Conditions were identical to those in fig. 1.

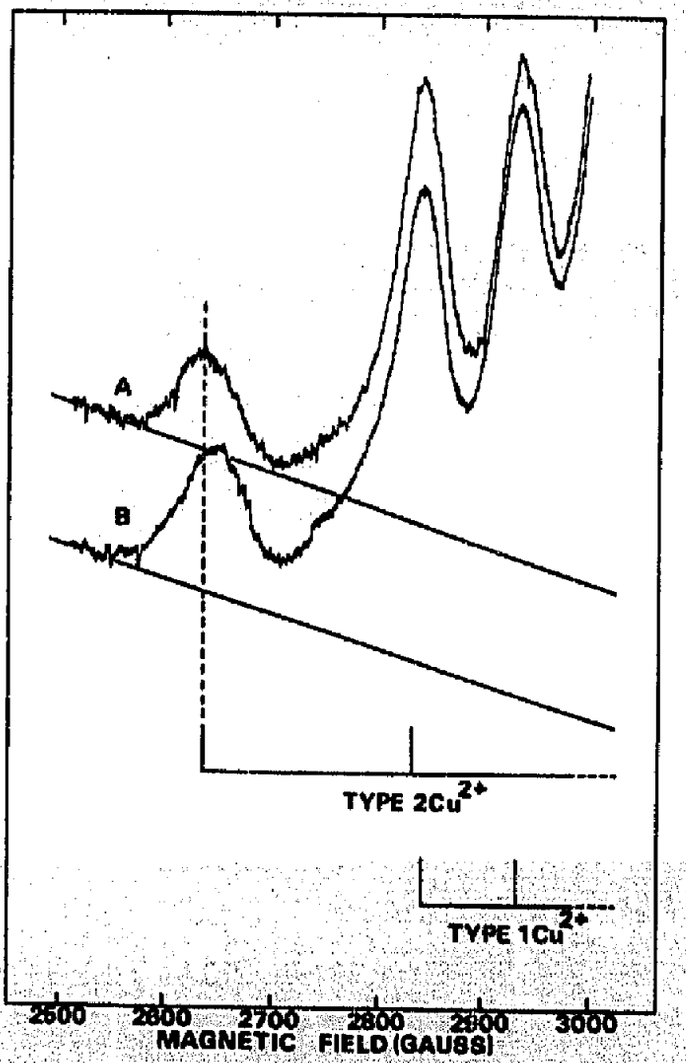

Fig. 3. Low-field part of rapid-freeze EPR spectra of laccase. The spectra were recorded at 77°K and 9.2 GHz. Spectrum A was obtained from a solution of oxidized laccase B in 0.1 M phosphate buffer, pH 5.5, mixed with equal amounts of buffer saturated with O_2 (1 atm.). For spectrum B an anaerobic solution of laccase B partially reduced with hydroquinone (2.3 electron equivalents per mole of enzyme) was mixed with the same buffer. The protein concentration after mixing was 300 μM and the reaction time about 50 msec.

Some results from rapid-freeze experiments, carried out with enzyme reduced to the same extent as in the stopped-flow measurements, are shown in fig. 3. It can be seen that the low-field line, associated with Type 2 Cu^{2+} [14], is shifted up-field about 20 gauss. With longer reaction times an EPR spectrum indistinguishable from that of the resting enzyme was obtained.

4. Discussion

It has earlier been proposed (see Introduction and [4, 5]) that laccase reduces oxygen in consecutive two-electron steps with the formation of bound H_2O_2 as an intermediate. To test this hypothesis we have here studied the reoxidation of partially reduced laccase. Under the conditions used one would expect any intermediate formed to decay slowly, as the additional reducing equivalents required to form water can only be provided through intermolecular reactions. The results indeed do show the formation of a new molecular species with a sufficient life time for a spectral characterization by the stopped-flow and rapid freeze techniques (figs. 1–3). It is notable that its optical and EPR properties are similar to those of the complex with Type 2 Cu^{2+} formed when H_2O_2 is added to the native enzyme [8]. An intermediate with a similar optical spectrum has been described for ceruloplasmin [10], so that the formation of such a compound may be a common property of the blue oxidases. It may be noted that the observed changes in the EPR spectrum of Type 2 Cu^{2+} cannot be related to the redox state of Type 1 Cu^{2+}, as this is almost completely oxidized in the experiment of fig. 3 while in an earlier rapid-freeze experiment (fig. 2 in [12]) there was no change in the low-field line when Type 1 Cu^{2+} was nearly fully reduced.

The results presented here provide additional circumstantial evidence for the laccase mechanism proposed earlier [4, 5]. A definite identification of the new species as an H_2O_2 intermediate formed during the reduction of oxygen will, however, require a much more detailed characterization of its spectroscopic properties as well as of its kinetics of formation and decay.

Acknowledgements

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