A PARAMAGNETIC INTERMEDIATE IN THE REDUCTION OF OXYGEN BY REDUCED LACCASE

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1. Introduction

The four-electron reduction of molecular oxygen in the catalytic reaction of laccase, like that of cytochrome *c* oxidase, poses great mechanistic problems [1,2]. With laccase, in particular, a mechanism involving four consecutive one-electron steps would appear unlikely, since the high oxidation–reduction potentials of the redox sites of the enzymes [3] would lead to a very high, positive free-energy change for the formation of the superoxide radical. This fact, together with the presence of a co-operative two-electron acceptor in the enzyme, has led to the suggestion [2] that the reaction of reduced laccase with oxygen comprises consecutive two-electron steps.

Spectroscopic evidence for an intermediate in the reaction of reduced laccase with oxygen has been presented earlier [4]. It was tentatively proposed that this species is H_2O_2 (or one of its ions) bound to Type 2 Cu^{2+}. However, recent experiments [5]with tree as well as fungal laccase indicate, that when the fully reduced enzymes react with oxygen, both the Type 1 copper and the two-electron acceptor become rapidly re-oxidized, while the Type 2 copper remains reduced. The intermediate so formed has optical properties similar to that described previously [4]. The e.p.r. spectrum at 77 K showed only the presence of Type 1 Cu^{2+}. As O_2 is an even-electron system and three electrons had been transferred from reduced enzyme-sites, the absence of another e.p.r. signal appeared inconsistent with the kinetic results. This prompted an e.p.r. study at lower temperatures, leading to the discovery of the paramagnetic intermediate described in this communication. While its chemical nature is unknown, it does not represent a normal Cu^{2+} complex or an ordinary free radical. Presumably the unusual spectrum originates from an oxygen radical, which possibly interacts with one or more of the metal ions in the enzymes. If this interpretation is correct, it means that the reduction of oxygen by the laccases must involve at least two one-electron steps.

2. Experimental

Fungal laccase A and lacquer tree laccase were prepared and freed from contaminating F^- as described previously [6,7]. The protein concentrations were determined spectrophotometrically with the known extinction coefficients [2].

The anaerobic and rapid freeze techniques were the same as used earlier [4]. The e.p.r. spectra were recorded at temperatures between 4.2 and 77 K at 9.15 GHz in a Varian E-9 spectrometer. The ^{17}O-enriched oxygen was produced electrolytically from water containing 40% ^{17}O.

3. Results

The e.p.r. spectra of the native laccases at temperatures down to 4.2 K were identical to those observed at 77 K. If the laccases are anaerobically reduced with four electron equivalents of ascorbic acid and mixed with O_2-containing buffer, a new type of e.p.r. signal

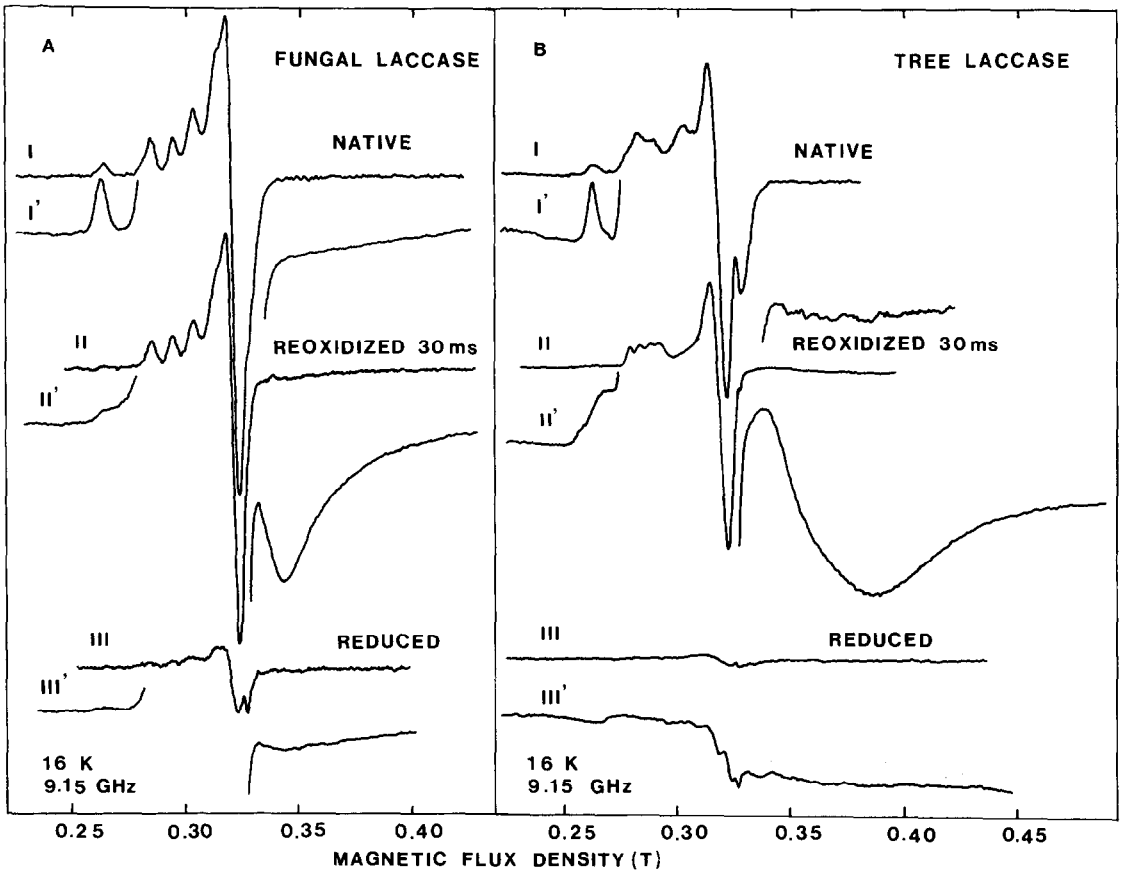

Fig.1. Rapid-freeze e.p.r. spectra of fungal (A) and lacquer tree laccase (B). Spectra 1A and 1B show the oxidized enzymes. For spectrum IIA and IIB anaerobic solutions of the enzymes reduced with 4 electron equivalents of ascorbic acid were mixed with O_2-saturated buffer, and the reaction quenched after 30 ms. Spectra IIIA and IIIB show the enzymes reduced anaerobically with 4 electron equivalents of ascorbic acid. The protein concentration and pH after mixing were for fungal laccase 400 μM and 5.5 and for tree laccase 300 μM and 7.4, respectively. The spectra were recorded at 16 K and 9.15 GHz. The microwave power was 20 mW for the primed traces and 0.02 mW for unprimed traces, which show the full Cu^{2+} spectra.

is obtained (fig.1). This is formed concomitantly with the complete reoxidation of Type 1 copper, while Type 2 copper remains e.p.r.-undetectable. The linewidth of the new signal produced with lacquer tree laccase is about twice that with fungal laccase. The new signal can only be observed at temperatures below 25 K (fig.2) and is not saturated at a microwave power of 20 mW at 16 K. Neither the native enzyme nor the fully reduced one show this type of signal (fig.1).

After about 1 s the intensity of the intermediate signal in fungal laccase has decreased to about 50% of the value at 30 ms, but in lacquer tree laccase the same decline takes about 18 s. If the reoxidation of fungal laccase is performed in the presence of a ten-fold excess of ascorbic acid in the O_2-containing buffer, the new e.p.r. signal has decreased to less than one fourth already 30 ms after mixing (fig.3). In an experiment, in which both O_2 (1 mM) and the reducing substrate (5 mM ascorbate) were mixed with oxidized tree laccase (150 μM, all concentrations after mixing), an intermediate signal corresponding to 60% of that in fig.1 was observed in the steady state.

It is not possible to observe the overall shape of

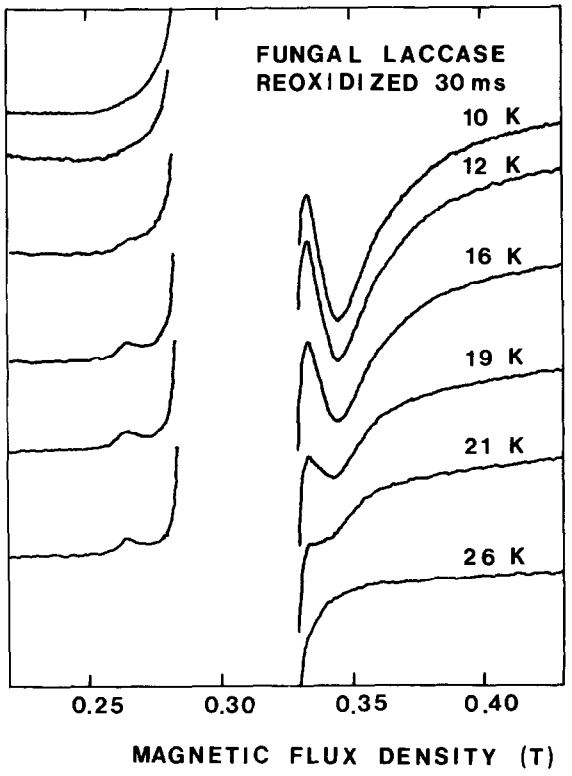

Fig.2. The e.p.r. spectrum II′ from fig.1 A recorded at different temperatures to show its temperature-dependent relaxation. Other instrumental settings were the same as in fig.1 (20 mW).

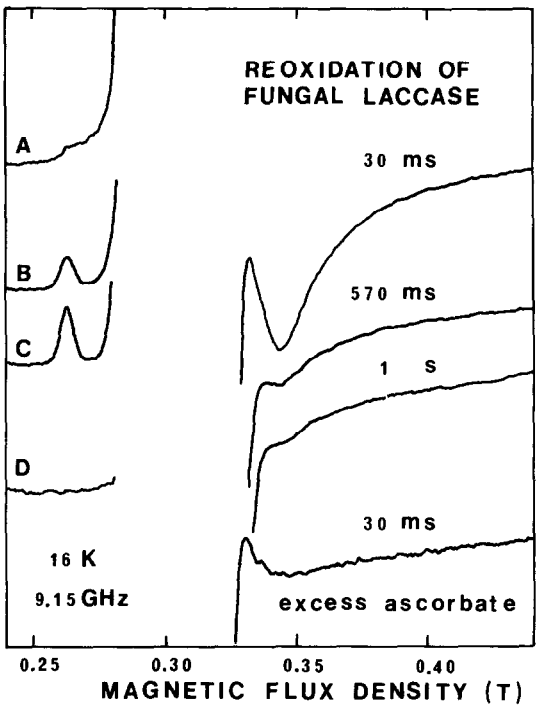

Fig.3. E.p.r. spectra of anaerobically reduced fungal laccase reoxidized for different times. The conditions were the same as in fig.1A, but different quenching times were used: (A) 30 ms; (B) 570 ms: (C) 1 s. Spectrum D represents an experiment, in which the O_2-saturated buffer contained a ten-fold excess of ascorbic acid, and the reaction was quenched after 30 ms. The instrumental conditions were the same as for the primed spectra of fig.1.

the new signal due to the strong overlap of the Type 1 Cu^{2+} signal (fig.1). Therefore, a determination of the number of unpaired spins corresponding to the new signal is rather uncertain. However, an attempt was made assuming that the signal is isotropic with a derivative shape of which essentially only the second half below the base line is observed. The g-value and peak-to-peak linewidth is taken as 2.0 and 35 mT for the signal observed with the fungal laccase (fig.1A) and 1.9 and 75 mT for tree laccase (fig.1B). It is also assumed that the signal arises from an isolated Kramers' doublet with $S = 1/2$. With these assumptions it was found that the integrated intensity of the new signal at its maximum appearance corresponds to roughly one unpaired spin per molecule for both the fungal and tree enzyme. For these calculations the Type 1 Cu^{2+} signal in the sample, recorded at non-saturating conditions, was taken as reference.

Samples showing the intermediate signal could be stored at 77 K for at least a couple of weeks without any changes in the e.p.r. spectrum as observed at low temperatures. However, it was found that the intermediate signal in fungal laccase irreversibly disappeared if a sample was kept at 230 K for about 10 min, while it was still stable at 180 K.

At temperatures lower than 30 K the Type 1 Cu^{2+} signal in samples of fungal laccase with the intermediate present was more difficult to power-saturate than in the native enzyme. However, no such difference could be observed at higher temperatures.

To test if the new e.p.r. signal arises from an oxygen intermediate, ^{17}O-enriched O_2 (40%) was used with fungal laccase. However, no changes in the 35 mT broad signal could be detected. Simulations showed that with the enrichment used it will be

experimentally impossible to detect a change in the signal by a hyperfine splitting of less than 6 mT.

Earlier experiments [8] showed that the hyperfine lines of Type 2 Cu^{2+} in oxidized fungal laccase are broader in the presence of ^{17}O-enriched water. Oxidized enzyme was therefore mixed with O_2-saturated buffer containing an excess of ascorbate. However, we could not observe a broadening of the Type 2 Cu^{2+} hyperfine lines after 30 ms, when ^{17}O-enriched O_2 was used.

The linewidth of the intermediate e.p.r. signal was not significantly different when D_2O was used instead of H_2O.

4. Discussion

Under the conditions used the new paramagnetic species described here forms rapidly but decays slowly. If it represents an intermediate in the reaction between the reduced laccases and molecular oxygen, it would consequently be expected to reach a concentration close to that of the enzyme. On the assumption that it represents an $S = 1/2$-system, it is indeed formed in an amount stoichiometric with that of Type 1 Cu^{2+} (see Results).

The kinetics of formation and decay of the new paramagnetic compound closely parallel those of the optical intermediate described elsewhere [4,5], indicating that this may not represent a peroxide complex with Type 2 Cu^{2+}, in contrast to an earlier suggestion [4]. The disappearance of the new compound would seem to be too slow for it to be an intermediate in the normal catalytic reaction, since with fungal laccase, for example, its half-time is 1 s, while the catalytic constant is about 30 s^{-1} [9]. This does, however, only reflect the artificial conditions used, in which O_2 reacts with the fully reduced enzyme. When excess reducing equivalents are present, as they always are during turnover, then the new e.p.r. signal disappears rapidly (fig.3). In addition, the signal could be observed in the steady state with tree laccase, and again the kinetic e.p.r. observations agreed with the optical results [5].

The fact that the paramagnetic intermediate decays at temperatures around 200 K, indicates that it can be reduced by intramolecular electron donation.

The spectra of Type 1 Cu^{2+} are essentially the same at 4.2 and 77 K and also in the presence of the intermediate. However, at temperatures at which the intermediate signal can be detected, the Type 1 signal is more difficult to power-saturate than in the native enzyme. This indicates that the properties of one site in the enzyme is affected by the state of other sites, possibly through a spin–spin interaction similar to that in xanthine oxidase [10].

The new e.p.r. signal has unusual characteristics. In particular, it is associated with a very short relaxation time, so that it does not easily saturate even at very low temperatures and cannot be observed above 25 K (fig.2). The only e.p.r. signal to be expected in the reactions of laccase should originate either from Cu^{2+} or from a free-radical intermediate. However, the characteristics of the signal are not consistent with either a normal Cu^{2+} complex or an isolated radical. Specifically, the signal does not have the properties associated with the oxygen radicals O_2^- [11] or OH [12]. The radical O^-, on the other hand, can have relaxation properties [13] similar to those of the intermediate. Another possibility is that the new paramagnetic species involves an interaction between an oxygen radical and one or more paramagnetic centres in the enzyme other than Type 1 Cu^{2+}. Such interacting systems could be characterized by short relaxation time.

In an attempt to associate the new signal with an oxygen intermediate, experiments with ^{17}O were performed. The lack of effect does not mean that the paramagnetic species is not associated with oxygen, as a small hyperfine splitting (< 6 mT) cannot be detected (see Results). In fact, the observed ^{17}O-splittings for the radical O^- is close to this limit [14]. The necessary stabilization of a reactive oxygen radical could be a factor in decreasing the hyperfine splitting due to ^{17}O. (In view of the negative ^{17}O results, the lack of effect of D_2O is not unexpected.)

The present results require a modification of an earlier suggestion that oxygen reduction in laccase occurs via two double-electron transfers [2]. Most likely the function of the two-electron acceptor is to allow the energetically unfavourable one-electron step from O_2 to O_2^- [2] to be by-passed, but apparently a third electron is added so rapidly to the presumed peroxide intermediate that this step cannot be kinetically resolved. An attractive possibility is

that the intermediate is O^-, the decay of which involves the addition of one electron and two protons, this process being rate-limiting in the overall reaction. However, a detailed description of the reaction must await further experimentation.

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