TYPE 2 COPPER(II) AS A COMPONENT OF THE DIOXYGEN REDUCING SITE IN LACCASE: EVIDENCE FROM EPR EXPERIMENTS WITH ^{17}O

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1. Introduction

Recently it has been shown that in the reduction of O_2 to H_2O by fully reduced fungal and tree laccase, an oxygen intermediate is rapidly formed [1,2]. The decay of the intermediate seems to be coupled to the reoxidation of the Type 2 copper, indicating electron transfer between the two species [3] with the formation of the ultimate product, water.

In the catalytic mechanism of laccases the Type 2 copper has been proposed to stabilize an intermediate in the reduction of O_2 to H_2O [4]. It has also been shown that this copper in fungal laccase, under anaerobic reduction conditions, can function as an electron-donor beside Type 1 and Type 3 coppers [5]. These findings indicate that Type 2 is part of the oxygen reducing site in the enzyme. In this communication we give more direct evidence for this relation between Type 2 copper and the oxygen site.

From studies with fungal laccase we know that H_2O is an exchangeable ligand to Type 2 Cu^{2+} [6]. Here we present evidence, using dioxygen enriched in ^{17}O, that the product water formed in the reduction of O_2 by fully reduced tree laccase, is indeed coordinated to Type 2 Cu^{2+}. In the oxidized protein this ligand exchanges very slowly with bulk water since most of it remains even after 30 min. Upon reduction, followed by reoxidation, water is exchanged, however.

2. Experimental

Fungal laccase A and lacquer tree laccase were prepared and characterized as described previously [7,8]. The anaerobic and rapid freeze techniques were the same as used earlier [9,10]. All kinetic experiments were done at 25°C. The EPR spectra were recorded at 77°K and 9.22 GHz in a Varian E-3 spectrometer. The ^{17}O-enriched O_2 (91.8%) was purchased from Miles Laboratories Ltd., Slough SL2 4LY, England.

3. Results

Tree laccase was anaerobically reduced with four equivalents of ascorbic acid and mixed with an equal volume of O_2-saturated buffer. At pH 4.4, in 50 mM sodium acetate buffer, about 40, 95 and 100% of the Type 2 copper is reoxidized after quenching times of 1 s, 5 s and 1 min, respectively. At pH 7.4, in 25 mM phosphate buffer, only 30% of the Type 2 copper is reoxidized after 5 s. In all the samples from these experiments the lowest-field EPR line of Type 2 Cu^{2+} shows the same broadening, in presence of ^{17}O-enriched O_2, relative to ^{16}O controls (Fig.1). The linewidth at half maximal amplitude increases from 5.0–7.0 mT. This broadening is completely consistent with an interaction of one ^{17}O ($I = 5/2$) with Type 2 Cu^{2+} and a hyperfine splitting of 1.1 mT. The calculated value for the hyperfine constant is in the order of the value found for one oxygen atom lying in the equatorial plane in smaller complexes [11]. The splitting due to an axial oxygen ligand is too small to be observed, so in our case we are unable to decide if a second molecule of water is coordinated to Type 2 Cu^{2+}. The linewidth of Type 1 Cu^{2+} is not affected.

In the resting and $^{16}O_2$-reoxidized enzyme at pH lower than seven, Type 2 Cu^{2+} shows very characteristic structure around the g_1 region (fig.2). Upon reoxidation with $^{17}O_2$ this structure is no longer observable

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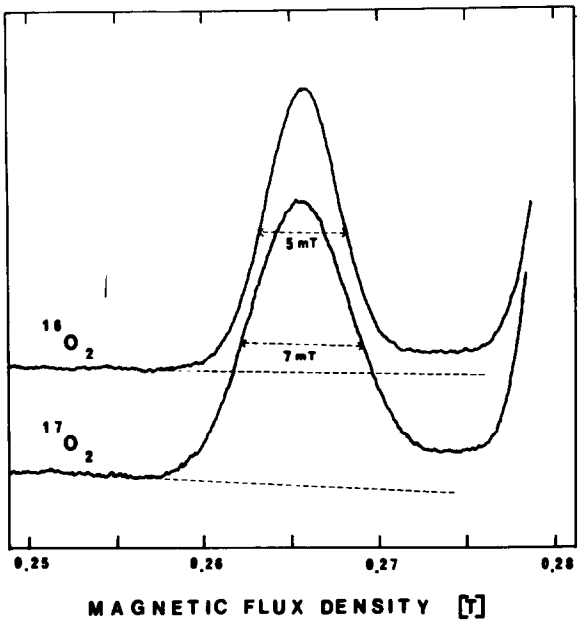

Fig.1. Low-field part of the EPR spectra of tree laccase. The enzyme was anaerobically reduced with 4.2 electron equivalents of ascorbic acid and mixed with an equal volume of O_2 saturated buffer, 100% ^{16}O and 91.8% ^{17}O, respectively. The reaction was quenched after 5 s. The final protein concentration was 500 μM in 50 mM sodium acetate buffer at pH 4.4. The spectra were recorded at 9.22 GHz and 77°K with a microwave power of 10 mW and a modulation amplitude of 2 mT.

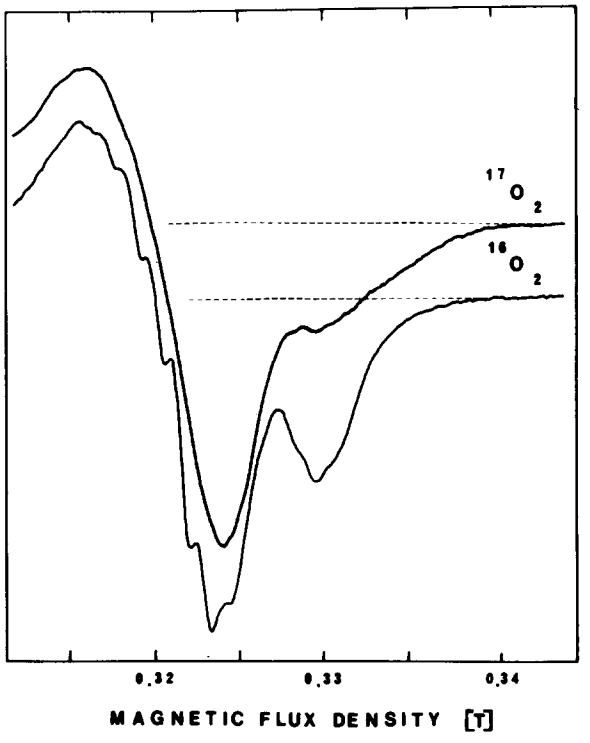

Fig.2. High-field part of the EPR spectra of tree laccase. The conditions were the same as in fig.1 but a modulation amplitude of 0.1 mT was used.

(fig.2), which is consistent with the broadening observed on the $g_{\parallel}$ region. An estimation of a ^{17}O hyperfine splitting is difficult in this region.

After a reaction time of 30 min, at pH 4.4, most of the broadening still remains. If, however, the $^{17}O_2$ reoxidized sample, having reacted for 1 min, was once more made anaerobic, reduced with ascorbic acid, mixed with $^{16}O_2$ and quenched 5 s after mixing (a process taking totally less than 5 min) the broadening was completely eliminated.

Fungal laccase was also reoxidized in presence of $^{17}O_2$, at pH 5.5, in 50 mM sodium acetate buffer but no isotope effect could be observed on the Type 2 Cu^{2+} in this case.

4. Discussion

The effect on Type 2 Cu^{2+}, by substituting $^{17}O_2$ for $^{16}O_2$ in the reoxidation of fully reduced tree laccase, gives conclusive evidence that at least one of the two water molecules formed in this reaction becomes coordinated to Type 2 Cu^{2+}. It is thus evident that this copper must be in or near the dioxygen reducing site of the enzyme.

In presence of the inhibitor F^-, which binds to Type 2 Cu^{2+}, the redox potential of the cooperative two-electron acceptor site (Type 3) is changed [12]. It has been suggested that this acceptor in the reduced state takes part in an initial two-electron step in the reduction of O_2 [4]. This, together with the findings in this report, indicates that Type 2 as well as Type 3 copper are located in the dioxygen reducing site of the enzyme.

The water formed in the reoxidation reaction exchanges much faster upon reduction of the enzyme, a situation similat to that for the inhibitor F^- which dissociates very slowly from the oxidized protein but is

released faster when the enzyme is reduced [13]. The result presented here supports the idea that the Type 2 copper plays an important role in the reduction of O_2 by the laccases. It also suggests that this copper is located in a cavity which can be either closed or open, depending on the redox-state of the different electron acceptors of the protein. The lack of an isotope effect on Type 2 Cu^{2+} in fungal laccase is not inconsistent with this proposal since it may only reflect a faster exchange of the water in this case, even if the cavity is closed. It shall be noted that the dissociation of F^- from fungal laccase is much faster than in tree laccase [13].

We here propose that this cavity is the dioxygen reducing site in which both Type 3 and Type 2 coppers are situated and we think that the property of the cavity being open or closed is important for the catalytic mechanism of the enzyme in stabilizing reactive oxygen intermediates.

Two water molecules are formed in the complete reduction of O_2, but for the moment we have only detected one of them. It is thus evident that a detailed description of the reaction must await much more work.

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