THE REQUIREMENT OF THE "NON-BLUE" COPPER (II) FOR THE ACTIVITY OF FUNGAL LACCASE

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1. Introduction

In a recent report from this laboratory [1], it was shown that fungal laccase (*p*-diphenol: O_2 oxidoreductase, EC 1.10.3.2) contains two non-equivalent Cu^{2+} in addition to two diamagnetic copper atoms. One type of Cu^{2+} (Type 1) is characterized by a narrow hyperfine splitting in the electron paramagnetic resonance (EPR) spectrum of the protein, is responsible for the intense blue color of the protein and possesses a high oxidation-reduction potential [2,3], while the second form (Type 2) has a broader hyperfine splitting in EPR and does not contribute significantly to the blue color. It has been presumed, on the basis of its rapid reduction by substrate and on analogy with the oxidase activity of ceruloplasmin, that Type 1 Cu^{2+} functions as a one-electron carrier in the oxidation of substrate [4], but the function of the Type 2 Cu^{2+} is not clear.

In this communication, we wish to report on the effect of inorganic anions on laccase. Kinetic studies have shown that anions are effective inhibitors. EPR characterization of the protein has led to the demonstration that the anion-binding site in the protein is Type 2 Cu^{2+} and has given information on the mode of binding of CN^-. These experiments are taken as a demonstration of the requirement of the Type 2 Cu^{2+} for the activity of fungal laccase.

2. Materials and methods

Fungal laccase A was purified as previously described [5]. Enzyme activity measurements were carried out as described by Broman [6] except that EDTA was omitted from the reaction mixture.

EPR measurements were made at 77°K in a Varian E-3 spectrometer at 9.15 GHz and at about 90°K in a Varian V-4503 spectrometer at 34.5 GHz.

Reagent grade chemicals were used in all experiments. $K^{13}CN$ of 61% enrichment was purchased from Bio-Rad Laboratories.

3. Results

3.1. *Inhibition with anions*

In inhibition studies with inorganic anions, it was found that anions which have strong copper-binding properties were effective inhibitors. Thus, azide, cyanide, cyanate, fluoride, and thiocyanate at concentrations between 10^{-6} and 10^{-4}M were found to inhibit laccase activity 50% in the standard assay. Other anions, such as chloride, phosphate, nitrate and sulfate also showed inhibition but the concentration used with these anions was at least 10^{-2}M. The inhibition with the copper-binding anions was of a complex nature and further kinetic experiments are planned to detail the behavior of these inhibitors.

3.2. *Effect of cyanide on EPR spectra of laccase*

The high-field part of the X-band EPR spectrum of the protein treated with CN^- is shown in fig. 1. The entire EPR spectrum obtained with $K^{12}CN$ has been previously described in ref. [1], fig. 7. Figs. 1A and 1B show the spectra resulting when the protein is treated as described in the legend with $^{12}CN^-$ and CN^- enriched in ^{13}C to 61%, respectively. Figs.

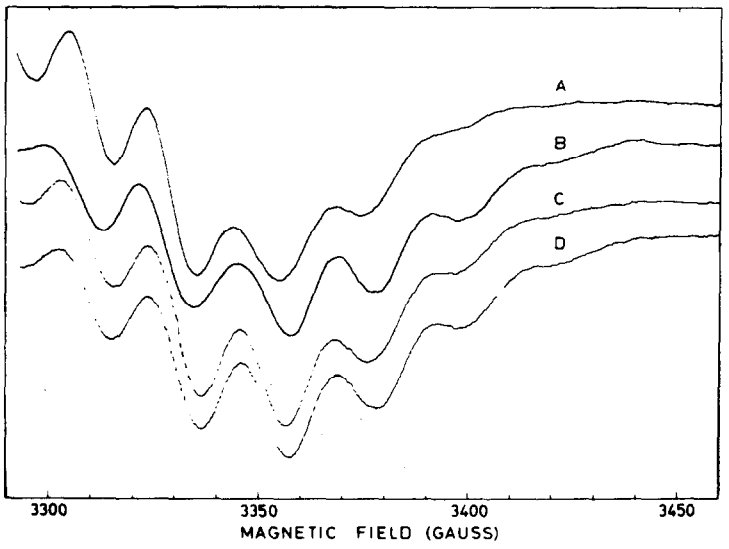

Fig.1. High-field portion of the EPR spectra of laccase A treated with $K^{12}CN$ and $K^{13}CN$. Laccase A (0.27 mM in 0.1 M potassium phosphate, pH 6.0) and 50 mM $K^{12}CN$ (A) or 61% enriched $K^{13}CN$ (B) (cyanide solutions were adjusted to pH 6.0) were allowed to react for 75 min at room temperature. Spectra (C) and (D) were synthesized from Spectrum (A), assuming interaction of one CN^- and two CN^-, respectively, and a ^{13}C splitting of 47 gauss, as described in section 4. Microwave frequency was 9.15 GHz, and the modulation amplitude was 8 gauss.

1C and 1D give synthesized spectra as described in section 4.

The reversible removal of the Type 2 Cu^{2+} from fungal laccase has been achieved (R.Malkin, B.G. Malmström and T.Vänngård, manuscript in preparation), and the reaction of CN^- with this copper-depleted protein has been tested. The native protein and two preparations of the copper-depleted laccase, both containing approximately one atom per mole of Type 1 Cu^{2+} but only 0.3 and 0.15 atoms per mole of other types of Cu^{2+}, respectively, were reacted with cyanide under conditions identical to those in fig. 1. In a comparison of the resulting X-band spectra, the two copper-depleted proteins showed a total intensity of only 15-20% of that in the case of the native protein. In addition, the signal after CN^- treatment of the copper-depleted proteins did not resemble that derived from the native protein.

3.3. *Effect of fluoride on EPR spectra of laccase*

In fig. 2A is the EPR spectrum of laccase recorded at 34.5 GHz. The four hyperfine lines in the z direction due to the two types of Cu^{2+} are indicated. The reason why the Type 2 signal is better resolved here than in the spectrum published earlier (ref. [1], fig. 2) is not clear, but this may be related to our finding that this signal is sensitive to changes in ionic strength buffer and pH. The EPR parameters differ somewhat from those given earlier [1].

Upon the addition of approximately one equivalent of F^- at pH 5.5, the signal from the Type 1 Cu^{2+} appears unchanged (fig. 2B), but the hyperfine lines around g_z of the Type 2 Cu^{2+} are shifted to lower field and split into doublets. The spectrum is complicated due to the presence of small extra components, mostly from the species giving the spectrum in fig. 2C. This obtains in the presence of a 20-fold excess of F^-, and clearly shows the splitting of each of the Cu hyperfine lines into a triplet with an intensity ratio of approximately 1 : 2 : 1. Again, the signal from the Type 1 Cu^{2+} shows no obvious change.

By comparisons between the 34.5 GHz spectra in fig. 2 and the spectra at 9.15 GHz of the same samples, it is clear that the splitting into doublets and triplets is a superhyperfine structure and not due to the presence of several complexes. The legend of fig. 2 gives some of the EPR parameters calculated on the basis of this assumption.

The same effects of F^- on the EPR spectra were observed when the reactions were carried out in D_2O.

4. Discussion

4.1. *Binding of cyanide*

Under treatment with CN^- the protein depleted of the Type 2 Cu^{2+} does not give the specific signal obtained with the native protein. This strongly suggests that this signal is due to one of the Cu^{2+} only, the Type 2 Cu^{2+}. Thus, for the following discussion of this signal, it can be considered to originate from one species only.

As is seen from figs. 1A and 1B, the spectra obtained after treatment with $^{12}CN^-$ and CN^- partially enriched in ^{13}C are different. For example, the peaks at 3352 and 3362 gauss are shifted to higher fields and the peaks at 3362 and 3400 have increased in intensity. At about 3420 gauss there is an indication of a new line appearing. These effects can be explained as a superhyperfine structure from the ^{13}C ($I = ½$) nucleus. Figs. 1C and 1D are spectra synthesized from fig. 1A under the assumption of isotropic coupling to ^{13}C. This is justified as the ligand hyperfine structure often has a dominant isotropic part and, furthermore, fig. 1 shows only part of the spectrum (the "overshoot" line of one of the Cu hyperfine lines). For fig. 1C interaction with one CN^- and a ^{13}C splitting of 47 gauss were assumed. The same ^{13}C splitting but interaction with two CN^- was used for fig. 1D. In the simulations a Varian C-1024 Time Averaging Computer was used into which the spectrum of fig. 1A was fed several times with gains and field center properly adjusted. The best agreement is obtained assuming interaction with two CN^-, when all the mentioned features of fig. 1B are reproduced. However, a higher ^{13}C enrichment is needed before definite conclusions can be drawn on the number of CN^- involved.

The high value of the ^{13}C splitting provides strong evidence that CN^- binding to the Cu^{2+} occurs via the carbon end. We have studied a mixed complex of Cu^{2+} with citrate and CN^- and found a ^{13}C splitting of about 50 gauss whereas the splitting due to the ^{14}N of the CN^- must be less than 8 gauss (unpublished work). This indicates that the splitting into many lines with a separation of about 17 gauss in the spectrum of cyanide-treated laccase that was attributed to coupling to 3-4 N [1] is not caused by the CN^-.

Thus, our results indicate that the Type 2 Cu^{2+} in cyanide-treated laccase is bound to 3-4 N supplied by the protein and to two CN^- coordinating through carbon. The fact that all these give superhyperfine structure simultaneously may be the result of a low symmetry of the complex which can, for example, cause mixing of the $d_{x^2-y^2}$ and d_{z^2} orbitals.

4.2. *Binding of fluoride*

The doublet resulting when laccase is treated with equivalent amounts of F^- (fig. 2B) is most likely due to coupling of the unpaired electron of the Type 2 Cu^{2+} to a ^{19}F ($I = ½$) nucleus. With excess F^- two ^{19}F nuclei interact equally with the same Cu^{2+}. Also, g_z increases with increasing number of F^- interacting, which is consistent with relative ionic character of bonding of F^-. Coupling to protons seems excluded on the basis of the D_2O experiment. In addition, the reported proton splittings in other Cu^{2+} complexes (about 5 gauss [7]) are much smaller than those found here (55 gauss). In fact, Chiang and Bersohn [8] observed a ^{19}F splitting of about 50 gauss in mixed complexes with amines and F^-. Thus, we conclude that the Type 2 Cu^{2+} in laccase can coordinate one or two F^- depending on the concentrations of the reactants. This binding does not affect the Type 1 Cu^{2+} as judged from its EPR spectrum.

4.3. *The nature of the anion-inhibition site*

The experiments in this work have demonstrated that two anions, CN^- and F^-, inhibit laccase by coordinating to one of the two Cu^{2+} present, namely that designated Type 2. Although the EPR effects of other anions have not been reported, preliminary experiments indicate that cyanate and thiocyanate also bind to Type 2 Cu^{2+} and these findings make it probable that all anion inhibitors have a common mode of action in binding to the Type 2 Cu^{2+}.

Curzon [9,10] has reported studies on the inhibition of ceruloplasmin-catalyzed oxidations by N_3^- and CN^-. On the basis of kinetic results alone, he has suggested that CN^- binds to a permanent cuprous atom and N_3^- to a valence-changing Cu^{2+}. Since ceruloplasmin has four Cu^{2+} per molecule, one

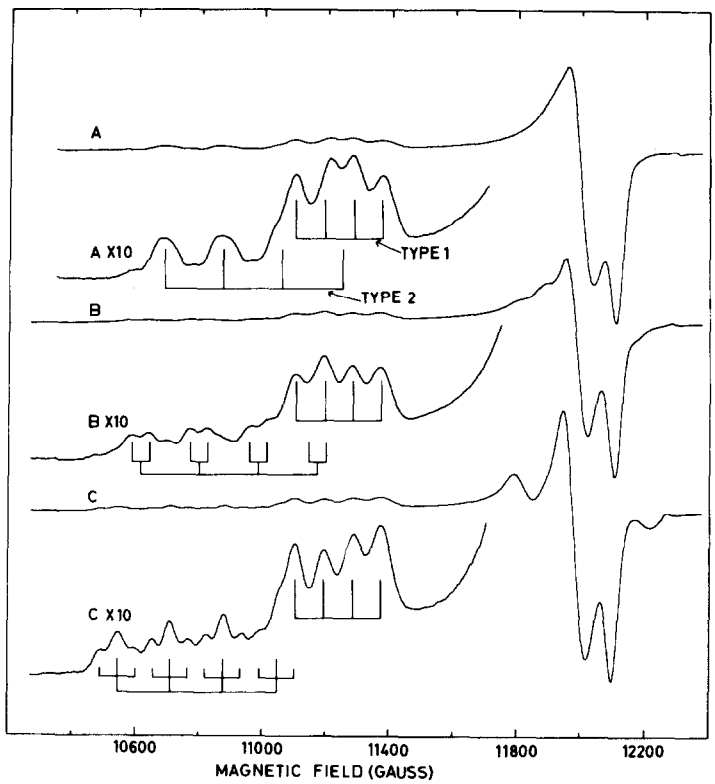

Fig. 2. EPR spectra of native and fluoride-treated laccase A at 34.5 GHz. Laccase A (0.7 mM in 0.1 M sodium acetate, pH 5.5) was treated as follows: (A) – no fluoride, (B) – 0.7 mM NaF, and (C) – 14 mM NaF. Samples were mixed at room temperature and immediately frozen. The low-field portion is also shown recorded at ten times higher gain (A, B and C X 10). The splitting due to the Cu nucleus in the g_z region of Type 1 and Type 2 Cu^{2+} is indicated. In (B) and (C), the superhyperfine structure of the Type 2 Cu^{2+} is also shown. The values of g_z and A_z(Cu) for the Type 2 Cu^{2+} are: (A) 2.243 and 185 gauss, (B) 2.261 and 185 gauss, and (C) 2.281 and 169 gauss. The superhyperfine splitting in (B) and (C) is about 55 gauss.

being similar to the Type 2 Cu^{2+} found in laccase [11], an alternative explanation for anion inhibition would be through interaction with this single Cu^{2+}. These possibilities are currently being investigated with ceruloplasmin as well as with the laccase from lacquer tree.

Although the interaction of anions with the Type 2 Cu^{2+} in laccase is an indication of the direct involvement of this form of copper in the catalytic reaction, its function is not yet clear. Further experiments are being undertaken to clarify the role of the different Cu^{2+} in the individual steps of laccase-catalyzed reactions.

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References

[1] B.G.Malmström, B.Reinhammar and T.Vänngård, Biochim. Biophys. Acta 156 (1968) 67.

[2] B.G.Malmström and T.Vänngård, J. Mol. Biol. 2 (1960) 118.
[3] J.A.Fee and B.G.Malmström, Biochim. Biophys. Acta 153 (1968) 299.
[4] L.Broman, B.G.Malmström, R.Aasa and T.Vänngård, Biochim. Biophys. Acta 75 (1963) 365.
[5] G.Fåhraeus and B.Reinhammar, Acta Chem. Scand. 21 (1967) 2367.
[6] L.Broman, Nature 182 (1958) 1655.
[7] H.A.Kuska, M.T.Rogers and R.E.Drullinger, J. Phys. Chem. 71 (1967) 109.
[8] T.Chiang and M.Bersoh, Chem. Phys. Letters 1 (1968) 521.
[9] G.Curzon, Biochem. J. 100 (1966) 295.
[10] G.Curzon, Biochem. J. 106 (1968) 905.
[11] T.Vänngård, in: Magnetic Resonance in Biological Systems, eds. A.Ehrenberg, B.G.Malmström and T.Vänngård (Pergamon Press, Oxford, 1967) p. 213.