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ARTICLE *in* FEBS LETTERS · NOVEMBER 1975

Impact Factor: 3.17 · DOI: 10.1016/0014-5793(75)80226-2 · Source: PubMed

^{17}O HYPERFINE INTERACTION IN THE EPR SPECTRUM OF FUNGAL LACCASE A

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Received 11 July 1975

1. Introduction

Laccase from *Polyporus versicolor* contains four copper atoms per molecule, one Type 1 and one Type 2 Cu^{2+}, both EPR-detectable [1], and a Cu^{2+} pair, which is EPR non-detectable [2]. The nature of the ligands to Type 1 and Type 2 Cu^{2+} is still rather uncertain. No ligand hyperfine coupling in the EPR spectrum of Type 1 has been reported earlier. However, ENDOR measurements on stellacyanin [3] which only contains one Type 1 Cu^{2+} indicate that at least one nitrogen co-ordinates to copper and that the metal is buried in the protein. In fungal laccase Type 2 Cu^{2+} is available for binding of various anions, and the affinity of Type 2 Cu^{2+} for fluoride ions is exceptionally high [4]. On addition of equimolar amounts of F^- each hyperfine line of the Type 2 Cu^{2+}-signal splits up into a doublet due to the coupling to the $I=1/2$ nucleus [5]. In a rapid-freeze reduction experiment ligand hyperfine coupling to nitrogen of Type 2 Cu^{2+} has also been detected [6].

Proton relaxation time measurements [1,7] have not provided any conclusive information on the possibility of H_2O co-ordination to any of the Cu^{2+} ions in the blue oxidases.

In the present study we provide EPR evidence using water enriched in ^{17}O ($I=5/2$) That H_2O is an exchangeable ligand to the Type 2 Cu^{2+} in fungal laccase, presumably positioned equatorially. The ^{17}O hyperfine interaction in fungal laccase is comparable to the splitting due to the equatorial ligands in Cu^{2+}acetylacetonate. F^- presumably replaces H_2O, since when one $F^-$is bound to Type 2 Cu^{2+}, ^{17}O in water no longer affects the Cu^{2+} EPR spectrum. The Type 1 Cu^{2+} signal is not affected by $H_2{}^{17}O$ or F^- and shows a splitting due to the interaction of at least one N.

2. Materials and methods

The fungus was grown in a medium containing isotopically pure ^{63}Cu to improve the resolution of the EPR spectrum of the laccase. The protein was prepared as usual [8].

The fluoride complex was obtained by addition of a slight excess of F^- to the laccase and dialysis overnight, followed by concentration. The concentrated protein was diluted in the EPR tube by addition of normal or deuterated water or water containing 50.22% ^{17}O and an unknown amount of deuterium. The final protein concentration was about 280 μM as determined by integrations of the EPR spectra. The final sodium acetate buffer concentration, pH 5.5 was 0.02 M or 0.13 M with the F^- complex.

EPR spectra were recorded at 9.15 GHz and 77°K in a Varian E-3 spectrometer. Line-shape simulations were performed assuming Gaussian line-shape.

The ^{17}O enriched water (0.5 ml) was a generous gift of Dr Israel Pecht, Weizmann Institute of Science, Rehovot, Israel.

3. Results and discussion

The X-band spectrum at 77°K of ^{63}Cu-fungal laccase A in presence of ^{17}O enriched water shows a significant broadening of the Type 2 Cu^{2+} signal without effects on Type 1. The broadening is

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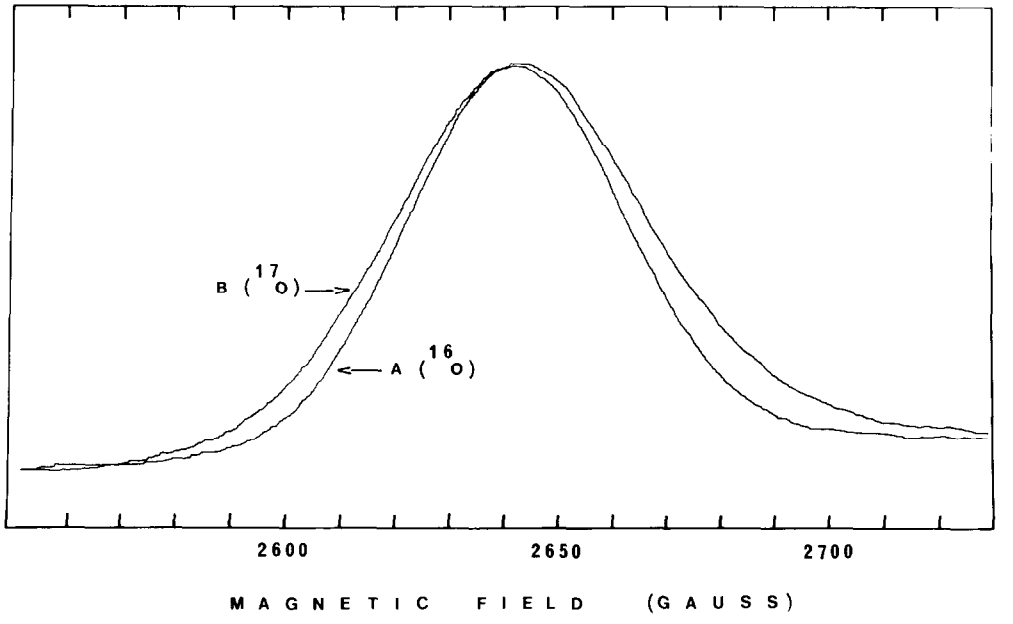

Fig.1. Low-field part of the EPR spectrum of ^{63}Cu fungal laccase A in (A) normal water (100%^{16}O) and (B) enriched water (40% ^{17}O). The spectra were recorded 10 min after preparation of the samples and dit not change on incubation overnight at room temperature. Microwave power: 20 mW; modulation amplitude 16 gauss. The amplitude of the sample with normal water was normalized to the sample with ^{17}O by adjustment of the gain.

accompanied by a decrease in amplitude. This broadening could not come from the unknown amount of deuterium in the enriched water since 80% $D_2{}^{16}O$ had no effect as compared to normal water. The increase in line-width is best seen on the low-field line of Type 2 Cu^{2+} (fig.1A and B) but can also be observed in the high-field part of the spectrum, where Type 2 Cu^{2+} contributes significantly to the amplitude [1].

In the presence of equimolar amounts of fluoride no line broadening by ^{17}O is seen.

Line-shape analysis of the low-field line of Type 2 $^{63}Cu^{2+}$ without ^{17}O present gives a full width at half the maximal amplitude of 41 gauss. Under the assumption that only one H_2O contributes to the broadening, simulation of the low-field line gives the best fit for a ^{17}O hyperfine splitting constant of 12.2 ± 0.3 gauss.

There are few reports on ^{17}O hyperfine interaction with Cu^{2+} in the literature, and as a model we therefore studied Cu^{2+}acetylacetonate [2]. One hour after preparation of the complex the spectrum with ^{17}O enriched water was still the same as with normal water. This indicates that ^{17}O in H_2O if present as an axial ligand must have a very small effect on the Cu^{2+} signal. However, after several days at room temperature a structure appears in the low-field line (fig.2A and B). The same spectrum is obtained after one week, indicating that equilibrium has been reached. The final line form can be analysed as resulting from the interaction of four equivalent equatorial ^{17}O nuclei with the Cu^{2+} in the $(CH_3.CO.CH_2.CO.CH_3)_2Cu^{2+}$ complex with a hyperfine splitting constant of 12 gauss (fig.2C). Evidently the carboxyl oxygens of acetylacetone have exchanged with the oxygen in the water [9] via the enol form of the free ligand present in excess. Our findings are in agreement with the effects reported for the $Cu^{2+}(H_2O)_6$ complex where the ^{17}O splitting could only be seen from the oxygen atoms lying in the equatorial plane [10]. The splitting due to the axial ligands were calculated to be less than 1 gauss [10].

From a comparison of the results for the protein and the smaller complexes we conclude that H_2O is

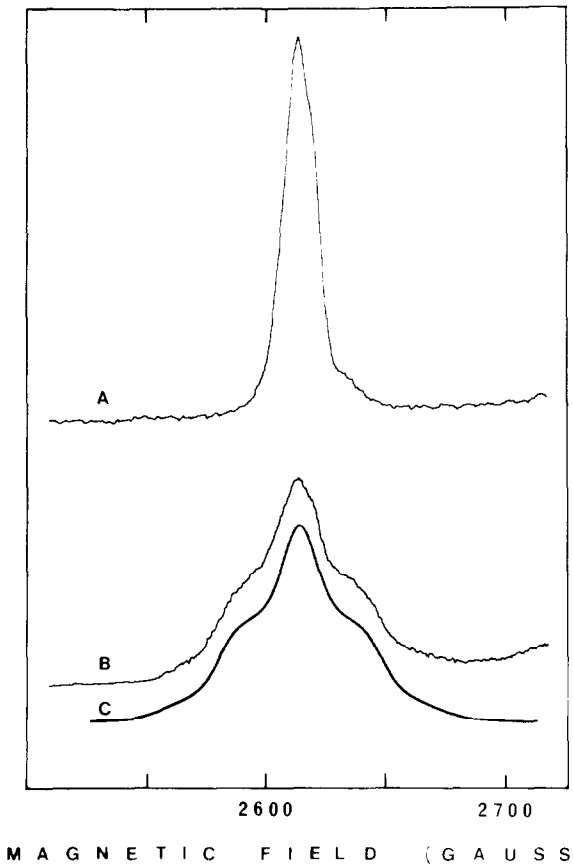

Fig.2. Low-field part of the EPR spectrum of $^{63}Cu^{2+}$acetylacetonate. The sample was 1.45 mM in $^{63}Cu^{2+}$ and 145 mM in acetylacetone in a 1:1 water:dioxan solvent at alkaline pH, in (A) normal water (100%^{16}O) and (B) enriched water (40%^{17}O). Spectrum (C)is a simulation of (B) assuming Gaussian line-shape with a full line width at half the maximal amplitude of 12.7 gauss, four equivalent oxygen nuclei and a hyperfine splitting constant for the interaction between the I=5/2 nucleus and Cu^{2+} of 12 gauss. Microwave power, 5 mW; modulation amplitudes, 5 gauss.

an equatorial ligand to Type 2 Cu^{2+}, which can be replaced by F^-. However, we cannot exclude that additional H_2O is bound, e.g. as axial ligands with a small hyperfine coupling, in particular since it is known that 2 F^- can bind to Type 2 Cu^{2+} [5]. Proton relaxation measurements [1,7] show that the co-ordinated water is not rapidly exchanging in the NMR time scale. Thus, the Type 2 Cu^{2+} does not seem to be on the outside of the protein but is more or less hidden, so that a steric hindrance slows down the exchange of water and reduces the rate of F^- binding [8]. It has been suggested [8] that intermediates in the reduction of O_2 to H_2O are stabilised by Type 2 Cu^{2+}. Some steric arrangement around Type 2 Cu might prevent these intermediates from being released into the solvent. To investigate this essential part in the catalytical process further studies with ^{17}O are in progress.

Finally, we have observed that a complicated structure on the g⊥ part of the EPR spectrum of fungal laccase can be seen (fig.3), since the resolution is improved with the use of the isotopically pure Cu

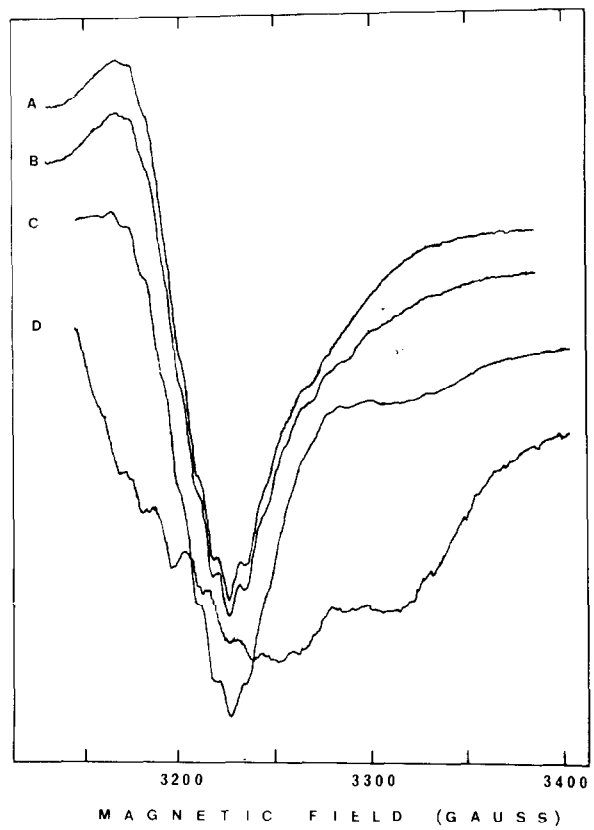

Fig.3. High-field part of the EPR spectrum of ^{63}Cu fungal laccase A in (A) normal water (100%^{16}O), (B) enriched water (40%^{17}O), (C) normal water with one equivalent F^-, (D) as sample (C) but after reduction of Type 1 Cu^{2+} with some solid ascorbate. Normalization as in fig.1. Microwave power, 5 mW; modulation amplitude 2 gauss. (D) is shown with a 4 times higher gain than in (C).

relative to normal Cu (cf. ref. 1). This structure is not affected by ^{17}O or F^-, and from a comparison with the spectrum observed with Type 1 Cu^{2+} reduced (fig.3D) clearly demonstrates, that the structure is not due to Type 2 Cu^{2+}. Some features of the structure may arise from hyperfine coupling to Cu, from the departure from axial symmetry ($\Delta g \approx 0.02$ [1]) and from the so called 'overshoot' line. However, the structure has so many lines that at least one nitrogen ligand must be bound to Type 1 Cu^{2+} in agreement with the earlier results for Type 1 in stellacyanin [3].

Acknowledgements

This study should not have been possible without the generous gift of 50.22% oxygen-17 enriched water from Dr Israel Pecht, Weizmann Institute of Science, Rehovot, Israel.

We are indebted to Miss Ann-Cathrine Carlsson who prepared the laccase from the fungal culture.

This study has been supported by grants from the Knut and Alice Wallenberg Foundation and the Swedish Natural Science Research Council.

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