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MAGNETIC SUSCEPTIBILITY OF LACCASES AND CERULOPLASMIN

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Summary

1. Recent magnetic susceptibility measurements on laccase (monophenol,dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) from the lacquer tree *Rhus vernicifera* showed a deviation from Curie behaviour above 50 K, which was taken as evidence for an antiferromagnetically coupled Cu(II)-Cu(II) pair in the oxidized enzyme. The magnetic susceptibility of this protein has been reinvestigated. Further measurements on laccase from the fungus *Polyporus versicolor* and human ceruloplasmin (iron(II):oxygen oxidoreductase, EC 1.16.3.1) are presented.

2. The magnetic susceptibility of fungal laccase and lacquer tree laccase can be accounted for by the EPR detectable copper ions in the temperature range 40—300 K.

3. If an antiferromagnetically coupled Cu(II)-Cu(II) pair exists in the laccases, then the coupling, expressed as $-J$, should be at least of the order of 300 cm^{-1}, as deduced from the Curie dependence of the susceptibility and the sensitivity in our measurements.

4. If an analogy with the laccases is assumed for the EPR invisible copper in ceruloplasmin then a limiting value of the coupling may be deduced also in this case, with $-J$ at least of the order of 200 cm^{-1}.

Introduction

The blue copper-containing oxidases laccase and ceruloplasmin contain three types of copper ions. Two of them, type 1 and type 2, are EPR-detectable. Type 1 is responsible for the strong blue colour in the enzymes and is also characterized by an unusually small EPR hyperfine splitting constant. Type 2 has a greater hyperfine splitting similar to those found for simple copper complexes. The third type of copper is not EPR-detectable and the mode of its binding to

the protein is not known. However, in each of the laccases it is believed to exist as a pair. Among several proposals [1] a pair of antiferromagnetically coupled Cu(II) ions acting as a two-electron acceptor seems to be the most attractive one [2]. There is no clear model for the EPR-invisible copper in ceruloplasmin but an analogy with the laccases cannot be excluded [3].

Previous measurements of magnetic susceptibility on blue oxidases have been used mainly to estimate the fraction of paramagnetic copper [4—6]. Recently, however, results from lacquer tree laccase have been presented showing a deviation from the Curie dependence of the susceptibility above 50 K [7]. This was taken as evidence for an exchange coupled cupric dimer in the enzyme.

We have reexamined the magnetic behaviour of lacquer tree laccase and extended the investigations to fungal laccase and human ceruloplasmin. Measurements have been performed over a wide temperature range using a magnetic balance of the Faraday type and at room temperature for the laccases using an NMR spectrometer.

Materials and Methods

Laccase from the lacquer tree *Rhus vernicifera* and the fungus *Polyporus versicolor* was prepared as described previously [8,9]. Ceruloplasmin was prepared according to Rydén [10].

Low temperature magnetic susceptibility measurements were done with a sensitive magnetic balance of the Faraday type constructed at the Department of Biophysics at Stockholm University. A commercial electronic microbalance modified with a precision variable voltage source was coupled to a tailed variable temperature cryostat. A conventional electromagnet equipped with field dial and special constant force pole caps was operated at a constant force field of 24.2 kG^2/cm. The temperature at the sample position was sensed by a calibrated platinum resistor. An electronic controller was used to regulate and measure temperature with an accuracy of better than ±0.1 K below and ±0.3 K above 90 K. Special attention was paid to the temperature equilibrium in the sample. Calibration of the instrument was performed using twice distilled water with $\chi_{mass} = -0.720 \cdot 10^{-6}$ cgs at 20° C. The estimated limit of resolution for this instrument is $\pm 2 \cdot 10^{-10}$ cgs for dilute enzyme solutions. An instrument of this type is particularly well suited to measure with high accuracy small variations on top of the total magnetic susceptibility. A more detailed description will appear elsewhere.

The paramagnetic contribution from physically dissolved oxygen in the low temperature measurements was eliminated by stirring about 0.2 ml of sample under a stream of deoxygenated humidified argon for 20 min at 20° C. After this procedure 0.1 ml of sample was transferred anaerobically to the deoxygenated delrin sample container. When still under argon the sample container was tightly closed with a screw lid. By means of a special sample transfer device the sample was frozen within 30 s under a helium/nitrogen atmosphere and transferred to the precooled cryostat. The position of the sample in the force field was properly adjusted and measurements could be started after careful evacuation of the sample space and refilling with helium gas to about 1.6 mmHg (213 N/m^2) via a liquid nitrogen trap.

The amount of blue copper in the samples was estimated from the absorbance maximum around 610 nm using extinction coefficients 5.7 $cm^{-1} \cdot mM^{-1}$ for lacquer tree laccase, 4.9 $cm^{-1} \cdot mM^{-1}$ for fungal laccase and 5.5 $cm^{-1} \cdot mM^{-1}$ per type 1 copper for human ceruloplasmin.

The EPR visible copper (the sum of type 1 and type 2 copper) was estimated from double integrated EPR spectra corrected for background contributions. Spin concentrations were calculated from double integrals using the method of Aasa et al. [11]. CuEDTA 1 mM was used as reference sample.

Room temperature magnetic susceptibility measurements were made with a Bruker 270 MHz NMR spectrometer giving a field parallel to the sample tube. The 'internal/external reference method' was used as described previously [12]. Experiments were performed in the same media as those used for the low temperature experiments except that 2% tertiary butanol (v/v) was added as reference.

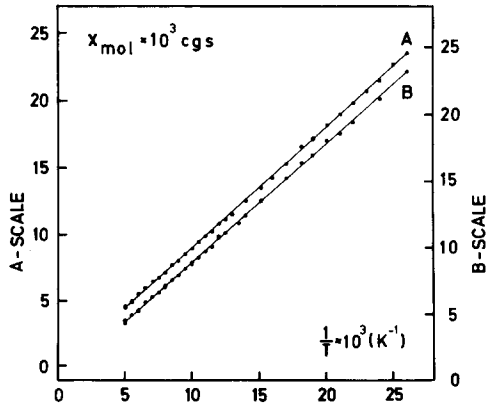
Fluoride complexes of the fungal and tree enzymes were prepared with a tenfold and 25-fold excess of fluoride, respectively.

The reference signal broadened approximately 1.5 Hz upon addition of either tree or fungal laccase and the width decreased 0.5 and 1 Hz after reduction of the proteins. Changes in linewidth of the reference could indicate the presence of other shift mechanisms than those due to a difference in the bulk susceptibility of the sample. However, as discussed below, such shift contributions are small in these experiments.

Reduction of samples was carried out under nitrogen atmosphere using an excess of minimally four electron equivalents of dithionite. Buffered solutions of dithionite were prepared anaerobically.

Results

Results from the low temperature magnetic susceptibility measurements are shown in Figs. 1 and 2, where changes in molar susceptibility are given as func-



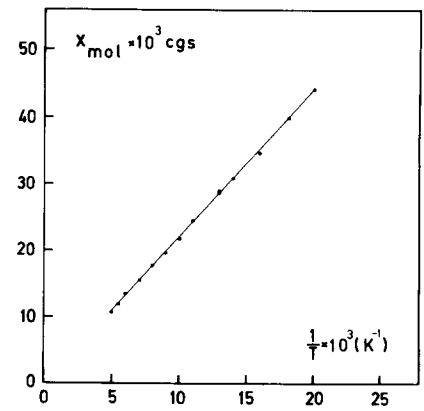


Fig. 1. Temperature dependence of the magnetic susceptibility of (A) laccase, 0.1 ml 2.33 mM, from the lacquer tree *Rhus vernicifera* in 0.1 M phosphate buffer pH 7.4, (B) laccase, 0.1 ml 1.88 mM, from the fungus *Polyporus versicolor* in 0.1 M phosphate buffer pH 6.0. Note that two lines for clarity have been separated by shifting the vertical scales to different zero points. Actually they are very nearly but not exactly coincident.

Fig. 2. Temperature dependence of the magnetic susceptibility of human ceruloplasmin, 0.1 ml 0.58 mM.

TABLE I

DATA FROM THE LOW TEMPERATURE EXPERIMENTS ON LACQUER TREE LACCASE, FUNGAL LACCASE AND HUMAN CERULOPLASMIN

Enzyme	Type 1 Cu light abs. (mM)	Type 1 + type 2 Cu EPR (mM)	$d(\chi_{mol})/d(\frac{1}{T})$ observed (cgs · K · mol^{-1}	$d(\chi_{mol})/d(\frac{1}{T})$ * calculated (cgs · K · mol^{-1})
Lacquer tree laccase	2.33 ± 2%	5.46 ± 7%	0.90 ± 3%	0.99 ± 7%
Fungal laccase	1.88 ± 4%	4.14 ± 9%	0.89 ± 3%	0.91 ± 9%
Human ceruloplasmin	1.15 ± 2%	2.16 ± 7%	2.20 ± 3%	1.56 ± 7%

* Protein concentrations were determined from the light absorbance maximum around 610 nm assuming one type 1 Cu per molecule in the laccases and two in ceruloplasmin. For extinction coefficients see Materials and Methods. $\chi_{mol}(SI) = \chi_{mol}(cgs) \cdot 4\pi \cdot 10^{-6}$.

tion of inverse temperature for lacquer tree laccase, fungal laccase and human ceruloplasmin. Background from sample suspension wire, sample holder and buffer solution has been subtracted. All three samples show Curie dependence of the susceptibility ($\chi \propto 1/T$) in the investigated temperature range. The straight lines have been fitted to the experimental points by a computer least squares procedure.

Analytical results from light absorption and EPR are shown in Table I together with the observed and calculated values of the slopes $d(\chi_{mol})/d(1/T)$. Volume susceptibilities were calculated using our analytical data and the *g*-values previously reported for the type 1 and type 2 copper ions [13]. Conversion to molar susceptibilities was obtained using the protein concentrations. No contribution was assumed from the EPR-invisible copper.

Table II summarizes the results from the NMR measurements at room temperature. Normalized chemical shifts and molar susceptibilities are given for the oxidized minus the reduced enzymes. The values obtained for the paramagnetic susceptibility are corrected for a shift due to physically dissolved oxygen [12]. Shifts due to the addition of dithionite, measured in a separate experiment, were found to be negligible. The figures represent the mean of three measurements. Theoretical values were calculated assuming one type 1 and one type 2 copper per molecule.

TABLE II

NORMALIZED CHEMICAL SHIFTS AND MOLAR SUSCEPTIBILITIES OF FUNGAL AND LACQUER TREE LACCASE (OXIDIZED-REDUCED) AT 298 K

Enzyme	$\Delta\nu$ (Hz · mM^{-1})	$\Delta\chi_{mol} \times 10^3$ observed (cgs · mol^{-1})	$\Delta\chi_{mol} \times 10^3$ * calculated (cgs · mol^{-1})
Lacquer tree laccase	2.9	2.6	2.8
Fungal laccase	3.2	2.8	2.8

* See comment to Table I.

Discussion

Table I shows for the two laccases that the amount of EPR-detectable copper is about twice the amount of blue copper. The observed paramagnetic susceptibility can be accounted for by the EPR-detectable copper. This is consistent with previous results [14,15,5] and leaves the EPR-invisible copper with a diamagnetic ground-state. Supposing an antiferromagnetically coupled Cu(II)-Cu(II) pair with, to a first approximation, no orbital angular momentum associated with the ground states of the interacting copper ions, the observed Curie dependence of the magnetic susceptibility allows us to put limits on the exchange coupling.

The exchange interaction can be represented by the spin Hamiltonian

$$\mathcal{H} = -2J\hat{S}_1 \cdot \hat{S}_2$$

where J is the exchange integral between the interacting ions (<0 for an antiferromagnetically coupled pair). Calculated in a standard manner (see for example ref. 16) with $S_1 = S_2 = 1/2$ the susceptibility of the interacting ions will be

$$\chi_{mass} = \frac{N\beta^2\bar{g}^2}{kT} \cdot \frac{2}{3 + e^{-2J/kT}}$$

where N is the number of EPR-invisible copper pairs in one gram of solution, β the Bohr magneton, k Boltzmann's constant and $\overline{g^2}$ the spatially averaged value of g^2. This contribution to the susceptibility is nonlinear in $1/T$.

At low enough temperatures the contribution is negligibly small and increases as the available thermal energy becomes sufficiently high to promote an increasing fraction of pairs to an excited paramagnetic state.

The observed Curie dependence of the magnetic susceptibility implies that the contribution to the mass susceptibility from the paired copper ions should be less than $4 \cdot 10^{-10}$ cgs at 200 K (corresponding to molar susceptibilities of the order of $2 \cdot 10^{-4}$ cgs). For the proposed model with $\overline{g^2} = 4$ and assuming one antiferromagnetically coupled pair per enzyme molecule we obtain $-J \geqslant 300$ cm^{-1} for lacquer tree laccase and $-J \geqslant 290$ cm^{-1} for fungal laccase.

The interpretation of the ceruloplasmin results is more complicated. The magnetic susceptibility shows Curie dependence over the temperature range studied, but the observed paramagnetism is higher than that expected for the EPR-detectable copper. The difference could be accounted for by the presence of 85 μM high spin ferric iron impurities in the sample. Copper analysis gives 3.24 mM concentration of total copper in the sample. Combined with the analytical data of Table I this indicates about equal amounts of the three types of copper per molecule in this preparation.

Though the relative amounts of different types of copper seem to be somewhat anomalous compared to previous results [17] conclusions about the EPR invisible copper may be extracted.

If in analogy with the laccases the EPR invisible copper is supposed to exist in antiferromagnetically coupled Cu(II)-Cu(II) pairs with orbital singlet ground

states then the Curie dependence of the magnetic susceptibility gives us a possibility to calculate a limit for the exchange coupling. In the same manner as described previously $-J \geqslant 195$ cm^{-1} is obtained.

The NMR results for the laccases are in good agreement with the low-temperature data, accounting for the EPR-detectable copper ions only. The limit for the exchange integral, as deduced from the accuracy of the NMR experiments, is $-J \geqslant 330$ cm^{-1} for both tree and fungal laccase.

The validity of the NMR results rests upon the assumption that other shift contributions than that from a change in the bulk susceptibility can be neglected. The most obvious extra contribution could arise from interaction between the reference substance butanol and the paramagnetic centres of the protein (i.e. type 2 copper). However, addition of fluoride, which complexes the type 2 copper, does not significantly affect the reference linewidth or the susceptibility, indicating that there is no substantial interaction.

Our results are not in agreement with $J = -85$ cm^{-1} (recalculated from $J = 170$ cm^{-1} corresponding to $\mathcal{H} = J\hat{S}_1 \cdot \hat{S}_2$) which Solomon et al. [7] obtained for lacquer tree laccase. Using their value for the exchange integral one finds that the antiferromagnetically coupled cupric ions would contribute significantly to the room temperature susceptibility and give substantial deviation from Curie dependence in our low-temperature measurements.

In fact, our measurements do not give any positive evidence for the existence of exchange coupled Cu(II)-Cu(II) pairs in the proteins investigated. Previously a disulphide in connection with a Cu(I)-Cu(I) pair has been excluded [1], and the only further possibility for the type 3 copper ions is a Cu(III)-Cu(I) pair, which also would have a diamagnetic ground state. However, this model can most likely be excluded as well [5]. Thus, if binuclear pair of Cu(II) exists, then the high sensitivity in our measurements gives lower limits for the coupling, expressed as $-J$, such that $-J$ should at least be of the order of 300 cm^{-1} for the laccases and 200 cm^{-1} for ceruloplasmin (alternatively expressed: the first excited level, i.e. the spin triplet with $S_1 + S_2 = 1$, should be at least of the order of 600 and 400 cm^{-1}, respectively, above the singlet ground state). Up till now molluscan oxyhemocyanin is the only copper protein for which a comparable value has been reported [7].

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