MAGNETIC SUSCEPTIBILITY STUDY OF THE LACCASE–PEROXIDE DERIVATIVE

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1. Introduction

Laccases are copper containing oxidases in which the 4 copper ions are bound in 3 distinct sites. Two of these are EPR detectable (type 1 and type 2) while the third site (type 3) most probably consists of an antiferromagnetically coupled Cu(II)–Cu(II) pair [1]. The magnetic susceptibility of native oxidized *Rhus* laccase can be quantitatively accounted for by the EPR detectable copper [2–4]. Thus, formulation of the type 3 site as a copper pair requires a lower limit for the coupling, expressed by the exchange integral, $-J$ of 250–300 cm^{-1} [3,4].

We have demonstrated the formation of a high affinity peroxy–laccase complex upon reacting native oxidized enzyme with H_2O_2 [5]. Primarily on the basis of the absorption and CD spectra it was proposed that the peroxide binds to the copper pair of the type 3 site [6]. We have shown spectral similarities between the peroxy–laccase complex and intermediates formed during the reoxidation of reduced laccase with O_2 [7].

These observations have led us to extend the study of the type 3 site, since the interaction with dioxygen or its derivatives might affect the exchange coupling of this copper pair. Therefore the magnetic properties of peroxy–laccase was measured by means of high resolution NMR technique. Indeed we have observed an increase in the magnetic susceptibility upon peroxide binding which is assigned to a decrease in the antiferromagnetic coupling of the type 3 copper pair.

2. Materials and methods

Laccase was prepared from the acetone powder made of the *Rhus Vernicifera* lacquer according to a modified version [7] of the procedure in [8]. Spectroscopic, EPR, and catalytic properties were in agreement with those in [8,9]. Protein concentrations were determined from the A_{614} (ϵ = 5700 M^{-1} cm^{-1}). EPR spectra were recorded on a Varian E-3 at liquid nitrogen temperature and on a Varian E-12 at liquid helium temperature.

Peroxy–laccase was produced by adding an equimolar amount of hydrogen peroxide to a 7.5×10^{-5} M laccase solution. The formation of the peroxy complex was followed spectrophotometrically [5] and after the reaction reached completion the protein was brought to the desired concentration by means of vacuum dialysis.

The magnetic susceptibility measurements were performed at 300 K with a Bruker 270 MHz spectrometer giving a field of 6.3 T parallel to the sample tube. The 'internal/external marker' method was applied [10] using coaxial tubes (Wilmad Glass Co., NY). The protein was placed in the inner tube (first native oxidized laccase and then peroxy–laccase of exactly the same concentration) together with 5% of the reference in 0.1 M potassium phosphate at pH 6.0. The concentration range for the protein was 1.0–3.2 mM. The outer tube contained 0.2% reference in 98% D_2O. As reference we used *tert*-butanol and ethylene glycol. The reference signal broadened ~0.6 Hz/mM protein with ethylene glycol and ~0.7 Hz/mM with *tert*-butanol.

The change in the molar susceptibility relative to the native oxidized enzyme was calculated from [10]:

$$\Delta\chi_{mol} = \frac{3\,\Delta\nu}{C\,\nu_0}$$

where $\Delta\nu$ is the difference in Hz between the refer-

ence signals for peroxy- and native oxidized laccase at concentration C and ν_0 the spectrometer frequency.

3. Results

Table 1 shows the results from the NMR measurements at 300 K. The chemical shifts are given for peroxy–laccase minus native oxidized laccase at the same concentration. No corrections were made for the oxygen present in the protein solutions, since the amount dissolved should be the same in both samples of corresponding peroxy- and native oxidized laccase.

In order to check if any paramagnetic impurities were present in the peroxy–laccase solutions the EPR spectra of all protein solutions were measured at liquid nitrogen or helium temperature. However, no differences were observed between the EPR spectra of peroxy- and native oxidized laccase in the 0.1–0.5 T region in accordance with the measurements in [5,7].

4. Discussion

Table 1 shows that the difference in the chemical shifts of the reference signal is larger with peroxy–laccase than with native oxidized laccase. It has been shown that there is no substantial interaction between *tert*-butanol and the paramagnetic centres in laccase [3]. Since the shift is the same for both *tert*-butanol and ethylene glycol and the lines broaden by approximately the same amount, the interaction of ethylene glycol with the paramagnetic copper sites can be assumed to be negligible as well.

Therefore, the change in chemical shift can be taken as evidence for a change in the bulk susceptibility. The molar susceptibility of native oxidized laccase is 3.52×10^{-5} SI units $\times$ mol^{-1} at 298 K [3] corresponding to 2 paramagnetic Cu^{2+}/molecule. Thus the increase in susceptibility for peroxy–laccase of 1.77×10^{-5} SI units $\times$ mol^{-1} corresponds to an enhancement of 50% which is equivalent to formation of one new paramagnetic site with $g \sim 2$. However, there are several reasons why such a species is unlikely. The production of one additional Cu(II) ion can be excluded since uncoupling of the type 3 site copper pair would produce 2 new paramagnetic ions per molecule. Further, the formation of a free radical can be ruled out for a variety of reasons: The existence of a long-lived (several days) radical produced by the interaction of μM levels of peroxide with the protein is highly improbable, and as demonstrated [5] the two extra oxidation equivalents of the peroxide are retained in the peroxy–laccase. Also a new paramagnetic species is expected to show up in the ESR spectrum. However, this was not the case, even at liquid He temperature. Therefore, to our knowledge the only explanation for the increase in susceptibility is a decrease in the antiferromagnetic coupling between the type 3 copper pair upon binding of peroxide. For such a dimer the susceptibility is given by [3]:

$$\chi_{mol} = \frac{L\beta^2\bar{g}^2}{3kT} \times \frac{2}{1 + \frac{1}{3}\,\mathrm{expt}(-2J/kT)}$$

where L is Avogadro's number, β the Bohr magneton, and J is the exchange energy defined by the spin Hamiltonian:

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

$\bar{g}$ was taken as 2.14 [1]. From this we calculate a coupling constant, $-J = 120 \pm 10$ cm^{-1}. This result would also explain the absence of any new EPR signal since the contribution of this site to the overall magnetic susceptibility would only be 10% at liquid nitrogen temperature and 0 at He temperature. Furthermore, a resonance line from a Cu(II) pair could be very much broadened because of the exchange coupling.

The hypothesis of partial or full uncoupling of

Table 1
Chemical shifts and molar susceptibility for peroxy-laccase (peroxy- minus native oxidized laccase) at 300 K

Reference	Protein (mM)	$\Delta\nu$ (Hz)	$\Delta\chi \times 10^5$ (SI units $\times$ mol^{-1})
tert-Butanol	1.2	2.04	1.89
tert-Butanol	3.2	5.25	1.82
Ethylene glycol	1.1	1.64	1.66
Ethylene glycol	1.0	1.55	1.72

Average: $\Delta\chi_{mol} = (1.77 \pm 0.12) \times 10^{-5}$

the copper pair agrees with our observations that the type 3 site behaves as a pair of single electron donors upon reoxidation with either H_2O_2 or O_2 [5]. Also strong reductants are able to induce a transition of the type 3 copper ions from a cooperative 2 e^- acceptor to a pair of 1 e^- acceptors [11].

It is of interest to compare the magnetic susceptibility of peroxy–laccase with that of oxyhemocyanin since the active site of the latter protein is also described as a pair of Cu(II) ions in an antiferromagnetically coupled state [4]. However, measurements of both *Megathura crenulata* and *Limulus* oxyhemocyanin show that the binuclear copper pair is diamagnetic up to room temperature which implies a coupling constant $J \leqslant -310$ cm^{-1} for the former and $J \leqslant -275$ cm^{-1} for the latter (recalculated from [4, 12]). Also the Cu(II) pair in methemocyanin from *Cancer magister* is reported to be diamagnetic at least below liquid N_2 temperature [13]. This different behaviour of the copper pair in oxyhemocyanin and in laccase is remarkable and most probably reflects their distinct functional roles in the two proteins: Reversible oxygen binding in the former versus an irreversible oxygen binding as a reduction intermediate in the latter.

If the increase in bulk susceptibility of peroxy–laccase really is the result of a decrease in the coupling constant, this would be the first direct demonstration of the type 3 site being an antiferromagnetically coupled Cu(II) pair in the oxidized enzyme. However, a proof of this hypothesis requires an extensive series of susceptibility measurements over a wide temperature range. This study is presently being carried out.

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