Multi-frequency e.p.r. studies of a mercury-containing mixed-metal derivative of laccase

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Multi-frequency e.p.r. studies of a derivative of laccase containing one mercury atom and three of copper were carried out at -150°C . The e.p.r. signal of the mercury derivative and fluoride-binding studies establish that a type-2-like copper centre is present. The signal suffers broadening, owing to g -strain, but at low frequencies (S-band) ligand hyperfine splitting can be resolved, and it can be explained in terms of coupling to three nitrogen atoms. The g values and the effect of solvent deuteration on the line width suggest that the fourth ligand in the equatorial plane is a water molecule. Simulations of the e.p.r. spectrum reveal that the site is slightly rhombic at -150°C , a finding in accord with the proposed N_3O donor set. Finally, it is emphasized that a structural reorganization of the type-2 copper site occurs with the binding of fluoride at low temperature. The reorganization may be linked to a conformational change which has previously been claimed to occur on cooling; however, this transition is not necessarily relevant to the temperature-dependence of fluoride binding.

INTRODUCTION

Oxidases which mediate the four-electron reduction of O_2 to H_2O without releasing peroxide intermediates form an interesting class of enzymes. They are usually complex structures which react with a variety of reductants, and they often contain multiple redox centers. Laccase, which can be isolated from the latex of the oriental lacquer tree, is a comparatively simple member of the class. Previous work has shown that laccase contains four copper atoms distributed among three different binding sites (Malkin & Malmström, 1970; Fee, 1975; Reinhammar, 1984). The type-1 site of laccase appears to be similar (Dooley *et al.*, 1979) to those of the small blue copper proteins plastocyanin (Freeman, 1981; Guss & Freeman, 1983) and azurin (Adman & Jensen, 1981; Norris *et al.*, 1981), whose crystal structures have been solved. The core of the donor set consists of two histidine nitrogen atoms and a cysteine sulphur atom. A fourth, weak, bond to a methionine sulphur atom defines a distorted tetrahedral binding site in plastocyanin and azurin, but this ligand may vary from protein to protein. The type-1, or so-called 'blue', copper site exhibits an intense visible absorbance around 600 nm, attributed to a $\text{S}(\text{Cys}) \rightarrow \text{Cu}(\text{II})$ charge-transfer band, and yields an e.p.r. spectrum with an unusually small hyperfine splitting in the low-field region.

The type-2 copper has no intense visible absorbance, but it exhibits an e.p.r. spectrum characteristic of a tetragonal co-ordination environment. E.p.r. studies in ^{17}O -enriched water have implicated an equatorial water ligand (Deinum & Vänngård, 1975; Brändén & Deinum, 1977). Spin-echo e.p.r. studies have indicated that it also has at least one nitrogen ligand (Mondovì *et al.*, 1977), although as many as three to four nitrogen ligands have

been assigned to the type-2 copper of other blue oxidases (Dawson *et al.*, 1979; Brändén & Reinhammar, 1975; Malmström *et al.*, 1968).

Finally, laccase contains two copper atoms in the type-3 site, which, as a result, functions as a two-electron acceptor (Reinhammar, 1972). This site is e.p.r.-non-detectable in both the oxidized and reduced forms, and magnetic-susceptibility studies indicate the two copper atoms are anti-ferromagnetically coupled in the oxidized state (Dooley *et al.*, 1978; Petersson *et al.*, 1978). The oxidized type-3 site gives rise to an optical absorbance around 330 nm, which may be due to a $\text{His} \rightarrow \text{Cu}(\text{II})$ charge-transfer transition (Solomon, 1983). A half atom of copper at a distance of 0.34 nm is required to model satisfactorily the outer-shell contribution to the copper e.x.a.f.s. (extended X-ray-absorption fine structure) spectrum of native laccase, and this has been interpreted as an indication of the copper separation within the type-3 site (Woolery *et al.*, 1984).

That the e.x.a.f.s. spectrum of native laccase must be interpreted in terms of the 'average' copper centre (Woolery *et al.*, 1984) illustrates a recurring problem in the characterization of the enzyme: spectral overlap complicates the analysis of signals from the individual copper centres. Chemical modifications of laccase which alleviate this problem are of obvious importance. The preparation of type-2-depleted laccase (Graziani *et al.*, 1976; Morpurgo *et al.*, 1980) afforded new avenues of study for the type-1 and type-3 sites (Reinhammar & Oda, 1979; Morpurgo *et al.*, 1981; Spira & Solomon, 1983). We previously reported the preparation of the first mixed-metal derivative of laccase, in which the experimental evidence indicates that $\text{Hg}(\text{II})$ is bound specifically to the type-1 site, while copper is bound to type-2 and type-3

sites (Morie-Bebel *et al.*, 1984). Consistent with this formulation, the mercury derivative (MDL) gives only one e.p.r. signal. The spectrum is typical of a tetragonal copper centre and has been assigned to the type-2 copper. To characterize the type-2 copper of MDL in more detail, we have undertaken the multi-frequency e.p.r. studies described below.

EXPERIMENTAL

The acetone-dried powder from the Chinese lacquer tree (*Rhus vernicifera*) was obtained from Saito and Co., Osaka, Japan, and extracted by the method of Reinhammar (1972), with only trivial modifications. The protein was repeatedly passed through ion-exchange columns until the A_{280}/A_{614} ratio was 20 ± 1 . The mercury derivative MDL was prepared as previously described (Morie-Bebel *et al.*, 1984). Copper was added as isotopically pure ^{65}Cu . All other chemicals were reagent grade and were used as obtained. Deoxygenated buffer solutions were obtained by purging with N_2 which had been passed through scrubbing solutions containing vanadous ion over zinc amalgam or passed through a column containing catalyst R3-11 (Chemlog no. 18-3000-00). Buffers were prepared from deionized water and passed through a Chelex-100 column to remove any trace metal-ion contaminants. The u.v.-visible spectra were obtained on a Cary-17D spectrophotometer. The pH was measured at room temperature on either an Orion model 601A digital ionalyser or a Radiometer PHM64 pH meter. S- and Q-band e.p.r. measurements were conducted at the National Biomedical ESR Center in Milwaukee. The S-band spectrometer included a microwave bridge operating at 2–4 GHz and an S-band loop-gap resonator. The X-band spectra were obtained on a Varian E-109 spectrometer. Simulations of the e.p.r. spectra were run on either a PDP11/34 or CDC-6600 computer with a program obtained from J. R. Pilbrow (Toy *et al.*, 1971) and modified in our laboratory.

RESULTS AND DISCUSSION

The g values and A values in the spectrum of MDL are in good agreement with those of the type-2 site in native laccase; however, many tetragonal complexes of copper exhibit similar e.p.r. parameters. Because the type-2 copper of laccase has an unusually high affinity for fluoride (Morpurgo *et al.*, 1983; Malkin *et al.*, 1968), fluoride-binding studies provide a further test of the integrity of the type-2 centre in MDL. Fig. 1 shows the X-band spectra of native laccase and MDL in the presence of excess fluoride at -150°C . Both spectra exhibit complex structure, and this is especially true of the native sample, because the spectra of the type-1 and type-2 copper are superposed. However, it is apparent from Fig. 1(b') that the $m_I = -\frac{3}{2}$ transition in the parallel region of the signal from the type-2 copper (the transition at the low-field end of the spectrum) is split into a multiplet with an intensity pattern of approx. 1:2:1, when excess fluoride is present. Similar splitting occurs in the spectrum of the e.p.r.-active copper of MDL (Fig. 1c'). At Q-band the fluoride hyperfine splitting is more clearly resolved (Fig. 2). When 1.5 equivalents of fluoride are added to the protein at pH 6 (Fig. 2a), the low-field lines of the predominant species are split into doublets,

Fig. 1. X-band e.p.r. spectra of laccase derivatives

(a) Native laccase; (b) native laccase in the presence of 0.1 M-fluoride; (b') a 4-fold amplification of (b); (c) the mercury derivative of laccase in the presence of 0.1 M-fluoride; (c') a 2-fold amplification of (c). Experimental conditions: microwave frequency = 9.08 GHz; microwave power = 40 mW; modulation amplitude = 0.1 mT; temperature = -150°C .

indicating coupling to a single ^{19}F nucleus ($I = \frac{1}{2}$). When excess fluoride is added (Fig. 2b), a 1:2:1 splitting pattern is observed in this region, indicating that two ^{19}F nuclei are bound in equatorial positions of a tetragonal Cu(II) center. Fluorine hyperfine splitting is also evident in the high-field, or perpendicular, region of the spectrum. The corresponding splitting is not well resolved in Fig. 2(a), perhaps because of overlap with the signals from other species, e.g. uncomplexed MDL.

Both $2s$ and $2p$ orbitals of fluoride overlap the metal's $d_{x^2-y^2}$ orbital, the orbital which contains the unpaired spin. Accordingly, the fluorine hyperfine interaction is anisotropic. Since the geometry is unknown and the principal axes of the ligand hyperfine tensor can be assumed to be rotated with respect to those of the g tensor, no attempt to simulate the spectra in Fig. 2 has been made. However, the ligand hyperfine splitting of the bis-fluoride adduct is readily measured in the low-field region, $A^F = 65(5) \times 10^{-4} \text{ cm}^{-1}$. (In a planar co-ordination environment, this would correspond to the perpendicular component of the ligand hyperfine interaction if the symmetry is axial.) The corresponding splitting of the

Fig. 2. Low-temperature Q-band e.p.r. spectra of MDL in the presence of fluoride at pH 6.0

(a) MDL + 1.5 equivalents of fluoride; (a') same spectrum at higher gain; (b) MDL + excess fluoride ($100\times$); (b') same spectrum at higher gain. Experimental conditions: microwave frequency = 35.1 GHz; microwave power = 10 dB; temperature = -150°C .

Fig. 3. X-band e.p.r. signal of MDL

The bottom spectrum is the experimental spectrum in phosphate, pH 6. Instrumental settings were as in Fig. 1, except the microwave frequency was 9.079 GHz. The top spectrum is a computer simulation on the basis of the g values in Table 1. Other parameters are $w_x = 1.0$ mT, $w_y = 1.5$ mT, $w_z = 1.8$ mT, $A_x^{\text{Cu}} = A_y^{\text{Cu}} = 1.5$ mT and $A^{\text{N}} = 1.5$ mT.

fungal laccase derivative appears to be somewhat smaller, $A^{\text{F}} = 59 \times 10^{-4} \text{ cm}^{-1}$ (Malkin *et al.*, 1968), but the difference is probably not significant in view of the error inherent in the field measurements. As measured at Q band, the g_z value of MDL increases from 2.25 to 2.28 on binding two fluoride ions. A similar effect has been observed with fungal laccase and has been attributed to the ionic character of fluoride binding (Malkin *et al.*, 1968). π -bonding effects may also be a factor.

Together with g and A^{Cu} comparisons, the fluoride-binding studies establish the integrity of the type-2 site in MDL, hence a detailed analysis of the e.p.r. spectrum is in order. Ligand hyperfine splitting is resolved in the perpendicular region of the X-band spectrum (Fig. 3). As this structure is retained in $^2\text{H}_2\text{O}$, interactions involving nitrogen-containing ligands or non-exchangeable protons are indicated. Unfortunately, in this region the parallel and perpendicular components of the e.p.r. spectrum overlap and the copper hyperfine structure is not well resolved. Therefore, simply counting the number of resolved lines is not necessarily a reliable guide to the number of nuclei involved. In the low-field region of the spectrum, the copper hyperfine structure is well resolved, but the ligand hyperfine structure is not.

The ligand hyperfine structure might be expected to be better resolved at lower frequencies (S-band) where g -strain broadening is decreased. These effects arise when small differences in the local environment of the paramagnetic ion occur in the sample. The resulting distribution in the ligand field splittings produces a spread in the g values and, hence, a heterogeneous broadening that obscures weak hyperfine interactions (Hyde & Froncisz, 1982). Theory predicts and experiments confirm that, at S-band frequencies, the ligand-hyperfine interactions of Cu(II) complexes are most easily analysed on the $m_I = -\frac{1}{2}$ transition of the copper hyperfine multiplet in the low-field, or parallel, region of the e.p.r. spectrum (Froncisz & Aisen, 1982; Antholine & Taketa, 1982; Taketa & Antholine, 1982; Antholine *et al.*, 1984).

Fig. 4 shows that the ligand hyperfine splitting is resolved at an S-band frequency. Three lines are clearly resolved on the low-field side of the central line, implicating at least a seven-line pattern. If the ligand hyperfine splitting is due solely to nitrogen atoms co-ordinated to copper, donor types which have previously been implicated (Mondovi *et al.*, 1977; Woolery *et al.*, 1984), the multiplet pattern requires the presence of at least three nitrogen donors. The simulation of the $m_I = -\frac{1}{2}$ transition is better if we assume there are 3, as opposed to 4, nitrogen atoms (Fig. 4), especially on the low-field side of the multiplet, where the asymmetry caused by strain phenomena is less pronounced (Rakhit *et al.*, 1985). As shown in Fig. 3, the ligand hyperfine structure in the perpendicular region of the X-band e.p.r. spectrum of MDL is also accounted for by the inclusion of 3 nitrogen atoms. Here the best fit is obtained when slightly rhombic g values are used, and the optimum parameters are listed in Table 1. The fact that g_x and g_y are not resolved in the Q-band e.p.r. spectrum of MDL sets a limit on the in-plane anisotropy such that $|g_x - g_y| < 0.02$. Until now, simulations of the type-2 copper signal have assumed axial symmetry (Reinhammar, 1984; Morpurgo *et al.*, 1973; Malmström *et al.*, 1970) because the signal from the type-2 copper of the native enzyme is not resolved in the perpendicular region of the spectrum.

In general, the simulations based on three equivalent nitrogen atoms give a reasonable fit to the experimental spectra; the disagreements may in large part reflect inadequacies in the theory employed. Thus we did not allow for strain artifacts, for possible misalignment of the g and A tensors or for unresolved splittings from other nuclei, e.g. non-exchangeable protons. A further complication is that signals from other paramagnetic species, e.g. a half-met type-3 site, may also be present. (Double

Fig. 4. The $m_1 = -\frac{1}{2}$ component of the S-band e.p.r. spectrum of MDL

Microwave frequency = 3.39 GHz; power = 12 dB; temperature = -150°C . ---- and show spectra simulated on the basis of three and four equivalent nitrogen atoms respectively; —, the spectrum obtained experimentally. The coupling constant assigned to nitrogen was 1.2 mT.

Table 1.

The g_x and g_y values were determined from simulations.

Frequency (GHz)	g_z	g_x	g_y	$10^4 \times A_z^{\text{Cu}} $ (cm^{-1})
2.54	2.249	2.045	2.060	197
9.08	2.245	2.045	2.060	200
35.1	2.254	2.050	2.065	199

integrations of MDL signals typically reveal 1.1 ± 0.2 e.p.r.-active copper atoms.) On the other hand, the fact that the agreement is imperfect means that other models, e.g. one that invokes splitting from non-exchangeable protons, are not entirely excluded. However, as discussed below, g -value comparisons are also suggestive of a donor set containing three nitrogen atoms.

Parenthetically, we note that different A^N values have been used in the simulations presented in Figs. 3 and 4. This is necessary because of anisotropy in the ligand hyperfine interaction, but, since the geometry is unknown, the individual components of the hyperfine tensor cannot be resolved.

Water is expected to be a ligand for the type-2 copper in MDL, as the presence of this group has been established for the native system (Deinum & Vännngård, 1975; Brändén & Deinum, 1977). Although splitting from the proton is not resolved, Fig. 5 shows that at the S-band the $m_1 = -\frac{1}{2}$ peak is broader in H_2O than in $^2\text{H}_2\text{O}$. This implies that at least one of the ligands, presumably water, is characterized by an exchangeable proton. Consistent with this argument, the resolved $g_z = 2.245$ value is in keeping with an in-plane N_3O donor set (Peisach & Blumberg, 1974; Barbucci & Campbell, 1976). Model studies have shown that, when two nitrogen atoms are present, g_z tends to lie in the range 2.27–2.29. In complexes with three nitrogen atoms, g_z is in the range

Fig. 5. Effect of solvent on the $m_1 = -\frac{1}{2}$ transition in the spectrum of MDL

Experimental conditions: microwave frequency = 2.53 GHz; microwave power = 6 dB; temperature = -150°C . Solvent: —, $^2\text{H}_2\text{O}$; - - -, H_2O .

2.23–2.25, and complexes with four nitrogen atoms typically have still lower g_z values (Barbucci & Campbell, 1976).

Altogether, the ligand hyperfine analyses, the g -value comparisons and the solvent deuteration studies argue strongly for an in-plane N_3O donor set which includes a water ligand. However, it should be noted that this conclusion pertains to the structure present at -150°C . This structure is not necessarily retained at room temperature; see below.

A previous structural proposal, based in part on n.m.r. studies, has been presented by Pecht and co-workers. They inferred that the equatorial donor set included a water ligand and an ROH ligand (from a serine or a threonine residue) as well as two histidine nitrogen atoms (Goldberg *et al.*, 1980). The ROH ligand was invoked to

account for the fact that the pH-dependence of the solvent relaxation rate, in the presence of protein, had two distinct protonation events, with pK values of 6.2 and 8.6. However, it seems quite unlikely that the same Cu(II) centre could support the heterolysis of two O-H bonds at such low pH values. More likely, the pH-dependence of the solvent relaxation reflects the water ligand and another group, perhaps one in the vicinity of the type-2 site, which influences the exchange rate between protons on the bound water and the bulk solvent (Pesando, 1975; Bertini *et al.*, 1978). Alternatively, two forms of laccase could be present in solution (Penner-Hahn *et al.*, 1984).

Pecht and co-workers also noted that the presence of fluoride had little effect on the room-temperature solvent relaxation, even though the electronic absorption spectrum indicated that fluoride was bound to the protein (Goldberg *et al.*, 1980). They concluded that fluoride binds axially. More recently, Morpurgo *et al.* (1985) have shown that, if fluoride binds at room temperature, it does not affect the e.p.r. spectrum of the type-2 copper.

In contrast the low-temperature e.p.r. experiments unequivocally demonstrate that two fluoride ligands bind in equatorial positions, since only equatorial donors give rise to significant hyperfine interactions in Cu(II) systems with a $d_{x^2-y^2}$ ground state (McMillin *et al.*, 1976). Since the type-2 copper centre retains tetragonal symmetry, fluoride binding must cause the dissociation of two of the original in-plane ligands, or relegate them to axial binding sites, if the orbital containing the unpaired electron is fixed in space at -150°C . Alternatively, the binding of fluoride may proceed with a concomitant reorientation of the tetragonal axis, in which case the g tensor will be rotated as well. Either way, a temperature-dependent structural reorganization of the type-2 site must attend the formation of the fluoride adduct at -150°C . This reorganization may be linked with the conformational transition which has previously been invoked to explain the marked temperature-dependence of the e.p.r. signal of laccase (Morpurgo *et al.*, 1981, 1983, 1985).

On the other hand, the temperature-dependence of fluoride binding may be inherent in the equilibrium constant for reaction. Studies have shown that in water the apparent equilibrium constant for the binding of polar substrates to transition-metal complexes can be several orders of magnitude larger (approx. 10^5 times) in frozen solution than it is in room-temperature solution (Murray & Margerum, 1982). Although this phenomenon is not well understood, changes in solvation as a function of temperature may be involved. In principle, a temperature-dependent binding equilibrium could induce the structural transition which is proposed to occur in laccase at low temperatures. Further work is needed if the basis of the temperature-dependence of the e.p.r. spectrum is to be understood in detail.

Conclusions

The fluoride-binding studies described herein provide convincing evidence that the type-2 copper centre of the mercury derivative (MDL) is analogous to the type-2 copper of native laccase, at least at -150°C . The ligand hyperfine structure resolved in the S-band e.p.r. spectrum of the mercury derivative and solvent-deuteration studies suggest that the equatorial plane of the tetragonal copper centre consists of an N_3O donor set at this temperature. The type-2 copper site exhibits a slightly rhombic e.p.r.

spectrum, a finding which is completely compatible with the proposed donor set. Finally, we have pointed out that the binding of fluoride at low temperatures requires a structural reorganization of the type-2 copper site. This reorganization may be linked with a conformational transition which is proposed to occur in laccase on cooling; however, the temperature-dependence of fluoride binding may be dictated by other factors.

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