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Type 2-depleted fungal laccase

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Although copper is quantitively removed from fungal laccase (*Polyporus versicolor*) by extended dialysis against high concentrations of cyanide, we have been unable to reconstitute the protein by addition of Cu(I) ions. However, two new methods for reversibly removing the type 2 Cu centre have been developed. The visible absorption at 610 nm, which is attributable to type 1 Cu, is unaffected by the precedure, but the absorbance of the type 3 Cu at 330 nm is decreased by 60 ± 10 %. The decrease is due, at least in part, to partial reduction of the binuclear type 3 centre, although there may be some change in the molar absorptivity of the oxidized chromophore as well. The change in the c.d. spectrum that occurs at approx. 350 nm may be explained in the same way, but it may also reflect the loss of a signal due to the type 2 Cu. Upon removal of the type 2 Cu an absorbance increase appears at approx. 435 nm, and it is assigned to the semi-reduced form of the type 3 pair. In the e.p.r. spectrum of the type 2-depleted enzyme the type 1 Cu signal exhibits well-resolved ligand hyperfine splitting, which can be simulated on the basis of contributions from two N and two H nuclei ($A^{H} \simeq A^{N} \simeq 25$ MHz). The H atoms are assumed to be attached to the β-carbon of the covalently bonded cysteine ligand. A signal from a semi-reduced form(s) of the type 3 site can also be resolved in the spectrum of the type 2-depleted enzyme, and on the basis of the second integral of the e.p.r. spectrum 40 % of the type 3 pairs are believed to be in a partially reduced state. The semi-reduced type 3 site is remarkably stable and is not readily oxidized by H_2O_2 or $IrCl_6^{2-}$ or reduced by $Fe(CN)_6^{4-}$. Intramolecular electron transfer is apparently quite slow in at least some forms of the type 2-depleted enzyme, and this may explain why the activity is at best 5 % of that of the native enzyme. Full activity returns when type 2 copper is restored.

INTRODUCTION

Laccase is a polyphenol oxidase which is found in certain plants and fungi. The fungal enzyme occurs in wood-rotting organisms and may be involved in lignin breakdown as well as certain invasive processes [1]. Fungal laccase oxidizes a variety of substrates, including polyphenols, via one-electron steps while it reduces O_2 to water, apparently without the release of partially reduced forms of O_2 [2,3]. To accomplish this the enzyme contains four Cu ions which are distributed among three different binding sites [4]. Of these, the type 1 Cu centre is responsible for the intense blue colour of the enzyme and a partially resolved e.p.r. signal with a narrow hyperfine splitting in the $g_{\parallel}$ region [4,5,6]. Like type 1 Cu, the type 2 Cu centre functions as a one-electron acceptor and also contributes to the e.p.r. spectrum [4]. The other two Cu ions are found in the type 3 site, which functions as a two-electron acceptor [4,7]. This site exibits an optical absorbance at 330 nm in its oxidized form, but is e.p.r.-silent in the oxidized and reduced forms [4,5]. However, an e.p.r. signal has been reported for a semi-reduced form of the type 3 site [8].

As a means of further characterizing fungal laccase, we have been interested in preparing chemically modified forms by removal and/or replacement of specific metal centres. This strategy has been effective in studies of other copper enzymes; for example, it has been possible to prepare a mixed-metal derivative of tree laccase, where the type 1 Cu is selectively replaced by Hg [9]. Previous studies of fungal laccase have also provided motivation. Thus Iwasaki *et al.* have reported that the apoenzyme can be partially reconstituted by incubation with CuCl [10], and Malkin *et al.* have described a method for reversibly removing the type 2 Cu centre [11]. In spite of the previous report, we have found that the type 1 and type 3 Cu centres are difficult to remove from fungal laccase. Although extended dialysis against high concentrations of cyanide does finally remove all Cu, incubation with Cu(I) or Cu(II) does not lead to reconstitution. In the course of our work, we have, however, developed new methods for removing the type 2 Cu centre, and we now report on the properties of the resulting derivative in detail.

EXPERIMENTAL

Materials

Laccase from *Polyporus versicolor* (chromatographic fraction A) was obtained by the method of Fåhraeus & Reinhammar [12]. Protein from two independent isolations was used. The ratios of the absorbance at 280 nm to that at 610 nm were 19 and 20 in the respective prepa-

Abbreviation: e.n.d.o.r., electron–nuclear double resonance.

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rations, both of which were derived from a ^{63}Cu-enriched medium. Stellacyanin was extracted from the acetone powder of latex from the Chinese lacquer tree (Saito and Co., Osaka, Japan) by the literature method [13].

All reagent-grade chemicals were used without further purification. L-Ascorbic acid (sodium salt) and guanidine hydrochloride were obtained from Sigma Chemical Co. Bathocuproinedisulphonic acid (disodium salt), hydroquinone (> 99% pure), and thiourea were obtained from Aldrich Chemical Co. KCN was a product of J. T. Baker Chemical Co., and $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$ were products of Mallinckrodt.

Methods

All buffers were prepared from deionized water and were passed through columns of (Bio-Rad) Chelex 100 resin (pre-equilibrated with the same buffer) to remove trace metal ions. Glassware was soaked for at least 12 h in strong acid. Completely demetalated laccase was prepared by anaerobic dialysis against 0.2 M-KCN at pH 10.3 for 48 h. We also tried dialysis against 0.2 M-KCN at pH 8 and pH 9.5, but not all of the Cu was removed even after 48 h. Dialysis of native laccase for 3–20 h against 0.10 M-imidazolium acetate buffer, pH 7.0, containing 50 mM-KCN and 1 mM-sodium ascorbate removed one Cu atom. Alternatively, one of the four Cu atoms could be removed by dialysis for 20 h against 0.05 M-ammonium acetate buffer, pH 4.0, containing 0.1 M-thiourea, 0.25 M-NaCl and 5 mM-sodium ascorbate. All dialyses were carried out at 5 °C under N_2 in a custom-built hollow-fibre device. Reconstituted protein was obtained by incubating the partially demetalated protein for 2 h at 5 °C with 2–3 equivalents of Cu in 0.5 M-sodium acetate buffer, pH 5.5, containing 0.1 M-NaCl and excess sodium ascorbate. (Ascorbate was present during reconstitution, since we have obtained better uptake of Cu by *Rhus vernicifera* laccase when the cuprous form was used [9].) In an attempt to produce the semi-reduced type 3 Cu site, 2 equivalents of ferrocyanide were anaerobically added to the partially demetalated protein [8].

Before analysis, protein samples were dialysed against 0.1 M-phosphate buffer, pH 6.0. Cu was determined spectrophotometrically by the method of Felsenfeld [14], and the protein concentration was determined by the biuret method [15] calibrated on the assumption that laccase contains exactly 4 Cu ions. With the same assumption we determined an apparent molar absorption coefficient of 82000 $M^{-1} \cdot cm^{-1}$ for the protein at 280 nm. Absorbance data were collected with a 1 cm path length, and the samples were typically centrifuged or filtered (0.22 μm pore size) before the measurement. The spin concentration was determined by double integration of the e.p.r. signal. A calibration curve was determined using a series of oxidized stellacyanin samples as standards, where the Cu concentrations were determined by the method described above.

Enzyme activities were measured with an oxygen electrode at 25 °C. The substrate was 2 mM- or 4 mM-hydroquinone in 0.1 M-sodium phosphate buffer, pH 6.0. Fresh solutions of hydroquinone were prepared before each experiment. The activity was expressed as the change in the percentage of dissolved O_2/min per mmol of protein.

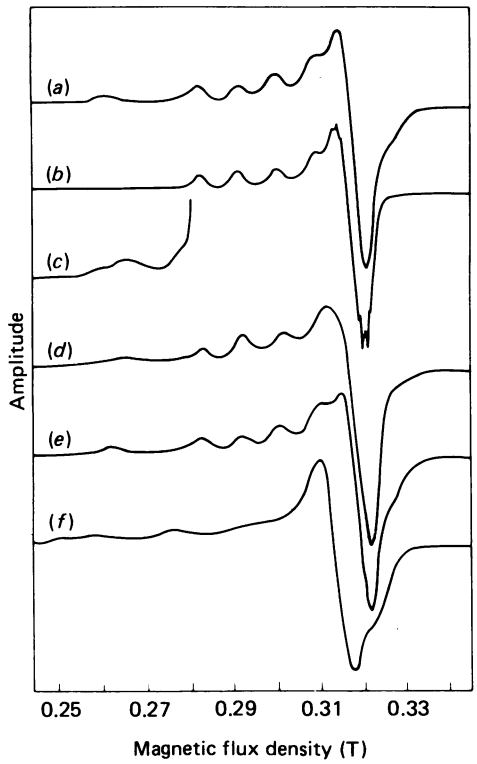

Fig. 1. X-band e.p.r. spectra of laccase derivatives in 0.1 M-phosphate buffer, pH 6.0

(*a*) Native laccase; (*b*) type 2-depleted laccase; (*c*) spectrum (*b*) at modulation amplitude of 1.0 mT; (*d*) type 2-depleted laccase by method of ref. [11]; (*e*) reconsitituted type 2-depleted enzyme; (*f*) 'reconstituted' apolaccase. Experimental conditions except as noted: microwave frequency 9.08 GHz; microwave power 40 mW; modulation amplitude 0.1 mT; temperature −150 °C.

Instrumentation

Visible and u.v. absorbance measurements were taken with a Cary 17D or a Perkin–Elmer Lambda 4C spectrophotometer. C.d. spectra were taken with a Cary 60 spectrophotometer. The oxygen electrode was a model 53 from Yellow Springs Instruments. The X-band e.p.r. spectra were recorded on Varian E-109 systems operating at around 9.1 GHz, either at Purdue University or at the National Biomedical E.S.R. Center at the Medical College of Wisconsin. S-band (2.4 GHz) data were recorded at the E.S.R. Center using a bridge designed and constructed at the Center. Spectra were obtained at both 77 K and 123 K. At the E.S.R. Center microwave frequencies were measured with an EIP model 331 counter, and the magnetic flux density was calibrated with an MJ-11OR Radiopan n.m.r. magnetometer.

A Masscomp 5600 computer performed the simulations described below. The simulation program [16,17] was obtained from J. R. Pilbrow, Monash University, Clayton, Vic., Australia, and was modified to include separate tensors for g values and the A^{Cu}, $A^{N}(1)$, $A^{N}(2)$, $A^{H}(1)$ and $A^{H}(2)$ hyperfine interactions, where the symbol $A^{X}(I)$ denotes the interaction with the I^{th} nucleus of type X. A Monte Carlo method was employed to minimize χ^2, which was calculated with respect to the experimental spectrum, by randomly varying spectral parameters within defined limits [17]. Visual inspection

Table 1. Analytical data

N, native protein; T2D, sample of type 2-depleted protein prepared by dialysis against 50 mM-CN^-, R, reconstituted form of T2D protein.

Sample no.	Total Cu (mol/mol of protein)	E.p.r.-active Cu (mol/mol of protein)	A_{280}/A_{610}
N 1	3.9	1.9	20
N 2	3.9	—	19
T2D 1	3.0	1.4	21
T2D 2*	3.0	1.2	22
T2D 3	3.1	1.3	22
T2D 4*	2.8	1.3	22
T2D 5	2.9	—	20
T2D 6*†	2.9	—	20
R 1	3.9	—	27
R 2	4.2	2.0	20

* Treated with peroxide.
† Prepared by the method of ref. [11].

of the fit is the final criterion for a good simulation. Initial g-values were taken from the literature [2]. Coupling to N and H nuclei was assumed on the basis of e.n.d.o.r. studies [18]. A_z^{Cu} and $A^N(I) = A^H(I)$ were estimated from the experimental data.

RESULTS

Preparation and product analysis

Although prolonged exposure to cyanide at relatively high pH removes all of the Cu from laccase, we have been unable to reconstitute the protein. When incubated in the presence of excess Cu(I), apolaccase took up about 2.6 mol of Cu/mol of protein, but there is no indication that significant amounts of Cu reside in native binding sites. In particular, the remetalated protein exhibits no appreciable absorbance at 610 nm, and the e.p.r. signals are quite distinct from those of either the type 1 or the type 2 Cu (compare Figs. 1*a* and 1*f*).

On the other hand, dialysis against 50 mM-cyanide at pH 7 for a few hours removes 1.0 ± 0.1 mol of Cu/mol of protein, and in this case the protein can be reconstituted. Time-resolved studies show that the Cu is lost within 3 h at pH 7 and 5 °C and that little or no additional Cu loss occurs if the dialysis against cyanide is continued for up to 20 h. As discussed below, the Cu is removed from the type 2 site, hence we refer to this derivative as type 2-depleted laccase. This same derivative can also be prepared by extensive dialysis against 0.1 M-thiourea and 0.25 M-NaCl in ammonium acetate pH 4, as indicated by spectral comparisons. The presence of both thiourea and Cl^- appears to be required for Cu removal.

In an attempt to purify the type-2 depleted sample, we adopted an ion-exchange procedure based on that used for the native enzyme [12]. The type 2-depleted protein elutes from an anion-exchange column as a single band when a concentration gradient of phosphate buffer, pH 6.0, is passed through the column (0.1–0.5 M). A small fraction of the type 2-depleted samples is eluted separately at high phosphate concentration, but native protein exhibits the same behaviour. This 'impurity', which

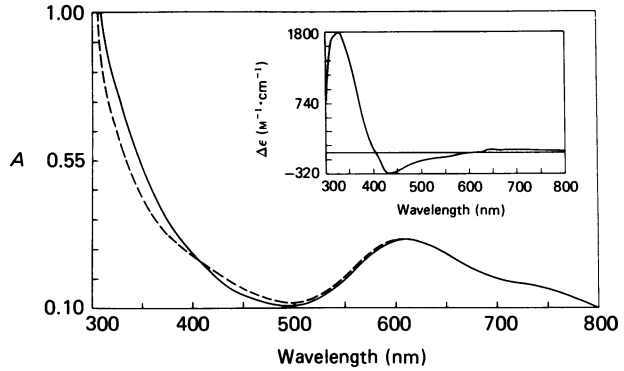

Fig. 2. Absorption spectra at 25 °C in 0.1 M-phosphate buffer, pH 6.0

——, Native laccase; – – – –, type 2-depleted laccase. Protein concentration for both samples is 69 μM. Inset: difference spectrum of the absorption spectra shown where the baseline represents a solvent blank.

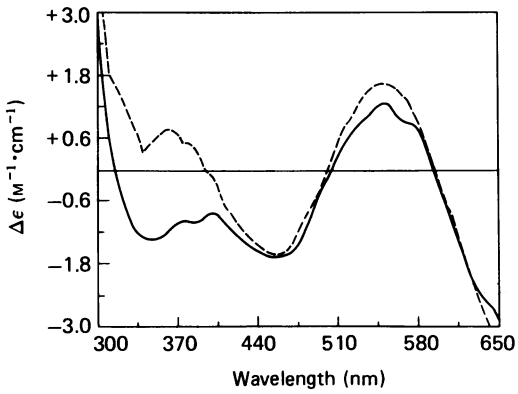

Fig. 3. C.d. spectra at 25 °C in 0.1 M-phosphate buffer, pH 6.0

– – – –, Native laccase; ——, type 2-depleted laccase. Protein concentration for both samples is 210 μM.

contains type 1 and type 2 Cu was not discovered until most of the extraction had been utilized. Fortunately, the second component is a minor one, and its presence does not significantly affect our analysis.

As noted previously, the type 2-depleted samples can be reconstituted. Two seperate preparations of the remetalated enzyme contained 3.9 and 4.2 Cu atoms per molecule (Table 1). Moreover, the native spectral properties were restored. Figs. 1(*a*) and 1(*e*) show how closely the e.p.r. spectra of reconstituted protein matches that of native. Although the type 2-depleted sample has only about 5% of the activity of the native enzyme, the reconstituted protein is fully active.

Electronic spectra

The type 1 site remains intact upon removal of the type 2 Cu, and, as monitored by the absorbance at 610 nm, is fully reoxidized within 40 h after exposure to air at 5 °C. It is obvious from the difference spectrum (Fig. 2 inset) that the molar absorptivity at 610 nm (ϵ_{610}) is the same for the native and the type 2-depleted proteins. On the other hand, Fig. 2 reveals that the absorbance shoulder at 330 nm is decreased in intensity in the type 2-depleted protein. The reoxidation of the type 3 Cu occurs more

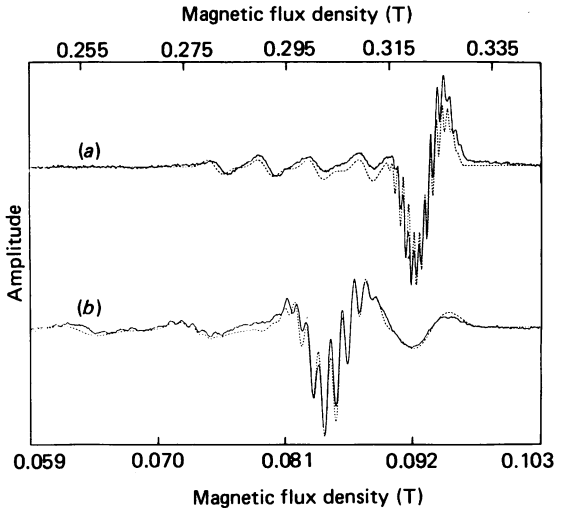

Fig. 4. Multifrequency e.p.r of type 2-depleted laccase in 0.1 M-phosphate buffer, pH 6.0

(*a*) (upper axis), Second derivative spectrum (——) of type 2-depleted laccase at 9.15 GHz from an average of four scans, and simulation (------) with hyperfine splitting from two N atoms and two H atoms; see Table 2 for parameters. Experimental conditions: microwave power 5 mW; modulation amplitude 0.5 mT; modulation frequencies 100 kHz and 1 kHz; temperature −150 °C. (*b*) (lower axis), Second derivative spectrum (——) of type 2-depleted laccase at 2.38 GHz from an average of four scans, and simulation (------) with hyperfine splitting from two N atoms and two H atoms; see Table 2 for parameters. Experimental conditions: microwave power 10dB; modulation amplitude 0.5 mT; modulation frequencies 100 kHz and 1 kHz; temperature −150 °C.

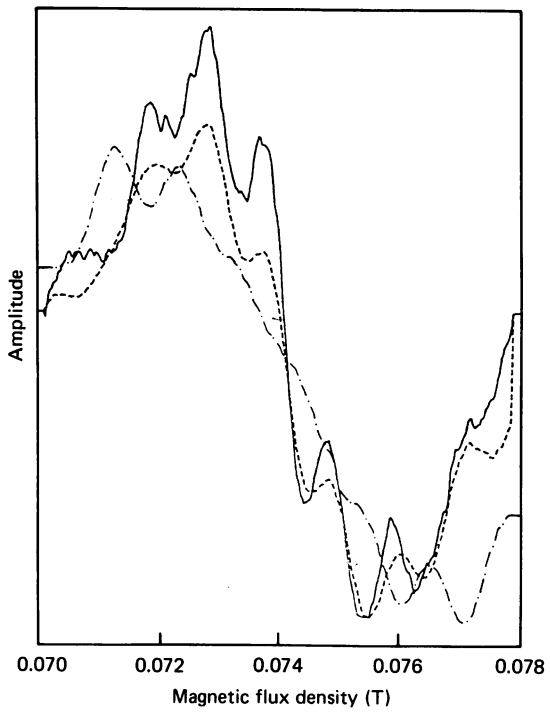

Fig. 5. $M_I = -\frac{1}{2}$ component of type 2-depleted laccase (presented as the second derivative signal obtained at 2.38 GHz)

The experimental (——) is an average of 50 scans of protein dissolved in 0.1 M-phosphate buffer, pH 6.0. A seven line pattern is clearly seen in $g_{\parallel}$ and is simulated (------) using two N atoms and two H atoms of approximately equal coupling constants (see Table 2 for parameters) and (–·–·–) using values from ref. [18]. Experimental conditions: microwave power 16 dB; modulation amplitude 0.5 mT; modulation frequencies 100 kHz and 1 kHz: temperature −150 °C.

slowly than that of the type 1 Cu because the absorbance at 330 nm, which is the signature of the oxidized form of the type 3 Cu, is still changing after 40 h at 5 °C. The 330 nm band of the type 2-depleted protein levels off when it is about 40 ± 10 % as intense as that of the native protein. Exposure to oxidizing agents such as ferricyanide or hexachloroiridate(IV) gives no further enhancement, whereas treatment with 3 equivalents of H_2O_2 led to a slight decrease in the absorbance at 330 nm. No chemical change in the type 3 Cu has occurred because the 330 nm absorbance is completely restored in the remetalated protein. Fig. 2 also reveals that there is a diminished absorbance above 650 nm for type 2-depleted protein relative to native. Finally, there is a new absorbance band at around 435 nm ($\Delta\epsilon \geqslant 320 \pm 50$ $M^{-1} \cdot cm^{-1}$) in the spectrum of the type 2-depleted protein which is not observed in the spectrum of native laccase.

The c.d. spectra of the native and the air-oxidized type 2-depleted proteins are shown in Fig. 3. The band positions obtained for native fungal laccase agree very well with a previous report [19], which attributed the visible transitions to the type 1 Cu. Although the c.d. spectra in Fig. 3 agree fairly well in the visible region, the c.d. spectrum of the type 2-depleted protein deviates strongly from that of the native enzyme in the near u.v. region, at around 350 nm.

E.p.r. spectrum of type 2-depleted laccase

The e.p.r. spectrum of the type 2-depleted protein is presented in Fig. 1(*b*). It shows that the e.p.r. signature of the type 2 Cu has vanished and that the spectrum is dominated by the signal from the type 1 Cu. A virtually identical signal is obtained when the type 2 Cu is removed by dialysis against thiourea and Cl^-. For comparison, the e.p.r. spectrum of the type 2-depleted derivative, prepared by the method of Malkin *et al.* [11], is presented in Fig. 1(*d*).

Double integration of the e.p.r. spectrum of a type 2-depleted preparation typically yields a value of 1.3–1.4 for the number of active spins per molecule of protein. Consistent with these results, careful examination of the e.p.r. spectrum reveals a second, rather broad, signal underlying the type 1 Cu signal. One component can clearly be seen in the low-field region of the spectrum by increasing the modulation amplitude (Fig. 1*c*). This signal appears to be very similar to that of the semi-reduced type 3 site [8]. Treatment of the protein with H_2O_2 does not seem to affect this signal significantly. Thus samples 2 and 4 of the type 2-depleted protein in Table 1 integrated for 1.2 and 1.3 mol of e.p.r.-active Cu(II)/mol of protein after addition of 1 and 3 protein equivalents of peroxide respectively. In an attempt to enhance the concentration of the semi-reduced type 3 site, we anaerobically treated the type 2-depleted protein with excess ferrocyanide. Although this procedure abolished the e.p.r. spectrum of the type 1 Cu, there was little, if any change in the intensity from the other component.

Table 2. Spin Hamiltonian parameters

S-band ligand hyperfine coupling constants in parentheses correspond to a second minimum in the error surface. The other minimum was chosen since the same fit is obtained at X-band.

Source	Nucleus	g_x	g_y	g_z	A_x (MHz)	A_y (MHz)	A_z (MHz)
X-band	^{63}Cu	2.028	2.054	2.190	22.5	19.5	270
	N(1)				27	23	25
	N(2)				27	26	24
	H(1)				27	26	25
	H(2)				27	23	25
S-band	^{63}Cu	2.038	2.062	2.190	18.0	17.7	273
	N(1)				26	24 (40)	25
	N(2)				25	27	25
	H(1)				26 (16)	28	24
	H(2)				26	28 (15)	24
Previous work	^{63}Cu	2.033*	2.051*	2.190*			270†
	N(1)						38
	N(2)						24
	H(1)						30
	H(2)						25

* Ref. [2]
† E.n.d.o.r. results [18].

Perhaps the most striking feature of the e.p.r. spectrum of the type 2-depleted derivative is the resolved superhyperfine structure. This structure is, by comparison, very poorly resolved in the spectrum shown in Fig. 1(*d*) (See also Fig. 2 in ref. [11].) The second derivative presentation greatly enhances the resolution of sharp peaks with respect to broad peaks and provides a more useful display (Fig. 4). As expected on the basis of previous work at S-band frequencies [20], the superhyperfine structure in the parallel region is best resolved on the $M_I = -\frac{1}{2}$ line of the Cu hyperfine. The spectra have been simulated with the assumptions of rhombic symmetry and superhyperfine contributions from two N ligands and two H atoms (see below). Fig. 5 shows the $M_I = \frac{1}{2}$ signal of the $g_{\|}$ region at S-band in expanded scale for both the simulated and experimental spectra. Parameters for the best-fit simulations are given in Table 2. Although the simulated spectra match the experimental spectra quite well, there are slight discrepancies in some of the peak positions and intensities. Several factors may be responsible for the disparities, including instrumental artifacts due to overmodulation and rapid passage effects. In addition, the corrections for the instrumental field settings may have been inadequate in the parallel region where large adjustments were necessary, or certain assumptions in the calculations, such as that of a Gaussian line shape, may have been faulty. Finally, the contribution of the semi-reduced type 3 Cu signal has been ignored in the calculated spectra.

Fig. 1 suggests that the type 2-depleted laccase sample obtained by the procedure of Malkin *et al.* [11] contains a higher proportion of semi-reduced type 3 Cu, and calculation of the appropriate difference spectrum supports this interpretation. However, the presence of the signal from the type 3 site is probably not responsible for the failure to resolve ligand hyperfine splitting, since samples obtained by the cyanide procedure exhibit ligand hyperfine even if the type 1 Cu is not fully reoxidized and a significant amount of semi-reduced type 3 site is present.

DISCUSSION

Failed reconstitution of apolaccase

The most significant finding with apolaccase is that incubation with Cu does not restore the native binding sites. More precisely, the spectral data demonstrate that the type 1 and type 2 Cu sites are not regenerated. Iwasaki *et al.* [10] have previously reported that the apoenzyme from *Lactarius piperatus* var. *pergameus* can be partially reconstituted by incubation against CuCl; however, this conclusion was apparently based solely on activity measurements. In particular, neither the metal analysis nor the spectral properties of the 'apolaccase' were reported. Since the Cu depletion was effected by 8 h dialysis against 0.1 M-CN^- at pH 7, it seems highly unlikely that all the Cu had in fact been removed.

The fact that the type 1 Cu site of the apoenzyme is not restored is interesting, since this site is readily reconstituted in a variety of other blue Cu proteins [21], including tree laccase [22]. This could imply that the fungal protein adopts a quite different structure when Cu is removed and that this process is not readily reversed. More likely, it may indicate that the protein has been chemically altered during Cu removal. Since CN^- can attack disulphide linkages, particularly at higher pH values [23,24] and since the fungal enzyme contains two disulphide linkages [25], one possibility is that the secondary structure of the protein has been altered. Alternatively, disulphide switching could occur under the conditions used. The latter has been observed as a pH-dependent process in Cu-depleted stellacyanin, although denaturing conditions were required [26]. In any event, the contrasting demetalation/remetalation

behaviour of the tree and the fungal enzymes is remarkable, given the fact that the two types of laccase exhibit so many parallels, e.g., Cu stoichiometry and site distribution, reaction mechanism, etc.

Removal of the type 2 Cu

At pH 7, only one Cu is lost, even after dialysis of up to 20 h against 50 mM-CN^-. Since the same derivative can also be prepared by dialysis against a solution of thiourea and Cl^- at pH 4.0, it seems unlikely that the Cu release is from random sites. On the assumption that it comes from a single site, it must be the type 2 site, as can be shown by a process of elimination. In the first place, the type 1 Cu is clearly retained on the basis of e.p.r. and absorbance data. It is worth noting in passing that the molar absorptivity at 610 nm is the same for type 2-depleted and native fungal laccase. Some investigators have reported that the corresponding absorptivity is also unchanged by removal of the type 2 Cu from tree laccase [27–29]; others report that there is a decrease in the absorption intensity [30,31].

Spectral analysis also confirms that type 3 Cu is present in the partially demetalated derivative of fungal laccase. Clearly, some of the type 3 Cu is present in the fully oxidized form, because the difference spectrum between native fungal laccase and the metal-depleted enzyme shows that 40 ± 10 % of the absorbance shoulder at 330 nm is retained, based on $\Delta\epsilon_{330}$ 3000 $M^{-1} \cdot cm^{-1}$ for the native enzyme [7]. Another 30 ± 10 % of the type 3 Cu exists in the semi-reduced (or half-met) form according to double integration of the e.p.r. spectrum (Table 1). Therefore, 70 ± 20 % of the type 3 Cu can be accounted for experimentally. A final piece of evidence that the type 2 Cu has been selectively removed is the fact that the derivative does not exhibit the type 2 e.p.r. signal, even after treatment with a number of oxidizing agents.

Although all of the type 3 Cu has not been spectroscopically identified; possibly because the absorptivity at 330 nm is decreased in the derivative the various observations described above provide persuasive evidence that our procedure selectively removes type 2 Cu. Stated differently the type 1 and type 3 Cu of fungal laccase appear to be much more difficult to remove than the type 2 Cu. This provides another contrast with the tree enzyme, where copper can easily be removed from all three binding sites by dialysis against cyanide at pH 7 [22].

Electronic absorption and c.d. spectra

In addition to the bands discussed above, there is an absorbance centred at about 730 nm (Fig. 2 inset) that is either absent or of decreased intensity in the type 2-depleted derivative. A recent study has assigned a similar band in the spectrum of *Rhus* laccase to the oxidized form of the type 3 site (R. Tamilarasan & D. R. McMillin, unpublished work). Since the type 2 Cu is absent, and since the 610 nm absorbance is unaffected, the same assignment seems evident here. The absorbance at around 435 nm in the spectrum of the type 2-depleted protein must be attributed to a chromophore that is not present in native laccase, presumably the semi-reduced type 3 site. A transient absorbance at this wavelength has previously been observed when the partially reduced enzyme was oxidized with O_2 [32] and when the native enzyme was exposed to H_2O_2 [33]. Although the latter signals were originally ascribed to a peroxide-bound intermediate, they could, in part, relate to the semi-reduced type 3 site, since it can sometimes be observed under turnover conditions [8,30], and partly to the oxygen intermediate state, which exibits absorption in this spectral region [34].

In the visible region, the c.d. spectrum is dominated by bands from the type 1 Cu centre at 550 nm and 455 nm [19,35]. Similar bands appear in the spectrum of the type 2-depleted enzyme, as shown in Fig. 3. However, the band at 550 nm appears with somewhat diminished intensity. Since the absorbance at 610 nm is unchanged, it seems unlikely that the change in $\Delta\epsilon$ at 550 nm is due to a pertubation of the type 1 Cu centre. Instead, it may indicate that there is a contribution from the type 2 or type 3 Cu at this wavelength. A similar postulation has been offered in a previous report involving studies of F^- and NO binding to both native *Rhus* and *Polyporus* laccases [35]. The relatively large difference between the spectra of native and type 2-depleted fungal laccase in the region of 345 nm presumably reflects the difference in oxidation state of the type 3 site and/or a contribution from the type 2 Cu. If so, there is a positive band in the c.d. spectrum at this wavelength due to the type 2 or the oxidized type 3 site.

E.p.r. signal of the type 1 Cu

There is little precedent for the observation of superhyperfine splitting in the e.p.r. spectra of type 1 Cu systems [3–5]. In our hands, the type 2-depleted derivative obtained by the procedure of Malkin *et al.* [11] does not yield the superhyperfine structure either (Fig. 1*d*). However, if the procedure is modified, and the guanidine hydrochloride and bathocuproinedisulphonate are removed by anaerobic dialysis, a spectrum very similar to that shown in Fig. 1(*b*) is observed.

Although the signal-to-noise ratio is smaller in the parallel region, it is advantageous to analyse the superhyperfine structure in this region, since the metal hyperfine is well resolved. A seven-line superhyperfine pattern is observed at S-band (Fig. 5), but unfortunately it is not possible to identify the donor atoms solely on the basis of hyperfine intensities. Thus the relative intensity pattern for the coupling of three equivalent N atoms is 1 : 3 : 6 : 7 : 6 : 3 : 1, whereas a 1 : 4 : 8 : 10 : 8 : 4 : 1 pattern is predicted if two N atoms and two equivalent protons are present and each nucleus exhibits the same coupling constant. These patterns are too similar to distinguish by simulation methods, although the possibility that only two inequivalent N atoms are present could be excluded.

In accord with recent e.n.d.o.r. results [18] we have assumed that there is coupling to two N atoms and two protons. Table 2 lists the coupling constants obtained from e.n.d.o.r. studies as well as our e.p.r. simulations. Good agreement is obtained between our data and those given in ref. [18] for coupling to the proton. But the coupling constants are large; so large that Desideri *et al.* have suggested that they cast doubt on the assignment [36]. However, theoretical calculations by a many-electron SCF-Xα-SW method suggest that the Cu–thiolate linkage is very covalent [37], hence it should be possible to generate considerable spin density on the β-H atoms of the cysteine ligand by spin polarization as well as directed delocalization. In accord with this assignment, Roberts *et al.* have demonstrated that non-exchangeable protons are involved [18]. The comparison between e.n.d.o.r. results and the e.p.r. data shows less

agreement as regards the N splittings. The e.n.d.o.r. results have been interpreted in terms of dissimilar N atoms, where one of the N atoms exhibits a coupling constant of approx 40 MHz [18]. As can be seen from Fig. 5, our e.p.r. results are inconsistent with this model, and they require the two N atoms to be approximately equivalent. It is worth emphasizing that our results do not uniquely define the nuclei involved; three N atoms would do as well as two N atoms and two H atoms. However, we have been able to refine the coupling constants for this rather novel set of ligand nuclei which have been identified by the e.n.d.o.r. technique [18].

Oxidation chemistry and mechanistic considerations

At least two possible functions have been envisaged for type 2 Cu in laccase, and the sharp decline in enzyme activity that attends removal of the type 2 Cu certainly demonstrates that this centre is involved in the catalytic process. Reinhammar & co-workers have presented evidence that the type 2 Cu has a role in the reduction of the type 3 pair [2,3]. During anaerobic reduction one of the electrons is transferred to the type 3 site via the type 1 site, as evidenced by the steady-state phase in the reduction of the latter. In the proposed mechanism, the type 3 pair is actually reduced via a simultaneous two-electron transfer, where the second electron is supplied by the type 2 Cu. And, in fact, inhibitor studies have revealed that the state of the type 2 Cu profoundly influences the rate of reduction of the type 3 pair [38]. Parenthetically we may note that our results are in keeping with the proposed intramolecular pathway for the reduction of the type 3 Cu site, since this site is not readily accessed by molecules of even moderate size, such as $IrCl_6^{2-}$.

Early on, it was also suggested that the type 2 Cu, along with the type 3 pair, forms the oxygen-reducing site of laccase [32,39]. The proposal was based on the idea that binding to the type 2 Cu could stabilize a putative peroxide intermediate and explain labelling studies, which indicate that one of the O atoms of the O_2 substrate remains bound at the type 2 Cu after combination with reduced laccase [39]. More recently, Reinhammar & co-workers have determined that the spectroscopically detectable reduced oxygen intermediate can formally be regarded as O^-, i.e. it appears after three electrons have been delivered to O_2 [34,38]. In the presence of excess reducing agent the fourth equivalent is channelled via the type 1 site; otherwise, the intermediate decays by a first-order process in which there is concomitant formation of the e.p.r. signal of the type 2 Cu. As the electron transfer between type 2 Cu(I) and the oxygen intermediate is much slower than the turnover rate of this enzyme, this electron-transfer step cannot be of catalytic importance. Reoxidation studies of the type 2-depleted tree laccase [30] have also cast doubt on the role of the type 2 site in the reduction of O_2.

However, Solomon & co-workers have recently rekindled the notion that the type 2 Cu is directly involved in the reduction of O_2 [40]. Their argument is based on spectroscopic evidence that small molecules such as azide (a model for peroxide?) can bridge the type 2 and type 3 Cu sites [41,42] as well as the fact that at least some preparations of type 2-depleted tree laccase retain a fully reduced type 3 pair even in air-saturated buffer [43]. It could be argued that the relatively slow auto-oxidation of the type 1 and type 3 centres in type 2-depleted fungal laccase provides additional evidence that the fully reduced type 3 site is not by itself competent to reduce O_2 efficiently. However, owing to traces of O_2 in our dialysis set-up, the results are not necessarily representative of the reoxidation of fully reduced type 2-depleted enzyme. Mechanistic inferences will have to be based on studies of type 2-depleted laccase that is precisely defined in terms of metal content, oxidation state etc. Reconciling results may not be easy; Pecht & co-workers argue that there are at least three different forms of type 2-depleted tree laccase [44].

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