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Optical Properties of Japanese-Lacquer-Tree (*Rhus vernicifera*) Laccase Depleted of Type 2 Copper(II)

INVOLVEMENT OF TYPE-2 COPPER(II) IN THE 330nm CHROMOPHORE

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1. Spectroscopic and functional properties of Japanese-lacquer-tree (*Rhus vernicifera*) laccase were re-investigated, with special emphasis on the relationships between the different types of copper centres (Types 1, 2 and 3). 2. On removal of the Type 2 Cu(II), a decrease of absorbance occurred in the wavelength region above 650nm ($\Delta\varepsilon_{750} = 300\,\text{M}^{-1}\cdot\text{cm}^{-1}$) and around 330nm ($\Delta\varepsilon_{330}$ up to $2200\,\text{M}^{-1}\cdot\text{cm}^{-1}$). 3. Reductive titrations with ascorbic acid or ferrocyanide showed that the electron-accepting capacity of the partial apoprotein is one electron-equivalent lower than that of the native protein, i.e. the protein two-electron acceptor is present in the oxidized state in spite of absorbance loss at 330nm. 4. The 330nm chromophore apparently depends on the presence of both the Type 2 and the Type 3 copper in the oxidized state. 5. This finding may have implications in the relative location of Type 2 and 3 copper centres and on the redox behaviour of laccase.

The laccase from the Japanese lacquer tree (*Rhus vernicifera*) contains two e.p.r.-detectable copper ions, the blue Type 1 and the Type 2 copper, and a copper pair undetectable by e.p.r., the Type 3 copper (Malmström *et al.*, 1970). The latter Type is likely to consist of antiferromagnetically coupled bivalent ions (Solomon *et al.*, 1976) and has been associated with an absorption band at 330nm (Reinhammar & Vänngård, 1971). This chromophore is titrated by redox reagents either with co-operative exchange of two electrons (Reinhammar & Vänngård, 1971) or with exchange of only one electron, depending on the reagent redox potential (Farver *et al.*, 1976, 1978).

We report in the present paper some new findings concerning the 330nm chromophore, which may help to elucidate its complicated redox behaviour and the fluorescence properties of the enzyme and may throw light on the interaction between the different copper centres determining some of the properties of this class of oxidases.

Materials and Methods

The acetone-dried powder from the latex of *Rhus vernicifera* obtained from Saito and Co., Osaka, Japan, was purified as described by Reinhammar (1970). The Type 2 Cu(II)-depleted protein (partial apoprotein) was prepared by the procedure of Graziani *et al.* (1976), modified as follows: 0.1 mM-laccase samples were dialysed anaerobically against 2mM-dimethylglyoxime and 2mM-potassium ferrocyanide in 0.05M-sodium acetate buffer, pH 5.2. After approx. 8h, 1mM-EDTA was added to the anaerobic solution. After 12h the protein solution was aerobically dialysed against several changes of 0.1M-potassium phosphate buffer, pH 6.0. All the treatment was repeated when removal of the Type 2 Cu(II) was unsatisfactory. Lower protein concentrations were used than in the previous method (Graziani *et al.*, 1976) to increase the dimethylglyoxime/protein ratio as this chelating agent is sparingly soluble in water. EDTA was added before removal of ferrocyanide to avoid some reduction of the partial apoprotein, which may occur in the presence of EDTA and is not reversed by air in the absence of a mediator such as ferrocyanide (Reinhammar, 1972).

The copper content was determined chemically with 2,2′-biquinolyl (Brumby & Massey, 1967). Absorption spectra were obtained with a Cary 14 spectrometer. X-band low-temperature e.p.r. spectra

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were recorded on an E-9 Varian instrument equipped with the Varian variable-temperature accessory. Fluorescence spectra were taken at room temperature with a FICA 55L corrected spectrofluorimeter. Anaerobic titrations were carried out in a fluorimeter cuvette sealed to a Thunberg-type apparatus. Solutions were de-aerated by three cycles of vacuum followed by flushing with purified argon. De-aerated solutions of the reductant were transferred to the reaction cuvette by an air-tight microsyringe through a serological cap.

C.d. spectra were recorded with a Cary 60 spectropolarimeter equipped with a 6001 attachment.

Results

Characterization of the Type 2 Cu-depleted laccase

In Table 1 are reported the relevant analytical data relative to some laccase samples treated as described in the Materials and Methods section. They show that over 40% of the e.p.r.-detectable Cu could be removed by this procedure, causing a significant decrease of A above 650nm and in the 330nm region (Fig. 1). The data of column 3 of Table 1, relative to Type 1 Cu, were calculated from the A_{614}, assuming the absorption coefficient of blue copper to be unchanged in the partial apoprotein, and show that the decrease of e.p.r.-detectable Cu is mainly due to removal of Type 2 Cu. The content of e.p.r.-non-detectable Cu, evaluated by the difference between total and e.p.r.-detectable Cu, was also lower than in the native samples. As the presence of systematic analytical errors is ruled out by comparison with the data relating to native protein samples, it appears that all types of copper were affected by the treatment, with far more extensive removal of Type 2 Cu. Reconstitution with copper under reducing conditions as described by Graziani *et al.* (1976) restored enzyme activity, the content of e.p.r.-detectable Cu and absorbance both at 330nm and above 650nm.

The decrease of A_{330} on removal of Type 2 Cu is greater than that reported by Graziani *et al.* (1976), and is not the same for all samples. This is difficult to explain, especially since the absorbance of native

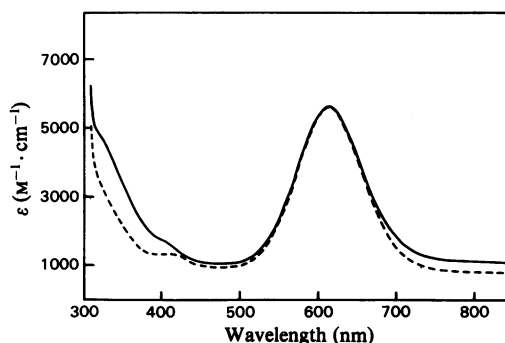

Fig. 1. *Optical-absorption spectra of native and Type 2-Cu(II)-depleted laccase*

0.44mM-Enzyme (from expt. 4 in Table 1) in 0.05M-acetate buffer, pH 5.2, was used. ——, Native laccase; ----, Type 2 Cu(II)-depleted enzyme.

Table 1. *Analytical data relating to samples of Type 2 Cu-depleted laccase*

Protein concentration was calculated from A_{280}, by using ε_{280} 93000$M^{-1}\cdot cm^{-1}$ (Malmström *et al.*, 1970); total copper was determined chemically with 2,2′-biquinolyl (Brumby & Massey, 1967); Type 1 and Type 2 copper was obtained by double integration of e.p.r. spectra, by using Cu–EDTA as standard solution; Type 1 Cu was measured from A_{614} by using ε_{614} 5700$M^{-1}\cdot cm^{-1}$ as in native laccase (Malmström *et al.*, 1970); Type 3 Cu was calculated as the difference between total and e.p.r.-detectable copper; $\Delta\varepsilon_{330}$ for the native enzyme reduced with ascorbate is 2800$M^{-1}\cdot cm^{-1}$ (Malmström *et al.*, 1970).

Sample	Content (mol/mol of protein)					
	Total copper	Type 1 and Type 2 copper	Type 1 copper	Type 2 copper	Type 3 copper	$\Delta\varepsilon_{330}$ ($M^{-1}\cdot cm^{-1}$)
1 Native	—	2.33	1.00	—	—	—
Partial apoprotein	2.88	1.09	0.92	0.09	1.79	−2200
Reconstituted	—	1.88	1.00	0.88	—	−300
2 Native	3.90	1.96	1.00	0.96	1.94	—
Partial apoprotein	2.56	1.12	0.81	0.31	1.44	−1650
Partial apoprotein	2.22	0.97	0.68	0.29	1.25	−2000
3 Native	3.92	1.90	1.00	0.90	2.02	—
Partial apoprotein	2.83	1.09	0.88	0.21	1.74	−1600
Partial apoprotein	2.80	1.04	0.85	0.19	1.76	−1500
4 Native	3.93	1.94	1.00	0.94	1.99	—
Partial apoprotein	2.88	1.21	0.96	0.25	1.67	−1550
5 Partial apoprotein	2.70	1.08	0.91	0.17	1.62	−2000
Reconstituted	3.78	1.72	0.80	0.92	2.06	+800

laccase is itself sensitive in this region to the previous history of the enzyme (Reinhammer, 1972; Farver *et al.*, 1976). It is apparent, however, that an A_{330} decrease does indeed occur and that it is not related to the loss of e.p.r.-non-detectable Cu.

Redox reactions and re-oxidation by oxygen

The Type 2 Cu-depleted laccase was reduced by ascorbic acid in a rather complicated way at pH 5.2, in 0.05 M-acetate buffer. Fig. 2 reports the behaviour of the 614 nm absorption band on subsequent additions of one electron equivalent of reductant (0.5 mol of ascorbic acid/mol of partial apoprotein). The first two additions caused an initial decrease of absorbance, followed by a slower increase (upper curves) very likely due to inter- or intra-molecular transfer of electrons from Type 1 Cu(I) to the two-electron acceptor. The increasing phase was in fact absent when the third equivalent was added (lower curve). Only minor absorbance variations occurred in the 330 nm region of the spectrum (Fig. 3), where ascorbic acid and its reduction products do not absorb significantly.

In the same buffer, reduction by ferrocyanide was much faster and could not be monitored by conventional techniques. Addition of about 5 mol of ferrocyanide/mol of partial apoprotein was required to obtain over 90% reduction of Type 1 Cu (Fig. 3). From the difference of A_{420} between the protein reduced with ferrocyanide and that reduced with ascorbic acid (Fig. 3), it was calculated that about 3 mol of ferricyanide were produced in the reaction. An absorbance coefficient of $1.0\,mM^{-1}\cdot cm^{-1}$ for ferricyanide asorbance at 420 nm was used.

When reduced partial apoprotein was re-exposed to air, re-oxidation of the ferrocyanide-treated samples was complete in a few minutes. In the case of ascorbate reduction, re-oxidation was partial and slower at pH 5.2, and no re-oxidation at all was observed in 0.05 M-phosphate buffer, pH 7.5. In any case, addition of less than stoichiometric ferricyanide restored the initial blue absorption.

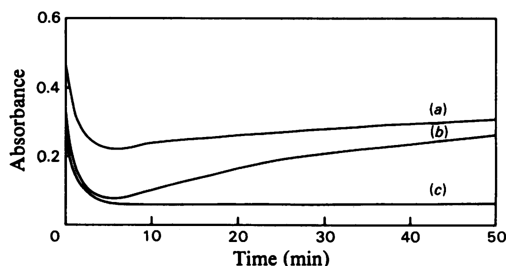

Fig. 2. *Reactions of Type 2 Cu(II)-depleted laccase with ascorbic acid, monitored by enzyme absorbance at 614 nm*
Enzyme (95 μM) in 0.05 M-acetate buffer, pH 5.2 was used: (*a*) 0.5 mol of ascorbic acid/mol of protein was added; (*b*) a further 0.5 mol of ascorbic acid/mol of protein was added; (*c*) a further 0.5 mol of ascorbic acid/mol of protein was added. The temperature was 25 °C, and samples were from expt. 3 in Table 1.

C.d.

Fig. 4 shows the c.d. spectra of the native and partial apoprotein (sample 4 of Table 1). Very similar results were obtained with different samples. It appears that removal of Type 2 Cu(II) did not substantially modify the rotatory power of the native protein in the 400–650 nm region of the spectrum.

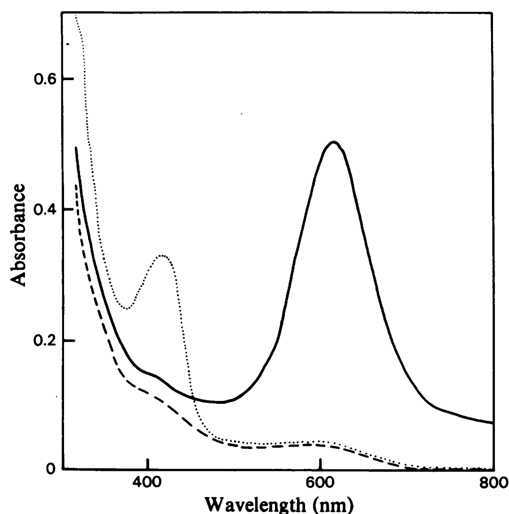

Fig. 3. *Optical spectra of reduced partial apoprotein*
——, 88 μM Partial apoprotein in 0.05 M-acetate buffer, pH 5.2 was used; – – – –, three electron equivalents of ascorbic acid were added; · · · ·, five electron equivalents of ferrocyanide were added. Samples were from expt. 3 in Table 1.

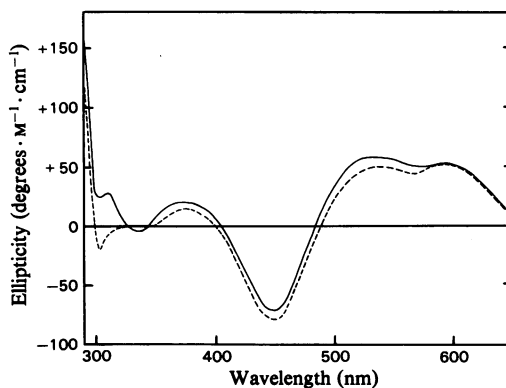

Fig. 4. *C.d. spectra of native and Type 2 Cu(II)-depleted laccase conditions were as in Fig. 1.*

Moreover, it is evident that the 330nm chromophore does not possess a high degree of chirality and that removal of the Type 2 Cu(II) caused the disappearance of two c.d. bands at 335 and 312nm, which both seem to contribute to the 330nm absorption. A small negative ellipticity appeared at 303nm that probably was also present in the native enzyme (see Fig. 4).

Fluorescence

In Fig. 5 are reported the fluorescence spectra of solutions of the holo- and partial apo-protein at similar concentrations, as determined from the A_{280}. The spectra were measured on sample 3 of Tables 1 and 2, and all the samples tested behaved in the same way. The fluorescence of the partial apoprotein is about 45% higher than that of the native protein and is independent of the content of Types 1 and 3 Cu, which is slightly different in the various samples. It is unaffected by anaerobic treatment with excess ascorbate, as checked by using both 280 and 295nm excitation energy (Table 2). In the latter case a protein concentration about 80μM was used, which permitted control of the 614nm absorption. Under similar conditions the holoenzyme fluorescence increased to about the same value as that of the partial apoprotein. It is important to recall that very little change at 330nm is produced by ascorbate reduction of the Type 2 Cu-depleted samples. Reconstitution with copper restored the fluorescence properties of the native protein.

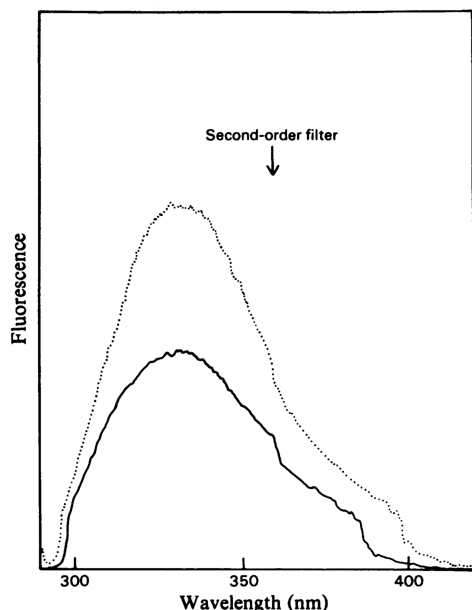

Fig. 5. *Fluorescence spectra of native and Type 2 Cu(II)-depleted laccase*

Enzyme (1.6μM) in 0.1M-phosphate buffer, pH6.0; ——, native laccase; ----, Type 2 Cu(II)-depleted enzyme. The shoulder on the long-wavelength side of the peaks is due to the insertion of the second-order filter in the optical path.

Discussion

C.d. measurements showed that the Type 2 Cu(II) of lacquer-tree laccase does not significantly contribute to the optical activity of the protein in the 400–650nm region, in agreement with previous data concerning this and other multicopper blue proteins (Bossa *et al.*, 1969; Falk & Reinhammar, 1972). On the other hand, it certainly contributes to the absorbance above 650nm; the A_{614}/A_{750} ratio increased from about 4.6 in the native protein to about 6.5 in the partial apoprotein. This corresponds to a $\Delta\varepsilon_{750} \approx 300\,\mathrm{M^{-1}\cdot cm^{-1}}$, quite a large value for a 'normal' type of Cu(II). A small contribution of Type 2 Cu(II) to the 614nm absorption is also possible, since the concentrations of the partial apoprotein, calculated by using the ε_{614} of the native protein, were always lower than those calculated at 280nm (Table 1), and it is not possible to state to what extent this is due to denatured or reduced protein molecules. C.d., optical-absorption and e.p.r. spectra of Type 1 Cu(II) are not substantially affected by removal of the Type 2 Cu(II). The same does not apply to Type 3 Cu(II), as shown by the decrease of A_{330}, hitherto assigned to this type of copper (Reinhammar & Vänngård, 1971), and by the disappearance of the two c.d. transitions at 335 and 312nm, which fall within the same envelope. The highest measured decrease of the 330nm absorption of $2200\,\mathrm{M^{-1}\cdot cm^{-1}}$ is close to the value of $2800\,\mathrm{M^{-1}\cdot cm^{-1}}$ observed on reduction with ascorbate of the native protein (Malmström *et al.*, 1970), and, in any case, very little further decrease was produced on reduction of the partial apoprotein with ascorbic acid (Fig. 3). The redox titrations show that the protein electron-accepting capacity is only one

Table 2. *Fluorescence of laccase and of the Type 2 Cu(II)-depleted enzyme*

Sample (see Table 1)	Fluorescence [arbitrary units (μM^{-1})]			Excitation wavelength (nm)
	Native	Depleted of Type 2 Cu	Reconstituted	
1	4.5	6.9	5.0	280
3	4.9	6.6		280
		6.8		280
	0.14	0.21		295
	(0.20) (reduced)	(0.21) (reduced)		295

electron equivalent lower than that of the native enzyme (Reinhammar & Vänngård, 1971), since three electron equivalents are required for full reduction of the Type 1 Cu either with ascorbic acid (Fig. 2) or with ferrocyanide (Fig. 3). Therefore it seems reasonable to associate the decrease of the 330nm absorption with removal of Type 2 Cu and to suggest that this affects the absorption from Type 3 Cu. Similar results were recently obtained (Avigliano *et al.*, 1979) with a related blue protein, ascorbate oxidase. Removal of Type 2 Cu caused a decrease of absorption at 330nm that was clearly unrelated to the content of Type 3 Cu very different in different samples.

The observation (Farver *et al.*, 1978) that the 330nm chromophore can be reduced with either a co-operative two-electron mechanism or a single-electron mechanism, or even with a combination of the two, depending on the reductant used, can now provide an alternative explanation to that of a non-equilibrium electronic distribution, as suggested by those authors. In fact the 330nm electronic transition appears to depend on both the Type 3 and Type 2 copper ions and may be considered to disappear when either copper type is reduced. Such a possibility had already been suggested for the ceruloplasmin 330nm chromophore (Deinum & Vänngård, 1973), which disappeared under certain conditions, together with the e.p.r. spectrum, by addition of only 0.5 reducing equivalent per copper ion. Besides possible reactions, such as substrate binding, that may cause changes of the copper redox potentials, the reduction of Type 3 Cu(II) may be prevalent over that of the Type 2 Cu(II) and vice versa, depending on the redox potential of the reductant. In fact from the Nernst equation relative to the single redox couples the following relationship can be derived:

$$\log\left[\frac{[Cu_2^{(II)}][Cu_3^{(I)}]}{[Cu_2^{(I)}][Cu_3^{(II)}]}\right] = \frac{2E_3^0}{0.059} - \frac{E_3^0}{0.059} - \frac{E^{\bar{0}}}{0.059} - \log\left(\frac{[ox.]}{[red.]}\right)$$

where E^0,, [ox.] and [red.] are the standard potential and the equilibrium concentrations of a one-electron redox reagent, and the subscripts 2 and 3 refer to the Type 2 and Type 3 copper. The latter is considered to exchange two electrons with a co-operative mechanism. The terms containing the standard potentials give a positive value varying within a few units for the reductants examined by Farver *et al.* (1978); the term log[ox.]/[red.] depends on E^0 and has a larger positive value the lower is E^0. Especially in the early stages of a reductive titration it may be large enough so that the right-hand side of the equation acquires a negative value, i.e., reduction of the Type 2 Cu(II) prevails over that of the Type 3 Cu(II). Under these conditions the 330nm band is therefore reduced with a single-electron mechanism. In the re-oxidation of laccase by O_2 and H_2O_2, the 330nm band is also reported to increase by a one-electron mechanism (Farver *et al.*, 1976). This agrees with the observation that the Type 2 Cu(II) is the last copper ion to be re-oxidized (Brändén & Reinhammar, 1975). Establishing how these findings fit into the above equation is more difficult, as it is not known which is the relevant redox process and which is the effect of a possible binding of the substrate to the protein (Aasa *et al.*, 1976).

Re-oxidation of the Type 2 Cu(I) is reported to be a very slow process, probably not involved in the turnover (Brändén & Reinhammar, 1975). Present data, however, show that the presence of the Type 2 Cu is essential to the process of reoxidation by air, as the partial apo-protein is only slowly reoxidized even if a mediator is present. In fungal laccase, re-oxidation by O_2 is inhibited by F^-, known by e.p.r. to bind to Type 2 Cu(II) (Malmström *et al.*, 1969; Andréasson *et al.*, 1973).

A chromophore depending on two metal-containing centres was also found in superoxide dismutase by Fee & Phillips (1975). They noted that the band of low intensity near 450nm in the native enzyme disappeared when it was brought to pH3.2 and when Zn(II) was removed. Unlike Fee & Briggs (1975), we believe that the band is due to a charge transfer to Cu(II) from the imidazolate group bridging Cu(II) and Zn(II) rather than a Cu(II) d–d transition, since it also disappears when Cu(II) is either reduced or removed from the protein (Rotilio *et al.*, 1977), at high pH, around pH11.5 (Rotilio *et al.*, 1971), and in the presence of cyanide (Fee & Gaber, 1972). In the two latter cases, as well as at pH3.2, when the Zn(II)–imidazolate bond is broken, a conformational change occurs at the Cu(II) site as its e.p.r. spectrum loses the rhombicity peculiar to the native spectrum and its redox potential decreases (Fee & DiCorleto, 1973; Rotilio *et al.*, 1973; Morpurgo *et al.*, 1976).

In a similar but not necessarily identical way, reactions occurring at one of the copper sites of laccase could affect a charge transfer occurring at the other site. The 330nm band (Brändén *et al.*, 1973) and the redox potential of both the Type 2 and Type 3 Cu(II) are affected to some extent by anion binding (Reinhammar & Vänngård, 1971; Reinhammar, 1972; Morpurgo *et al.*, 1974), which is generally believed to occur at the Type 2 Cu(II) site. The same occurs in fungal laccase (Brändén *et al.*, 1973; Andréasson *et al.*, 1973).

Fluorescence experiments showed that the increase of fluorescence caused by reduction of the native enzyme is related to the disappearance of the

330 nm absorption, rather than to the 614 nm band (Goldberg & Pecht, 1974). The same conclusion was reached previously on a different experimental ground (Avigliano *et al.*, 1978). The mechanism of fluorescence quenching in the holoprotein thus appears to be a non-radiative energy transfer between the tryptophan residues and the 330 nm chromophore. The relevant new result of the present study is that the Type 2 Cu(II) as well as the Type 3 Cu(II) may be involved in the quenching. Since removal of the Type 2 Cu(II) only affects the intensity of the fluorescence spectrum and not its shape, it seems reasonable to attribute the two c.d. transitions at 335 and 312 nm to the protein metal ions rather than to induced effects on the aromatic side chains.

Note added in Proof (Received 19 February 1980)

A paper has recently appeared (Reinhammar & Oda, 1979) reporting that removal of the Type 2 Cu from lacquer-tree laccase caused a decrease of A_{614}, while A_{330} remained unchanged. The discrepancy with the results of the present work is at least in part due to a difference of pH. We found that, at pH 7.4 under the conditions used by Reinhammar & Oda (1979), the Type 2 Cu-depleted laccase undergoes a slow autoreduction with decrease of A_{614}.

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