SELECTIVE REMOVAL OF TYPE 2 COPPER FROM *RHUS VERNICIFERA* LACCASE

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1. Introduction

Rhus vernicifera laccase contains, like fungal laccase, one Type 1, one Type 2 and two Type 3 copper ions [1], which can all be removed by cyanide at pH 8.0 in a reversible process [2]. In the case of fungal laccase a method is described [3] which allows reversible removal of only the Type 2 copper. In view of the many analogies presented by these two proteins we have investigated the possibility of obtaining, also in the case of *Rhus vernicifera* laccase, selective removal of Type 2 copper. A method for the preparation of a protein depleted of Type 2 copper is reported in this communication.

2. Materials and methods

Rhus vernicifera laccase was purified according to the method of Reinhammar [4]. The last stage of purification involving a DEAE-Sephadex A-50 column was repeated until a ratio $A_{330}/A_{614} = 0.8$ was obtained, as this ratio was found to be rather sensitive to the presence of impurities. Optical spectra were obtained with a DK-2A Beckman spectrophotometer and X-band EPR spectra with a V-4502 Varian spectrometer. Copper content was determined by atomic absorption spectroscopy, using a Hilgher & Watts Atomspek Model H 1170.

The laccase depleted of Type 2 copper was prepared by 24 h anaerobic dialysis of 0.5 mM native protein against a solution containing 2×10^{-3} M dimethylglyoxine (DMG) and 5×10^{-2} M ferrocyanide in 0.05 M acetate buffer pH 5.0, followed by a 24 h dialysis against 1×10^{-3} M EDTA in the same acetate buffer. Finally the protein solution was dialyzed against two or three changes of phosphate buffer pH 7.0.

3. Results and discussion

The data relative to a typical preparation of laccase depleted of Type 2 copper are collected in table 1. The ferrocyanide and *p*-phenylendiamine-oxidase activities [5] of laccase are very low in the treated protein samples and are recovered in the reconstituted protein. Figure 1 shows the EPR spectra of native laccase (a), of the protein treated with DMG (b) and with EDTA (c). The spectrum (b) shows the appearance of a new copper species, which is likely to be the DMG-Type 2 copper complex, the spectrum (c) is practically devoid of the Type 2 copper signal and closely resembles that obtained by Reinhammar [6] upon reduction of Type 2 copper, that is the spectrum of the laccase Type 1 copper [1]. In fact the corresponding optical spectrum (fig.2) shows that the 614 nm band, due to the Type 1 copper, is nearly unaffected by the treatment. Figure 2 also shows that, upon removal of the Type 2 copper, a decrease of absorbance occurs in the region above 700 nm of the optical spectrum. Though small, this decrease is always relatively much higher than that occurring at 614 nm and therefore can not be all related to small losses of Type 1 copper or to dilution. It seems more likely to be due to the loss of Type 2 copper, since this type of copper shows some analogy (EPR spectrum, reactivity toward inhibitory anions) with the copper of superoxide dismutase and of Cu(II) substituted carbonic anhydrase [7], which

Table 1
Properties of *Rhus vernicifera* laccase native, and depleted of Type 2 copper

Protein	Oxidase activity[e]		A/ϵ (mM)			Copper content (mM)			
	Ferrocyanide	PPD	280 nm	330 nm	614 nm	Total[a]	Type 1[b]	Type 2[c]	Type 3[d]
Native	100	100	0.25	0.28	0.26	1.18	0.26	0.28	0.64
DMG-treated	24	42	0.24	0.26	0.25	0.87	0.25	0.13	0.49
EDTA-treated	17	31	0.24	0.24	0.24	0.80	0.24	0.02	0.54
Recombined	98	100							

[a]From atomic absorption.
[b]From optical absorption intensity at 614 nm.
[c]From EPR double integrated intensity.
[d]From (a) minus EPR detectable copper.
[e]The ferrocyanide-oxidase activity was followed by recording the absorbance increase at 425 nm due to the ferricyanide produced. Reaction mixture contained 0.5 μM laccase, dissolved in 20 mM acetate buffer pH 5.0 and 3.3 mM ferrocyanide, in a final volum of 1 ml. The *p*-phenylendiamine-oxidase activity was followed at 30°C by recording the absorbance decrease at 340 nm due to reoxidation of NADH, according to [5] with minor modifications.

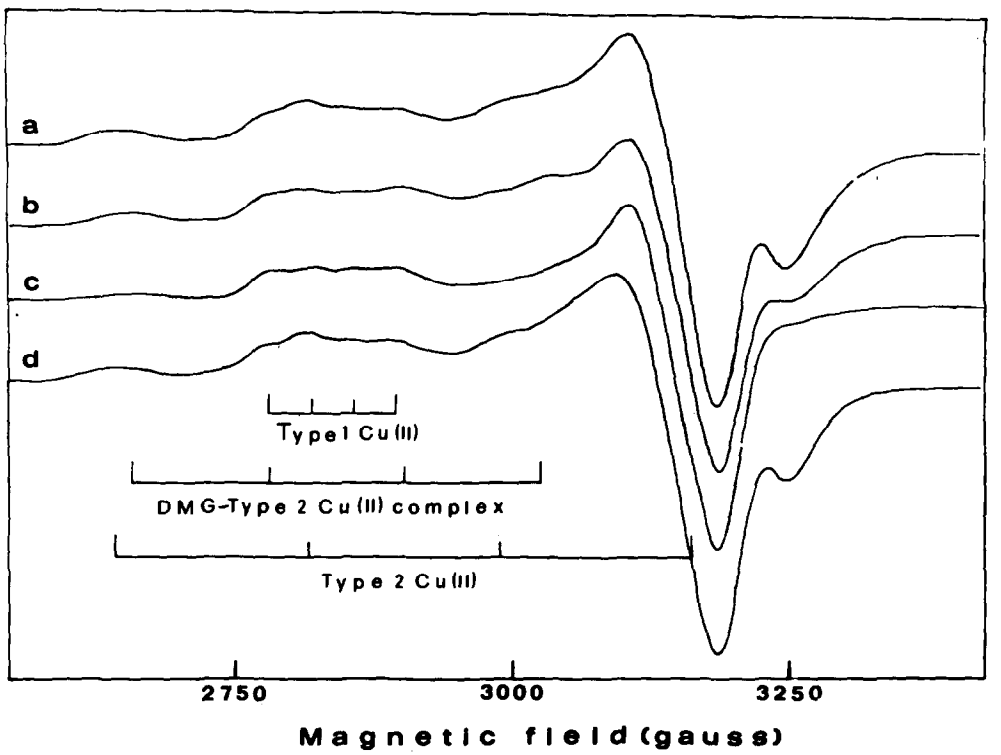

Fig.1. X-band EPR spectra of *Rhus vernicifera* laccase: (a) 0.25 mM laccase in 0.05 M phosphate buffer pH 7; (b) as in (a), after treatment with DMG; (c) as in (b), after treatment with EDTA; (d) as in (c), after anaerobic addition of 0.2 mM $CuSO_4$ in presence of excess ascorbate. For other details see text. EPR conditions: modulation amplitude, 10 G microwave power, 6 mW; temperature, −150°C.

absorb at 680 nm and 770 nm respectively. A similar decrease was not appreciable in fungal laccase [8] depleted of Type 2 Cu(II). In some samples a decrease of absorbance was observed in the 330 nm region after DMG/EDTA treatment. In these cases, a low content of Type 3 copper was also found from the difference between the total copper and the EPR-detectable copper (table 1), as described in ascorbate oxidase [9]. However the data were scarcely reproducible and too scattered to allow a definite correlation.

Reconstitution of the Type 2 Cu(II) (fig.1, (d) and table 1) was obtained by anaerobic addition of about two moles of copper (as the sulfate), per mol of protein, in the presence of excess ascorbate, and subsequent dialysis against EDTA to remove the excess copper. Some reconstitution, resulting in a slightly less active protein, could be obtained by anaerobic dialysis against very diluted $CuSO_4$ in the presence of ascorbate.

The fact that treatment of laccase with DMG only leaves some residual Type 2 Cu(II) and that EDTA alone has no effect on the native protein copper, even after four days dialysis under the same conditions, deserves some comment. DMG and EDTA are both Cu(II) chelating agents of comparable strength [10]. The solubility of DMG, however, as well as the stability of its Cu(II) chelate, increases in solvents of lower dielectric constant such as dioxan-water mixtures [10]. These properties and possibly the smaller molecular size of DMG may facilitate the approach to the protein Type 2 copper and may give rise to adsorption of the copper complex on the protein surface. This may explain the appearance of the new EPR spectrum of fig.1, (b). EDTA is probably only active in the extraction of copper from this complex. However no inhibitory effect by DMG was observed on the oxidase activity of laccase.

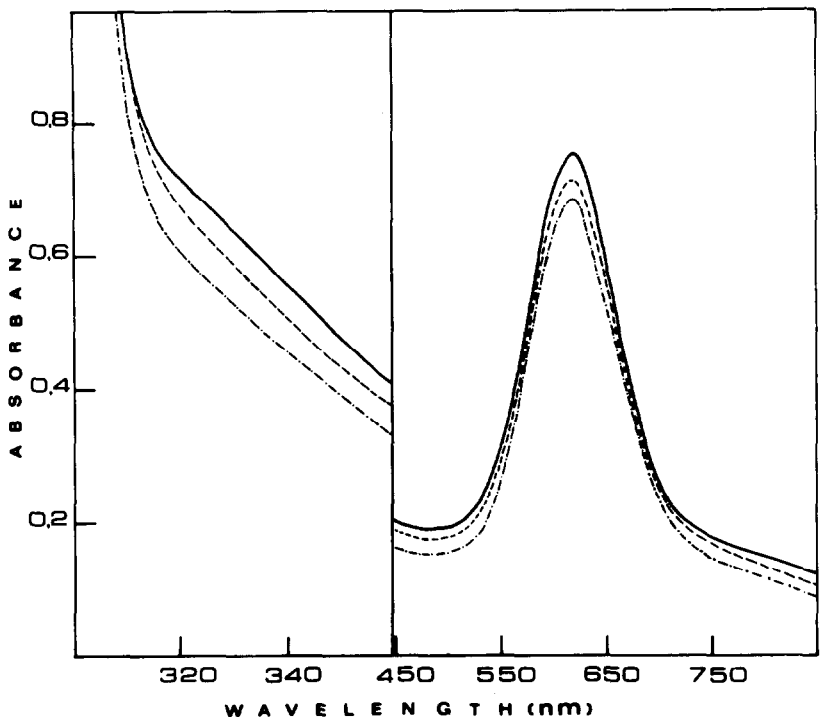

Fig.2. Optical spectra of *Rhus vernicifera* laccase. (——) 0.25 mM laccase in 0.05 M phosphate buffer pH 7.0 (– – – –) after treatment with DMG. (· – · – · –) after treatment with DMG and EDTA. Optical path: 0.5 cm.

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