pH DEPENDENCE OF REDOX PROPERTIES OF THE TYPE 2 Cu-DEPLETED TREE LACCASE

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1. Introduction

The laccases are the best characterized copper oxidases having three types of copper centres [1]. They contain one blue paramagnetic copper (Type 1), one non-blue paramagnetic copper (Type 2), and one copper pair undetectable by EPR (Type 3) [1]. Furthermore they can be selectively depleted of Type 2 Cu, resulting in an inactive enzyme which can be reactivated by reconstitution with copper ions [2,3]. Some discrepancies exist in the literature on the properties of the Japanese *Rhus vernicifera* laccase selectively depleted of Type 2 Cu [4,5]. They concern the spectroscopic properties of the remaining Type 1 and Type 3 Cu and the redox behaviour of the enzyme. We have now re-examined such properties, with particular attention to their dependence on pH, since different pH conditions had apparently been used in previous reports [4,5]. A definite pH dependence was found of the redox behaviour and the stability of Type 2 Cu-depleted laccase.

2. Materials and methods

Rhus laccase and Type 2 Cu-depleted enzyme were prepared as previously described [5]. In the procedure for the removal of Type 2 Cu the dialysis time was increased up to 2 days, as suggested by Reinhammar and Oda [4] in order to abolish the enzyme activity almost completely. Longer incubation times were found to have no further effect. Optical and EPR spectra and the anaerobic experiments were performed as previously described [5].

3. Results

3.1. *Optical and EPR properties of Type 2 Cu depleted laccase*

In fig.1 are shown the optical spectra in 0.05 M sodium acetate buffer pH 4.8 of native laccase and of a sample depleted of Type 2 Cu, before and after anaerobic reduction with three equivalents of ascorbic acid. Fig.2 shows the EPR spectra of native and Type 2 Cu-depleted enzyme. The lower curve a', obtained at larger instrumental gain, shows that removal of Type 2 Cu is not complete, as a hyperfine line of low intensity is still detected at lowest magnetic field. Such a line however appears to be shifted to higher field with respect to the native laccase (lower curve

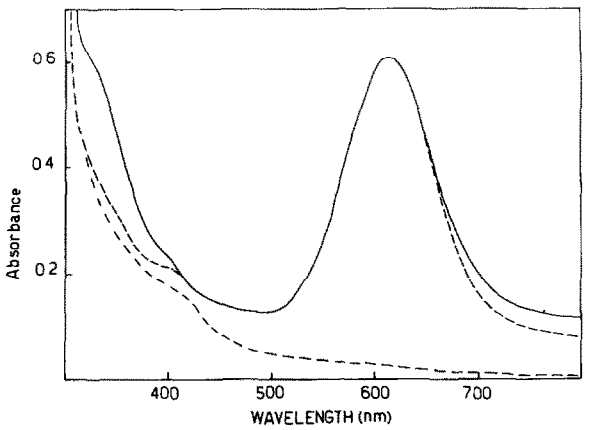

Fig.1. Optical spectra of native and Type 2 Cu-depleted tree laccase. 0.11 mM enzyme (sample 2 of table 1) in 0.05 M sodium acetate buffer pH 4.8: (——) native; (– –) depleted of the Type 2 Cu; (–·–) the latter anaerobically reduced with three equivalents ascorbic acid.

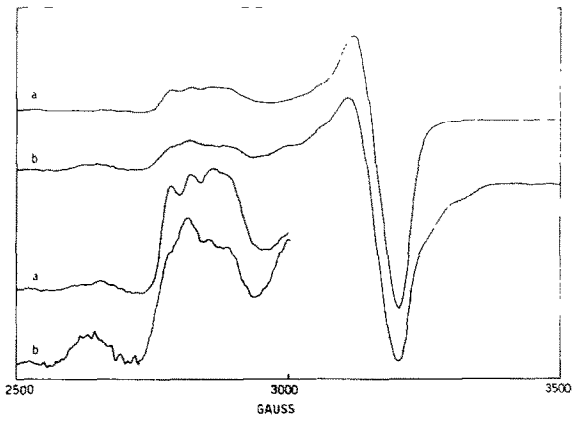

Fig.2. EPR spectra of native and Type 2 Cu-depleted tree laccase. (a) 0.23 mM Type 2 Cu-depleted laccase (sample 2 of table 1) in 0.01 M sodium acetate buffer pH 4.8; (a') a five-fold enlargement; (b) 0.11 mM native laccase in 0.01 M sodium acetate buffer pH 4.8; (b') a five-fold enlargement. Setting conditions: microwave frequency 9.15 GHz; microwave power 20 mW; temperature 77° K.

b'). A coincident line was already observed [3] in samples treated with dimethylglyoxime, in which as much as 50% of Type 2 Cu had remained bound to the protein. The measured $A_{\parallel}$ splitting of Type 1 Cu was of $35 \cdot 10^{-4}$ cm^{-1} [4].

Alcalinization of a Type 2 Cu-depleted laccase sample up to pH 7.4 by addition of concentrated Na_2HPO_4, led to a slow loss of the blue colour, 20% of the initial absorbance being lost in ~3 h. A parallel decrease of intensity was observed in the EPR spectrum. Such a bleaching was faster at higher pH and 80% of the 614 nm absorbance was lost in ~30 min at pH 9.4. Most of the blue colour could slowly be recovered by re-acidification of the solutions and much faster by both re-acidification and addition of ferricyanide. The reversibility of the process was increasingly less at higher pH values or longer incubation times at high pH. The solutions were otherwise stable at around 4–7 pH. Following these observations the data of table 1, relating to two different batches of laccase, were calculated as in previous papers [3,5] assuming ϵ_{614} to be unaffected by removal of Type 2 Cu. Together with the spectra of fig.1, they confirm previous results [5], i.e. that some Type 1 and Type 3 Cu are also lost in the treatment and that there is a consistent decrease of absorbance in the regions about 330 nm and above 650 nm. The decrease measured at 330 nm amounts to about 70% that observed on reduction with ascorbate of the native protein ($\Delta A = 2800$ M^{-1} cm^{-1} [6]). Reduction with ascorbate of the Type 2 Cu-depleted laccase samples caused a further 12–15% decrease (fig.1).

3.2. *Redox properties of Type 2 Cu-depleted laccase*

At pH 4.8, in 0.05 M sodium acetate buffer, the blue colour of laccase samples depleted of the Type 2 Cu, was in part rapidly bleached (fig.3) by anaerobic addition of two reducing equivalents (1 mol ascorbic acid/mol of protein), then a slower internal electron transfer occurred, apparently from the Type 1 to the Type 3 Cu, as shown by the partial recovery of blue colour on standing. Such a recovery was not observed in the parallel experiment carried out at pH 7.4 (fig.3). At both pH 4.8 and 7.4, however, a third reducing equivalent was required to achieve a

Table 1
Analytical data of native and Type 2 Cu-depleted tree laccase

Protein		Content (mol/mol enzyme[a])						
		Total copper[b]	Type 1 and Type 2 copper[c]	Type 1 copper[d]	Type 2 copper[e]	Type 3 copper[f]	$\epsilon^{g}_{330\,nm}$ (M^{-1} cm^{-1})	Activity[h]
(1)	Native	4.14	2.04	1.08	0.96	2.10	5100	100
	Type 2 Cu-depleted	2.80	1.17	0.94	0.23	1.63	3100	< 2
(2)	Native	3.85	1.85	1.00	0.85	1.85	5000	100
	Type 2 Cu-depleted	2.40	1.01	0.90	0.11	1.40	3100	< 1

[a]determined from $A_{280\,nm}$ and $\epsilon = 93.500$ M^{-1} cm^{-1} [6]; [b]determined with 2,2'-biquinolyl [12]; [c]determined by double integration of EPR signals; [d]determined from $A_{614\,nm}$ and $\epsilon = 5700$ M^{-1} cm^{-1}; [e]from[c] minus[d]; [f]from[b] minus[c]; [g]determined using protein concentration as in[a]; [h]determined as the ferrocyanide oxidase activity in 0.05 M acetate buffer pH 5.2 and 3.0 mM ferrocyanide at 25°C.

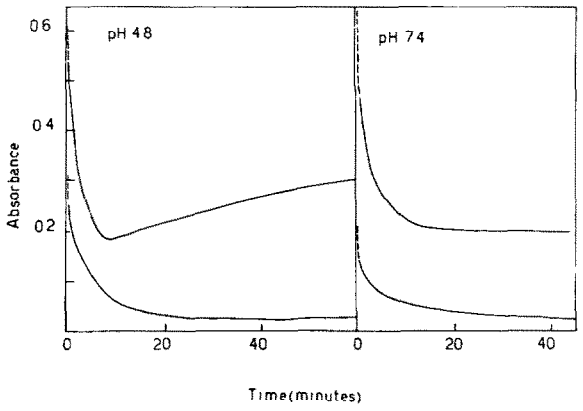

Fig.3. Reduction of the Type 2 Cu-depleted tree laccase by ascorbic acid. 0.11 mM enzyme anaerobically reacted with 0.11 mM ascorbic acid (upper curves); 0.055 mM ascorbic acid added (lower curves). pH 4.8: 0.05 M sodium acetate buffer; pH 7.4: 0.05 M potassium phosphate buffer.

complete and stable bleaching of the blue colour (lower curves). At pH 6.5, in 0.05 M phosphate buffer, the behaviour was as at pH 4.8.

The extent and kinetics of reoxidation in air of the reduced enzyme showed some variability in different samples. As a general trend recovery of the blue colour was complete at pH 4.8 and 6.5 in 2–3 h, whilst only 80% of the initial blue colour was recovered at pH 7.4 after one night standing in air at 5°C to limit denaturation. At any examined pH value a rapid identical absorbance decrease was observed at 330 nm on reduction (fig.1), that was about 15% that observed on reduction of the native enzyme. Such an absorbance was immediately recovered on admission of air into the cuvette and no further change was observed during the slow reoxidation of the blue chromophore. The presence of a parallel fast change at 614 nm, as reported by Reinhammar and Oda [4], could not be detected with our instrumentation, but is not excluded.

4. Discussion

These results confirm previous ones [5] that removal of Type 2 Cu from laccase has no effect on the optical properties of Type 1 Cu at low pH. Above pH 7 the blue colour is bleached somewhat depending on the pH, due to auto-reduction of the enzyme. On standing at alkaline pH, the protein tends to denaturate, that is the bleaching is no longer reversed by re-acidification, indicating that Type 2 Cu has a stabilizing effect on its structure. The other copper ions probably become more exposed to solvent perturbations when Type 2 Cu is removed. Recent NMR experiments [7] have shown that a considerable portion of the laccase proton relaxivity remains after removal of Type 2 Cu, indicating some interaction of Type 1 and/or Type 3 Cu with solvent protons. Bleaching at alkaline pH values was also reported for azurin [8] and plastocyanin [9].

A definite pH dependence was observed for the redox behaviour of Type 2 Cu-depleted laccase. The transfer of electrons from reduced Type 1 to Type 3 Cu, that is apparent in the acid pH range, is not observed at pH 7.4, although the protein still required three equivalents for complete reduction of the blue copper, indicating that reduction of Type 3 Cu does occur also at this pH. A dependence on pH of the mechanism of reduction of Type 3 Cu had also been observed in native laccase [10] and had been attributed to deprotonation of an enzyme group with $pK \simeq 7.4$, supposedly a water molecule bound to Type 2 Cu [10]. This is clearly not possible in the Type 2 Cu-depleted enzyme, where some other deprotonating group must be involved. Although the transfer of electrons from Type 1 to Type 3 Cu can take place also in the absence of Type 2 Cu, under certain conditions, the process is so slow that it is still possible to state that the Type 2 Cu has an important role in the process of electron transfer in the native enzyme, probably related to the possibility of simultaneous transfer of two electrons to the Type 3 Cu [4,10]. The presence of the Type 2 Cu also appears to be important in the reoxidation process, since it is also very slow in its absence. It may be noted in this contest that no spectroscopic effect was detected on addition of stoichiometric H_2O_2 to the Type 2 Cu depleted laccase (Morpurgo L., unpublished results) such as that observed for the native enzyme [11] suggesting that Type 2 Cu may actually be a binding site for some reduced oxygen intermediate.

The fast redox process connected with the residual absorbance at 330 nm may be related to a fraction of molecules containing paramagnetic copper other than Type 1, as shown in table 1 and fig.2a′. Such a copper however is not the native Type 2 Cu, as shown by the different position of the first hyperfine line at

low magnetic field and by the very low activity of the samples. The suggestion [4] that the fast reoxidation phase is related to a fraction of molecules containing reduced Type 3 Cu seems improbable, since there is no reason to believe that only a fraction of the Type 3 Cu was reduced (see fig.3 and [5]). Any variation of absorbance at 330 nm imputable to redox processes was fast, relatively small and unrelated to phenomena observed in the bulk solution.

The discrepancy with the data of Reinhammar and Oda [4] as far as the absorbance of the blue copper is concerned, may also be explained by the presence of paramagnetic copper other than Type 1. Together with some bleaching of Type 1 Cu at pH 7.4, this may be an additional factor leading to their underestimation of Type 1 Cu extinction coefficient.

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