Titration with Ferrocyanide of Japanese-Lacquer-Tree (*Rhus vernicifera*) Laccase and of the Type 2 Copper-Depleted Enzyme

INTERRELATION OF THE COPPER SITES

Laura MORPURGO,* Maria Teresa GRAZIANI,† Alessandro DESIDERI,‡ and Giuseppe ROTILIO†
*National Research Council Molecular Biology Centre and †Institute of Biological Chemistry, University of Rome, Rome, Italy, and ‡Physics Department, University of Calabria, Arcavacata, Italy

(Received 28 September 1979)

1. Redox titrations are reported of the metal centres in Japanese-lacquer-tree (*Rhus vernicifera*) laccase with ferrocyanide. 2. The redox potential of Type 1 Cu was found to increase with ferrocyanide concentration up to a limiting value similar to that for the Type 1 Cu in Type 2 Cu-depleted enzyme (which is independent of ferrocyanide concentration). 3. The redox potential of the two-electron acceptor (Type 3 Cu) is also independent of ferrocyanide concentration in Type 2 Cu-depleted enzyme and lower than values reported for the native enzyme. 4. The two-electron acceptor is present in the oxidized state in the Type 2 Cu-depleted enzyme, though the latter lacks the 330 nm absorption band. 5. The redox potential of Type 2 Cu also depends on ferrocyanide concentration, at least in the presence of azide. 6. The redox potentials are affected by freezing the solutions and/or addition of azide, the latter binding to Type 2 Cu with affinity dependent on the redox state of the two-electron acceptor.

An essential feature of the oxidases that form water on reduction of molecular oxygen in their catalytic cycle is the presence in their molecule of at least four metal redox centres (Malmström *et al.*, 1975). In the case of the 'blue' copper oxidases, two of the metal ions are closely associated with each other and show strong magnetic coupling (Solomon *et al.*, 1976), but there is evidence for interaction of the two other metal ions as well, either between each other or with the magnetic coupled pair [Holwerda *et al.* (1976) and references therein]. In the present paper we report new data on the interdependence of the copper sites obtained in the study of the reactions with ferrocyanide of Japanese-lacquer-tree (*Rhus vernicifera*) laccase and of the enzyme depleted of one of its copper ions, the Type 2 Cu [see the preceding paper (Morpurgo *et al.*, 1980)]. The four copper ions of laccase are referred to as Type 1 Cu, characterized by a very high molar extinction coefficient at 614 nm and narrow hyperfine splitting constant $A_{\parallel}$ in the e.p.r. spectrum, Type 2 Cu, with more 'normal' optical and e.p.r. parameters, and the Type 3 Cu, the magnetically coupled pair usually associated with a two-electron acceptor absorbing at 330 nm (Fee, 1975). This absorption has recently been reported [the preceding paper (Morpurgo *et al.*, 1980)] to be also affected by removal of Type 2 Cu. Here we show that the Type 2

Cu-depleted enzyme, although missing the 330 nm absorption band, still contains an acceptor of two electrons with a co-operative mechanism.

Materials and Methods

Japanese-lacquer-tree (*Rhus vernicifera*) laccase and the Type 2 Cu-depleted enzyme were obtained as described previously [the preceding paper (Morpurgo *et al.*, 1980)]. Analytical details about the samples of Type 2 Cu-depleted laccase are given in the legend to Fig. 1. The anaerobic titrations were carried out in an optical cell or e.p.r. tube sealed to a Thunberg-type apparatus. To the solutions, de-aerated by three cycles of vacuum-flushing with purified argon, de-aerated ferrocyanide solutions were added by means of a microsyringe, through serological caps.

Optical spectra were recorded on a Cary 14 spectrophotometer. E.p.r. spectra were recorded on an E-9 Variant apparatus at 100 K.

Results

Redox titrations

Native laccase and the Type 2 Cu-depleted enzyme were titrated with ferrocyanide under identical conditions. At any stage of the titration the following equation should hold

$$E_{\text{Cu}}^0 + 0.059 \log \{[\text{Cu(II)}]/[\text{Cu(I)}]\} = E_{\text{Fe}}^0 + 0.059 \log \{[\text{Fe(III)}]/[\text{Fe(II)}]\} \quad (1)$$

where E_{Cu}^0 and E_{Fe}^0 are the standard potentials of Type 1 Cu(II)/Cu(I) and ferri-ferrocyanide redox couples respectively. The type 1 Cu(II) concentration was calculated by using an ϵ_{614} value of $5700 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for both the native and Type 2 Cu-depleted enzyme [the preceding paper (Morpurgo *et al.*, 1980)], ferri-cyanide concentration was calculated from the absorption increase at 420 nm by using an ϵ value of $1000 \text{ M}^{-1} \cdot \text{cm}^{-1}$, the concentrations of copper- and iron-reduced species were obtained as the difference from the respective total concentrations at each stage of the titration. In Fig. 1 are reported the values of E^0 (Type 1 Cu) calculated by means of eqn. (1), as a function of the amount of added ferrocyanide, at two different pH values. Approximately constant values were obtained for the Type 2 Cu-depleted protein, whereas the values for the native protein were found to be dependent on ferrocyanide concentration. On increasing ferrocyanide concentration, E^0 (Type 1 Cu) approached the value calculated for the Type 2 Cu-depleted protein. Fig. 1 also reports two sets of values measured in the presence of NaN_3 , that show a trend similar to that of the native enzyme, though the values of the redox potential are lower.

As for the native enzyme, in the Type 2 Cu-depleted enzyme the amount of ferricyanide formed at each stage of the titration was larger than the amount of reduced Type 1 Cu, indicating that some other electron acceptor was present in the enzyme molecule. If this is in fact the two-electron acceptor, as previously suggested [the preceding paper (Morpurgo *et al.*, 1980)], the following equation should also hold:

$$E_{\text{Fe}}^0 + 0.059 \log \{[\text{Fe(III)}]/[\text{Fe(II)}]\} = E^0 + \left(\frac{0.059}{2}\right) \log \{[\text{ox.}]/[\text{red.}]\} \quad (2)$$

where E^0 , [ox.] and [red.] refer to the two-electron acceptor or Type 3 Cu. The concentration of the reduced form was calculated to be one half the difference between ferricyanide formed and reduced Type 1 Cu, and then subtracted from the total Type 3 Cu concentration to obtain the concentration of the oxidized form. By this procedure, approximately constant values were obtained for E^0 around the half-point of each titration. Average values are reported in Table 1, together with the values for the Type 1 Cu(II)/Cu(I) couple. The values of 410 and 418 mV were used for E_{Fe}^0 in acetate and phosphate buffer respectively (O'Reilly, 1973).

Dependence of redox equilibria on freezing

It is already known that the e.p.r. spectrum of a ferrocyanide-treated laccase solution in anaerobic

Fig. 1. Redox potential of Type 1 Cu as a function of ferrocyanide concentration

In the native enzyme (●); in the native enzyme in the presence of NaN_3 (●); in Type 2 Cu-depleted enzyme from two different stock solutions containing respectively 0.61 mM Type 1 Cu, 0.72 mM e.p.r.-detectable Cu, 1.90 mM total Cu, 0.66 mM-enzyme measured from A_{280} (○), and 0.44 mM Type 1 Cu, 0.55 mM e.p.r.-detectable Cu, 1.32 mM total Cu and 0.46 mM-enzyme measured from A_{280} (□). The protein concentration was about 0.1 mM in 0.05 M-phosphate buffer, pH 7.4 (a), and in 0.05 M-acetate buffer, pH 5.2 (b).

conditions only shows the signal due to Type 1 Cu, whereas the Type 2 Cu signal is absent, very likely due to reduction of this copper ion (Reinhammar, 1972; Morpurgo *et al.*, 1974). The Type 1 Cu signal persisted even in the presence of a large ferrocyanide excess, over 10-fold the enzyme molar concentration, i.e. under conditions in which the blue absorption of the Type 1 Cu was completely abolished in the room-temperature spectrum. As a matter of fact a change of colour of the solutions from yellow to green occurred on freezing. On thawing the change was reversed.

Reactions in the presence of NaN_3

The change of colour on freezing was not observed when the reaction with ferrocyanide was carried out in the presence of NaN_3 or NaCN . Under these conditions the e.p.r. spectra only showed a modified Type 2 Cu(II) signal (Morpurgo *et al.*, 1974; Desideri *et al.*, 1979) characterized by very narrow A_{11} , $106 \cdot 10^{-4} \text{ cm}^{-1}$ and

Table 1. Redox potential of Type 1 Cu and of the two-electron acceptor of native and of Type 2 Cu-depleted lacquer-tree laccase

Protein	pH	Ferrocyanide concn.	Redox potential (mV)		Reference
			Type 1 Cu	Two-electron acceptor	
Native	7.5	Less than protein	394	434	Reinhammar (1972)
	7.5	Excess over protein	434	483	Reinhammar (1972)
Type 2 Cu-depleted	7.4	Independent of	430*	420*	The present study
	5.3	Independent of	462†	448†	The present study

* In 0.05 M-phosphate buffer, $E_{Fe}^0 = 418$ mV (O'Reilly, 1973).

† In 0.05 M-acetate buffer, $E_{Fe}^0 = 410$ mV (O'Reilly, 1973).

$139 \times 10^{-4} \text{ cm}^{-1}$, in the presence of N_3^- and CN^- respectively. In the attempt to establish whether the Type 2 Cu(II)- N_3 complex was in the oxidized or reduced state at room temperature in the presence of excess ferrocyanide, some laccase solutions were anaerobically titrated with ferrocyanide in the presence of NaN_3 . In Fig. 2 are reported some significant spectra recorded during a titration in 0.05 M-sodium acetate buffer, pH 4.0. The results were qualitatively similar in the pH range 4.0–7.5, but the optical changes at 500 nm after the formation of laccase- N_3 complexes were better observed at lower pH, because of higher affinity of laccase for N_3^- (Morpurgo *et al.*, 1974). In Fig. 3 are reported the absorbance changes during the same titration at wavelengths that allow simultaneous control of the redox state of Type 1 Cu (614 nm), Type 2 Cu(II)- N_3 complex (500 nm), Type 3 Cu (330 nm) and ferricyanide (420 and 330 nm). The assignment of the 500 nm absorption to a Type 2 Cu(II)- N_3 complex is discussed below. In the enzyme depleted of Type 2 Cu, NaN_3 did not affect either optical or e.p.r. spectra in the pH range 4–7.5.

Discussion

The results of the titration with ferrocyanide of Type 2 Cu-depleted laccase confirm a previous suggestion [the preceding paper (Morpurgo *et al.*, 1980)] that this protein derivative contains a two-electron acceptor in the oxidized state, in spite of the absence of the 330 nm band. Its redox potential (Table 1) is close to that reported for native laccase (Reinhammar, 1972), has a similar pH-dependence (Nakamura, 1958), but, unlike native laccase, is independent of ferrocyanide concentration. In the enzyme depleted of Type 2 Cu, a similar behaviour is also observed for Type 1 Cu. Its redox potential has the same pH-dependence as in the native enzyme (Nakamura, 1958; present Table 1) and is independent of ferrocyanide concentration, unlike in native laccase (Reinhammar, 1972). The two redox sites differ in that the redox potential of Type 1 Cu is

Fig. 2. Absorption spectra measured during an anaerobic titration with ferrocyanide of laccase in the presence of NaN_3

(a) 0.11 mM-Laccase in 0.05 M-acetate buffer, pH 4.0; (b) 0.33 mM- NaN_3 added; (c) 0.33 mM-ferrocyanide added; (d) 0.88 mM-ferrocyanide added.

Fig. 3. Absorbance changes at certain wavelengths on addition of ferrocyanide to a laccase solution containing NaN_3

Conditions were as for in Fig. 2. The wavelengths are written alongside the curves.

close to the highest reported value for native laccase, whereas the redox potential of the two-electron acceptor is close to the lowest reported value for the native enzyme (Table 1). Such an affect is comparable with that produced by fluoride on native laccase (Reinhammar & Vänngård, 1971). The data of Fig. 1 show that the redox potential of Type 1 Cu in the Type 2 Cu-depleted enzyme represents the upper-limit value approached in a titration of native laccase with ferrocyanide. In the presence of azide, the redox potential of Type 1 Cu is decreased to some extent, but its dependence on ferrocyanide concentration is unchanged. Figs. 2 and 3 show in addition that, in the presence of azide, the decrease in A_{614} , related to reduction of Type 1 Cu, parallels a decrease in the A_{500} . The latter originates partly on addition of azide to native laccase (Morpurgo et al., 1974; present Fig. 2) and partly on a reaction with ferrocyanide very likely involving reduction of Type 3 Cu (Figs. 2 and 3). In fact in the early stages of the titration the A_{500} increases together with the A_{420} , due to ferricyanide formed. The absence of a parallel increase at 330nm, where ferri- and ferro-cyanide also absorb, indicates that it is compensated for by reduction of the two-electron acceptor. When the titration is carried out without previous addition of azide or when azide is added to Type 2 Cu-depleted laccase, no optical changes are observed at 500nm. It seems therefore reasonable to assign this absorption to a Type 2 Cu(II)-N₃ complex. Its formation appears to be favoured by reduction of Type 3 Cu, then it is reduced, on increasing ferrocyanide concentration, as the Type 1 Cu. These findings suggest: (i) that the higher affinity for azide of the partially reduced enzyme previously observed (Morpurgo et al., 1974; Holwerda & Gray, 1974) is related to reduction of the Type 3 Cu rather than of Type 1 Cu; (ii) that the optical effects of azide on oxidized laccase are only related to binding of the anion to Type 2 Cu(II), as they increase when Type 3 Cu is reduced; (iii) that the redox potential of Type 2 Cu is probably also dependent on ferrocyanide concentration, at least in the presence of azide, as it is for the other two copper sites. The nature of ferrocyanide interactions with laccase, affecting the

redox potential of the copper sites, is not easy to understand. It is certainly related to the presence of Type 2 Cu in the protein, but is unaffected by azide binding to the metal ion. It may involve binding of ferro- or ferri-cyanide to some protein site affecting the conformation of the copper centres. A change of conformation of the protein metal centres could also explain the different redox behaviour in the frozen state. Changes of metal co-ordination properties on freezing have been reported to occur in Co(II)- and Cu(II)-substituted carbonic anhydrase in the presence of excess cyanide (Haffner & Coleman, 1975).

It may be concluded that redox centres in laccase are interrelated in an equilibrium system, critically dependent on specific interactions occurring at each site, such as anion binding and redox reactions and on conformational states of the protein, such as that artificially induced by freezing.

References

- Desideri, A., Morpurgo, L., Rotilio, G. & Mondovi, B. (1979) *FEBS Lett.* **98**, 339–341
- Fee, J. A. (1975) *Struct. Bonding (Berlin)* **23**, 1–60
- Haffner, P. H. & Coleman, J. E. (1975) *J. Biol. Chem.* **250**, 996–1005
- Holwerda, R. A. & Gray, H. B. (1974) *J. Am. Chem. Soc.* **96**, 6008–6022
- Holwerda, R. A., Wherland, S. & Gray, H. B. (1976) *Annu. Rev. Biophys. Bioeng.* **5**, 363–396
- Malmström, B. G., Andréasson, L. E. & Reinhammar, B. (1975) *Enzymes 3rd Ed.* **12B**, 507–579
- Morpurgo, L., Rotilio, G., Finazzi-Agrò, A. & Mondovi, B. (1974) *Biochim. Biophys. Acta* **336**, 324–328
- Morpurgo, L., Graziani, M. T., Finazzi-Agrò, A., Rotilio, G. & Mondovi, B. (1980) **188**, 361–366
- Nakamura, T. (1958) *Biochim. Biophys. Acta* **30**, 44–52
- O'Reilly, J. E. (1973) *Biochim. Biophys. Acta* **292**, 509–515
- Reinhammar, B. (1972) *Biochim. Biophys. Acta* **275**, 245–259
- Reinhammar, B. & Vänngård, T. (1971) *Eur. J. Biochem.* **18**, 463–468
- Solomon, E. I., Dooley, D. M., Wang, R. H., Gray, H. B., Cerdonio, M., Mogno, F. & Romani, G. L. (1976) *J. Am. Chem. Soc.* **98**, 1029–1031