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OXIDATION–REDUCTION POTENTIALS OF THE ELECTRON ACCEPTORS IN LACCASES AND STELLACYANIN

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SUMMARY

1. Laccase (*p*-diphenol:O_2 oxidoreductase, EC 1.10.3.2) obtained from the fungus *Polyporus versicolor* and laccase and stellacyanin from the lacquer tree *Rhus vernicifera* were titrated with reducing and oxidizing agents in an anaerobic optical cell equipped with a combined metal electrode for simultaneous optical and potentiometric titrations of the chromophores absorbing at about 610 nm in all three proteins and the 330-nm chromophores in the laccases.

2. The oxidation–reduction potential of the "blue" copper atom in stellacyanin was found to be 184 mV when the protein was dissolved in a sodium phosphate buffer, pH 7.1 and ionic strength 0.3.

3. The oxidation–reduction potential of Type 1 copper and the two-electron acceptor, connected with the 330-nm chromophore, in *Rhus* laccase was found to be dependent on the concentration of hexacyanoferrate which was used as an electron mediator in the titrations. With about three times excess of this substance, compared to the enzyme concentration, the oxidation–reduction potentials of Type 1 copper and the two-electron acceptor were 434 mV and 483 mV, respectively, when the enzyme was dissolved in sodium phosphate buffer, pH 7.5 and ionic strength 0.2.

If the concentration of hexacyanoferrate was only about one third the concentration of enzyme, under otherwise similar conditions as above, the oxidation–reduction potentials of Type 1 copper and the two-electron acceptor decreased to 394 mV and 434 mV, respectively. The increase in oxidation–reduction potential of the Type 1 copper and the two-electron acceptor in the presence of excess hexacyanoferrate seems to be due to a specific reduction of Type 2 copper by the hexacyanoferrate (II).

4. For *Polyporus* laccase the oxidation–reduction potential of Type 1 copper was extremely high. A value of 785 mV was obtained for an enzyme solution in sodium phosphate buffer, pH 5.5 and ionic strength 0.2, which also contained small amounts of some metal cyanides as electron mediators.

The 330-nm chromophore was found to be a two-electron acceptor with an oxidation–reduction potential of 782 mV under these experimental conditions.

In the presence of excess of the electron-mediating metal cyanides or with about 100 times excess of the inhibitor NaF the oxidation–reduction potential of Type 1 copper was about the same as above. The potential of the two-electron acceptor decreased to 570 mV, however, when fluoride was present.

INTRODUCTION

Many copper-containing proteins take part in oxidation–reduction reactions. For example, the laccases (*p*-diphenol:O_2 oxidoreductase, EC 1.10.3.2), obtained from the fungus *Polyporus versicolor* and from the lacquer tree *Rhus vernicifera*, catalyze the oxidation of various compounds, including certain diphenols and aryldiamines, with concomitant reduction of oxygen to water. These enzymes have been found to contain four copper atoms which can be classified into three different forms[1,2]. There are two different paramagnetic copper atoms according to, among other things, spectral properties. One gives rise to the intense blue colour of these enzymes and electron magnetic resonance (EPR) spectrum with narrow hyperfine splitting. This copper atom is designated Type 1. The other paramagnetic copper atom, designated Type 2, does not seem to have any detectable optical absorption in the visible or near ultraviolet region and has a more normal EPR spectrum. These two copper atoms are reduced when substrate is added anaerobically and reoxidized by oxygen. For these laccases it has been shown that a strong optical absorption band at 330 nm, which also was reduced by substrate and reappeared when oxygen was introduced again, was not associated with the paramagnetic copper atoms but belonged to another electron acceptor[3,4]. It has also been shown that these enzymes can accept four electrons before the absorption band at 330 nm and the EPR signals vanish. For tree laccase the 330 nm chromophore was found to be associated with a two-electron acceptor[4] and indirect evidence suggests that this chromophore in fungal laccase is also a two-electron acceptor[3,5]. It has been proposed that the two remaining copper atoms are associated with this chromophore in both laccases[3,4]. The properties of the electron acceptors in the laccases seem to hold also for ceruloplasmin, although this enzyme contains a multiple of two of each type of these copper atoms[6,7].

Other blue copper proteins, *e.g.* stellacyanin (also called *Rhus vernicifera* blue protein), contain only one copper atom which has Type 1 character and can be reduced by and reoxidized by certain agents but can not be reoxidized by molecular oxygen[2]. The biological function of these copper proteins is not known but they probably act as electron carriers in oxidation–reduction systems.

The mechanism of electron transfer is especially complicated for the laccases. All copper ions in these enzymes are probably involved in the transfer of electrons from substrate to oxygen though this does not necessarily mean that electrons flow through all these copper ions. Investigations on the role of these electron acceptors in the catalytic mechanism are currently in progress in this laboratory for fungal laccase[21]. To establish the oxidation–reduction potentials of these electron acceptors as well as the number of electrons each acceptor can take up we have performed potentiometric and optical oxidation–reduction titrations of fungal laccase, tree laccase and also with stellacyanin. The results are compared with earlier determinations of the oxidation–reduction potential of the electron acceptors in tree laccase[1] and fungal laccase[8], which were obtained under somewhat different experimental conditions and generally with more indirect techniques.

MATERIALS AND METHODS

Proteins and chemicals

Fungal laccase B was prepared from culture filtrates of the fungus *Polyporus*

versicolor as described previously[9]. Contaminating fluoride was removed from the enzyme according to a dialysis method[10]. The enzyme was stored in sodium phosphate buffer, pH 5.5, ionic strength 0.2, at —30 °C. Tree laccase and stellacyanin were prepared from acetone powder obtained from the lacquer of *Rhus vernicifera* according to the previously described method[11]. Both proteins were stored in water at —30 °C. Spectral properties of the three proteins used in this study were identical to those earlier published (for fungal laccase see refs 1 and 9, for tree laccase and stellacyanin see ref. 2).

Ascorbate was purchased from British Drug Houses, Ltd (Poole, England) and was used without further purification. Quinol was obtained from May and Baker, Ltd (Dagenham, England) and was recrystallized before use. Analytical grade potassium hexacyanoferrate (II) and (III) and NaF were obtained from Merck (Darmstadt, Germany). Potassium octacyanotungstate (IV) was prepared according to the procedure of Heintz[12] and potassium octacyanomolybdate (IV) was prepared by the method of Furman and Miller[13], both substances were obtained from Dr James A. Fee. All solutions were prepared from deionized distilled water. Other chemicals used in the experiments were of analytical grade.

Spectral measurements

Electron paramagnetic resonance (EPR) spectra were made at 77 °K and about 9 GHz in a Varian E-3 spectrometer. Optical absorption measurements were made at 25 °C in a Zeiss PMQ 20 A spectrophotometer. Visible spectra were obtained with a Zeiss M4Q II recording spectrophotometer.

Anaerobic oxidation–reduction titration technique

Oxidation–reduction titrations of the three copper proteins were carried out in a cell assembly of the type shown in Fig. 1. The cell was made anaerobic by alternatively decreasing the pressure to about 20 mm Hg and flushing with nitrogen gas which had been passed through three columns of vanadyl sulphate for removal of oxygen[5].

Addition of titrant was done through the rubber gasket (Fig. 1, F) with a Hamilton microsyringe with the needle reaching down into the solution. To obtain good homogeneity in the titrated solutions they were stirred by a small magnetic bar at the bottom of the cell. This bar was driven by a magnetic stirrer placed under the cuvette holder inside the thermostated cuvette house.

Potentiometric measurements

Oxidation–reduction potentials of the titrated copper protein mediator solutions were detected by combined metal electrodes obtained from Dr W. Ingold AG, Zürich, Switzerland. Two kinds of electrodes were used namely platinum–silver/silver chloride and platinum–calomel. Electrolytes in the silver/silver chloride and the calomel reference electrodes were a 3 M and a 1 M KCl solution, respectively. The electrodes were repeatedly tested against three different concentrations of anaerobic potassium hexacyanoferrate (II) and (III) at equal amounts and no significant changes in their electrode potentials were found during the time they were used in this study. The mean values of the electrode potentials of the calomel electrode and the silver/silver chloride electrode thus obtained were $+279.5 \pm 0.2$ mV and $+207.8 \pm 0.4$ mV,

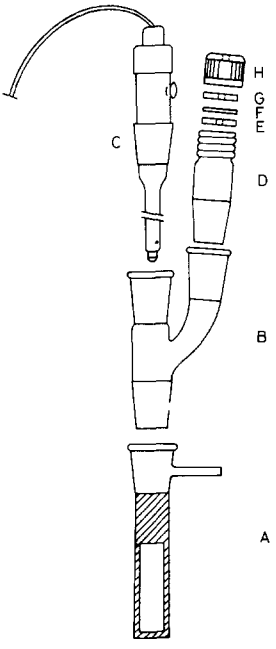

Fig. 1. Cell assembly for anaerobic optical and oxidation–reduction potential measurements. A, 10-mm light path optical quartz cuvette with a ground-glass socket and side arm for evacuation. B, connecting adaptor C, combined metal electrode with ground-glass cone (NS 14.5/15) D–H, gasket assembly made from a Quickfit screw-thread joint (D and H) two teflon washers (E and G) and a silicon rubber gas chromatography injection gasket (F). Sockets and cones with NS designation 14/23 were obtained from Quickfit. All joints were thoroughly ground with fine carborundum powder in mineral oil.

respectively. As these values are very close to the literature values of +280.7 mV and +208.3 mV[14], referred to the standard hydrogen electrode, we have used the literature values in the calculations of the oxidation–reduction potentials for the electron acceptors in the proteins. The sign convention used, is that the couple which accepts electrons from another couple is the more positive and is the oxidizing couple.

Oxidation–reduction potentials were measured with a PHM 26 potentiometer from Radiometer, Copenhagen. For the determination of the pH of the solutions the same potentiometer was used in combination with a Gk 2301 B glass electrode from the same manufacturer.

Experimental values were corrected for the dilution of protein concentration on adding titrant, the remaining absorbance when the titrated chromophore was fully reduced, and the absorbance of the oxidized and the reduced forms of the mediators used.

RESULTS

Oxidation–reduction potential of the copper atom in stellacyanin

The following mediator composition was found to give satisfactory electron transferring rate between the platinum electrode, the protein and the titrant (potentials of the oxidation–reduction chemicals under the conditions used within paren-

theses): potassium hexacyanoferrate (III)–(II) (425 mV)[15], 2,6-dichlorophenolindophenol (224 mV), EDTA and ferri–ferrous chloride (117 mV)[16]. Mediators were added in their oxidized form in concentrations listed in the legend to Fig. 2.

In Fig. 2 the results from a reoxidation experiment are shown in the form of a Nernst plot. The points follow a line with the slope of 1.0 which crosses the abscissa at 184 mV. Correction was made for 7 % loss of absorption at 605 nm apparently due to denaturation. (The loss of absorption was detected when the protein was fully reoxidized.) This apparent denaturation seemed to take place during the reduction of the protein, and the successive loss of absorption at 605 nm due to apparent denaturation during the reduction could not be corrected for and therefore the points obtained from the reduction experiment were excluded.

Optical and potentiometric equilibria of the titrated solutions were attained 20 min after each addition of reducing agent. Reoxidation equilibria were reached within 2 min after each addition of oxidant.

To test the possibility that the electron-mediating substances might have an effect on the potential of the copper ion we excluded all mediators except 2,6-dichlorophenolindophenol which was used in much less than stoichiometric amounts. However, the same oxidation–reduction potential of the copper ion was obtained although the equilibria were reached somewhat slower.

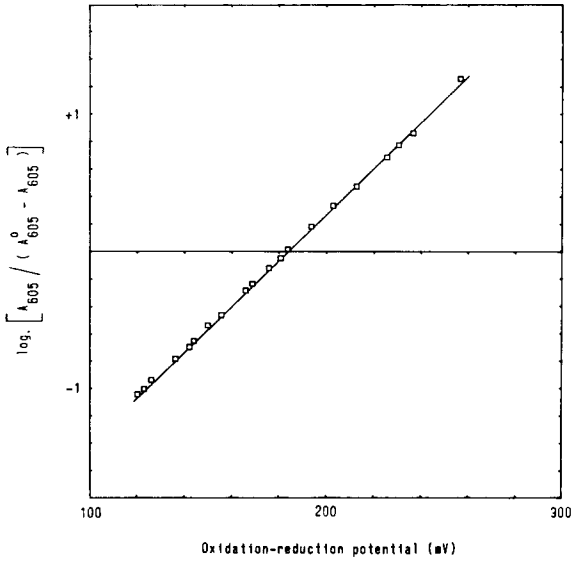

Fig 2. Oxidation–reduction potential of stellacyanin at pH 7.1, I 0.3, and 25 °C. Nernst plot of $\log A_{605}/(A^{\circ}_{605}-A_{605})$ against oxidation–reduction potential in mV. A°_{605} represents the corrected optical absorption at 605 nm of the fully oxidized protein at the beginning of the titration and A_{605} stands for the corrected absorption at this wavelength during the titration. The reaction mixture contained 3.5 ml of 0.12 mM stellacyanin, 0.09 mM potassium hexacyanoferrate(III), 0.26 mM ferrichloride, 8.6 mM EDTA and 1.4 μM 2,6-dichlorophenolindophenol in sodium phosphate buffer, pH 7.1, with a final ionic strength of 0.3. Titrations were performed at 25 °C in a cell assembly described in Fig. 1. Reduction was made with additions of small amounts of 10 mM anaerobic ascorbate in the above buffer. Reoxidation was obtained with successive additions of 3 mM anaerobic potassium hexacyanoferrate(III) in the same buffer. Correction was made for the absorption of oxidized 2,6-dichlorophenolindophenol which extinction coefficient at 605 nm was estimated to 16250 $M^{-1}\cdot cm^{-1}$. □, experimental points from the reoxidation experiment. The line is drawn with a slope of 1.0.

In some experiments about 3 % (compared to the concentration of stellacyanin) *Rhus vernicifera* laccase was added in order to reduce any oxygen that might leak into the cell. No change in the oxidation–reduction potential of the stellacyanin copper was found, however, under these conditions.

In view of the oxidation–reduction potential of the copper ion found here it is remarkable that other investigators[17] report that the copper ion in stellacyanin was rapidly reduced by quinol under similar experimental conditions as used by us. As the oxidation–reduction potential of the quinone–quinol system is about 270 mV[16] under the experimental conditions used we conclude that the stellacyanin prepared by them has an oxidation–reduction potential significantly higher than 184 mV.

Oxidation–reduction potential of the electron acceptors in Rhus vernicifera laccase

In an earlier publication from this laboratory[4] the oxidation–reduction potential of three electron acceptors in *Rhus vernicifera* laccase was estimated according to the following procedure. The titration patterns of Type 1 copper, Type 2 copper and the two-electron acceptor from reductive titrations, allowed the determination of the oxidation–reduction potential of the Type 2 copper and the two-electron acceptor, once the oxidation–reduction potential of the Type 1 copper had been determined (which was done in separate experiments with use of the oxidation–reduction buffer, potassium hexacyanoferrate (III) and (II)). The value obtained for Type 1 copper

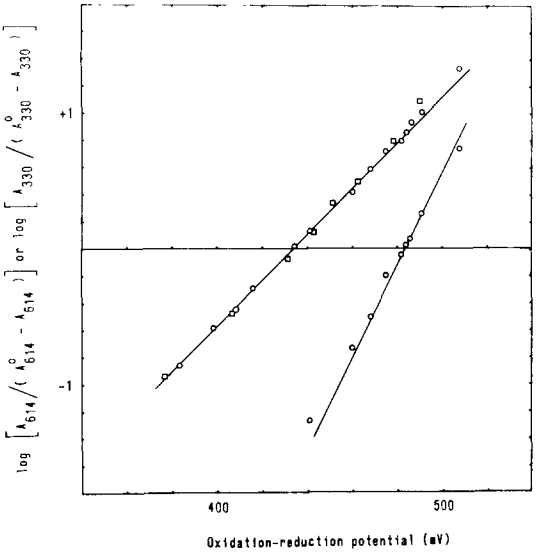

Fig. 3. Oxidation–reduction potential of electron acceptors of *Rhus* laccase in the presence of excess of hexacyanoferrate. Nernst plot of $\log A_{614}/(A°_{614}-A_{614})$ or $\log A_{330}/(A°_{330}-A_{330})$ against the oxidation–reduction potential in mV. A_{614}, $A°_{614}$, A_{330} and $A°_{330}$ stand for optical absorption at these wavelengths according to the same convention as used under Fig. 2. The cell contained 2.6 ml of a mixture of 97 μM laccase, and 290 μM hexacyanoferrate(III) in a sodium phosphate buffer, pH 7.5, ionic strength 0.2. Temperature was 25 °C. The extinction coefficients for the oxidized and the reduced hexacyanoferrate at 330 nm, used in the corrections, were estimated to 870 and 310 $M^{-1}\cdot cm^{-1}$, respectively. The lines drawn through the points have slopes of 1.0 and 2.0 for the absorbance at the 615-nm and 330-nm bands, respectively. ○, experimental points from the reduction of laccase; □, points representing reoxidation experiment

was 432 mV at pH 6.8 and ionic strength 0.35 at 25 °C. In these experiments the concentration of potassium hexacyanoferrate (III) and (II) was about 100 times as high as the protein concentration. The potentials of Type 2 copper and the two-electron acceptor were then estimated to be 30 mV lower and 40 mV higher, respectively, compared to the potential of Type 1 copper.

In the present potentiometric titrations, combined metal electrodes were used for determining the oxidation–reduction potential in the enzyme–mediator solutions. Potassium hexacyanoferrate was used as electron mediator and the estimated oxidation–reduction potential of this substance was 418 mV[15] under the experimental conditions used. Enzyme, mediator and titrants were dissolved in sodium phosphate buffer, pH 7.5, with a final ionic strength of 0.2. In Fig. 3 the results from a reductive titration with anaerobic ascorbate and an oxidative titration with oxygen-saturated buffer are shown (in some experiments quinol was used as reducing agent). The concentration of the mediator potassium hexacyanoferrate was 3 times the laccase concentration. In close agreement with the earlier determination of the oxidation–reduction potential of Type 1 copper[4] the value now obtained was 434 mV. The two-electron acceptor was found to have the oxidation–reduction potential of 483 mV under these conditions. On reoxidation of laccase the absorption at 330 nm increased more than expected, probably due to some denaturation of the protein. This unexpected increase of absorption was difficult to correct for, and was the reason for no points from reoxidation of the 330 nm chromophore being included in the plot.

If the concentration of the mediator potassium hexacyanoferrate was decreased to about one tenth of the protein concentration, the oxidation–reduction potential of Type 1 copper decreased by 40 mV to 394 mV and the potential of the two-electron acceptor decreased to 434 mV. Experimental conditions were otherwise as in the experiment described above. Optical and potentiometric equilibria were reached within 10 min after each addition of titrant.

The nature of this apparent interaction between *Rhus* laccase and hexacyanoferrate (III) and (II) is not yet known. However, the following experiments were performed in order to examine if the paramagnetic copper atoms in laccase were involved in this interaction; *Rhus* laccase, in the same phosphate buffer as in the experiment mentioned above, and 10 times excess of both hexacyanoferrate (III) and (II) were mixed under anaerobic conditions in a modified Thunberg tube. The result from EPR spectroscopy is shown in Fig. 4. Apparently, the Type 2 copper EPR spectrum disappears. The same result was obtained if the inhibitory ion fluoride in 100 times excess over protein was added before mixing the enzyme with the metal cyanides. If only hexacyanoferrate (II) was added to laccase the same effect on the EPR spectrum was obtained while hexacyanoferrate (III) alone had no effect on the EPR spectrum. Type 2 copper was slowly reoxidized when air was let in at the end of the experiments.

From these experiments it became obvious that there was an interaction, most likely a reduction of Type 2 copper. However, the interaction might be more complex and more experiments might have to be performed before an explanation of this phenomenon can be given.

In Table I the results from earlier anaerobic reductive titrations[4] and from the potentiometric titrations presented in this paper are summarized together with results from another laboratory[18]. It is to be noted that the oxidation–reduction

Biochim. Biophys. Acta, 275 (1972) 245–259

TABLE I

OXIDATION–REDUCTION POTENTIALS OF ELECTRON ACCEPTORS IN LACCASES AND STELLACYANIN

Sodium phosphate buffers were used in all experiments. For details about the electron mediators used in the different experiments, see the text. Oxidation–reduction potentials have been determined with the use of three different methods. P, B and R stand for potentiometric titrations, use of redox buffers and reductive titrations, respectively.

Protein	*pH*	*I*	*Concn. ratio, mediator to protein*	*Oxidation–reduction potential (mV)*			*Method*	*References*
				Type 1 copper	*Type 2 copper*	*Two-electron acceptor*		
Stellacyanin	7.1	0.3	*	184			P	This paper
Rhus laccase	7.5	0.2	0.1	394	≈365	434	P, R	This paper and (4)
	7.5	0.2	3	434	—	483	P	This paper
	6.8	0.35	100	432	—	—	B	(4)
	6.8	0.56	100	432	—	—	B	(18, 4)
+ 10 mM NaF	7.5	0.41	100	436	—	—	B	(4)
	7.5	0.3	0.07	390	≈390	≈390	P, R	This paper and (4)
Polyporus laccase	5.5	0.2	6.4	775	—	—	P	This paper
	5.5	0.2	0.3	785	—	782	P	This paper
	6.25	0.2	≈40	767	—	—	B	(8)
+ 1 mM NaF	5.5	0.2	0.3	780	—	570	P	This paper

* Mediator composition could be changed without effect on the potential.

potential of Type 1 copper was not significantly different, despite the differences in ionic strength, pH or the presence of excess of fluoride, in the experiments where excess hexacyanoferrate was present.

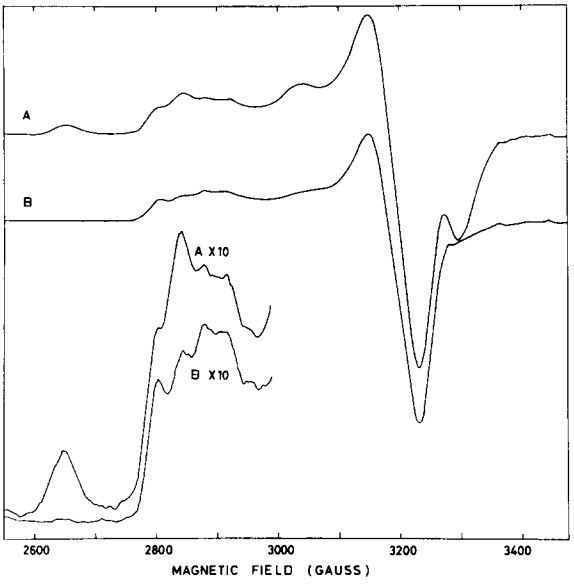

Fig 4. Effect of hexacyanoferrate(II) on the electron paramagnetic resonance spectrum of *Rhus* laccase recorded at 77 °K and 9.20 GHz. (A) Spectrum of 0.15 mM native laccase in sodium phosphate buffer, pH 7.5 and ionic strength 0.2. (B) Spectrum of the sample used in (A) frozen immediately after mixing of 2 mM of both hexacyanoferrate (II) and (III). A modified Thunberg tube with a 3-mm bore quartz tube attached was used in these anaerobic EPR titrations. Laccase was added to the bottom and the cyanides were contained in the bulb at the top The system was made anaerobic according to the procedure described under Materials and Methods After mixing the solutions for about 10 s the mixture was quickly frozen in liquid nitrogen. The difference in intensity of the EPR signal from Type 1 copper (between (A) and (B)) is due to dilution on adding the cyanide solution.

Oxidation–reduction potential of fungal laccase B

It was earlier found by workers in this laboratory that Type 1 copper in fungal laccase had an unusual high oxidation–reduction potential. When the enzyme was mixed under anaerobic conditions with various proportions of potassium octacyanomolybdat (IV) and (V), in about 30–40 times excess over protein, a potential of 767 mV was calculated for the Type 1 copper[8].

In view of the results with tree laccase (see above), where hexacyanoferrate (II) was found to have such a marked effect on the potentials of Type 1 copper and the two-electron acceptor, associated with the 330 nm chromophore, we have reinvestigated the fungal enzyme with respect to the oxidation–reduction potential of both these electron acceptors in the presence of excess, or less than stoichiometric amounts, of some metal cyanides.

In these potentiometric titrations of fungal laccase the following electron mediators, covering a potential range from about 400 to 800 mV, were found to give satisfactory electron-mediating capacity. Oxidation–reduction potentials of the redox couples at the experimental conditions used are given within parentheses.

Potassium hexacyanoferrate(III) (II) (418 mV)[15], potassium octacyanomolybdate (V)(IV) (778 mV)[19] and potassium octacyanotungstate(V)(IV) (510 mV)[20].

In the presence of a total excess of mediators, of a factor 6.4 compared to the enzyme concentration, the Type 1 copper titrated with a slope of 1 in a Nernst plot and with a midpoint potential of 775 mV. (The ratio between the three metal cyanides was the same as in the experiment described in the legend to Fig. 5.) This potential is close to the previously published value[8], which, on the other hand, was obtained at pH 6.2 and with only octacyanomolybdate (V) and (IV) present. With the concentration of the mediators used in the present experiment the absorption in near ultraviolet increased so much, depending on the absorption of the added mediators, that it was not possible to obtain a reliable value of oxidation–reduction potential of the 330 nm chromophore. Potential and optical absorbance equilibria were accomplished within seconds after addition of oxygen-saturated buffer and reduction equilibria were restored within 5 min after each addition of ascorbate.

When the total mediator concentration was decreased to about one third of

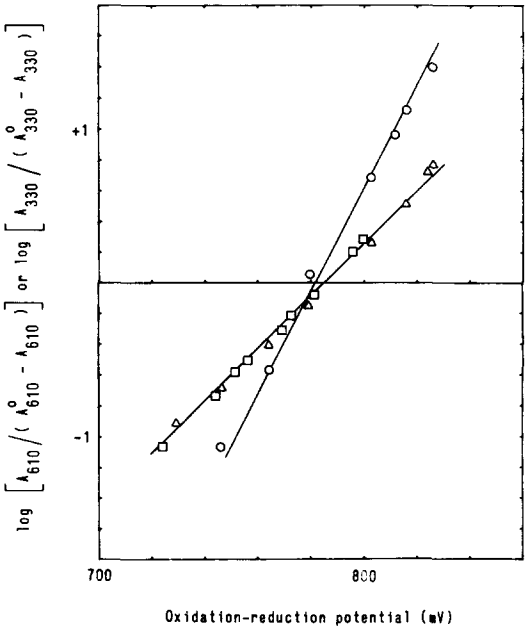

Fig. 5. Oxidation–reduction potentials of Type 1 copper and the two-electron acceptor in fungal laccase B. Nernst plot of log $A_{610}/(A°_{610}-A_{610})$ or log $A_{330}/(A°_{330}-A_{330})$ against the potential in mV. For the explanation of the symbols used see under Fig. 2. The anaerobic cell (see Fig. 1) contained 2.3 ml of a mixture of 0.13 mM fungal laccase B, 0.02 mM of each of octacyanomolybdate (V) and octacyanotungstate (IV) and 0.01 mM hexacyanoferrate (III) dissolved in sodium phosphate buffer with an ionic strength of 0.2 and pH 5.5. Temperature was maintained at 25 °C. Reduction was made by adding small amounts of 10 mM anaerobic sodium ascorbate with a microsyringe. Ascorbate was dissolved in the same buffer as mentioned above. Reoxidation was obtained by adding oxygen-saturated buffer ○, experimental points from the reduction of the 330-nm chromophore; △, experimental points from the reduction of Type 1 copper; □, experimental points obtained on reoxidation of Type 1 copper. The extinction coefficients at 330 nm for the oxidized and the reduced octacyanomolybdate, used in the correction of observed absorbances, were assumed to be 800 $M^{-1}\cdot cm^{-1}$ and 150 $M^{-1}\cdot cm^{-1}$, respectively. Lines with slopes of 1.0 and 2.0 are drawn through the experimental points obtained for the 610-nm and the 330-nm chromophores, respectively.

the enzyme concentration the oxidation–reduction potential of Type 1 copper was slightly higher, namely 785 mV (Fig. 5). The 330 nm chromophore was found to be a two-electron acceptor, as in the tree laccase, and this has previously been suggested on the basis of indirect evidence[3,5]. This two-electron acceptor has an oxidation–reduction potential of 782 mV. Apparently, the finding from previously performed reductive titrations that these two chromophores in fungal laccase were reduced together[3] is explained by the fact that their oxidation-reduction potentials are so similar, and is not due to a cooperativity between these electron acceptors as has been suggested[5].

With this concentration of electron mediators the equilibria were obtained within 15 min after each addition of oxidant and 20 min after each addition of reductant, respectively.

Upon reoxidation of the enzyme the absorbance at 330 nm increased somewhat more than expected, probably due to some denaturation. Therefore no points from the reoxidation of the 330-nm chromophore were included in the plot in Fig. 5.

In the presence of ten times excess of the inhibitor fluoride and the same concentration of the electron-mediating metal cyanides and enzyme as in the experiment shown in Fig. 5, there was a remarkable change in the potential of the two-electron acceptor. The results from one such experiment are shown in Fig. 6. The oxidation–reduction potential of the two-electron acceptor decreased to 570 mV while the potential of Type 1 copper was still in the same range as in the absence of fluoride, namely 780 mV. These results confirm earlier reductive titrations in the presence of fluoride, where it was found that Type 1 copper was completely reduced before the

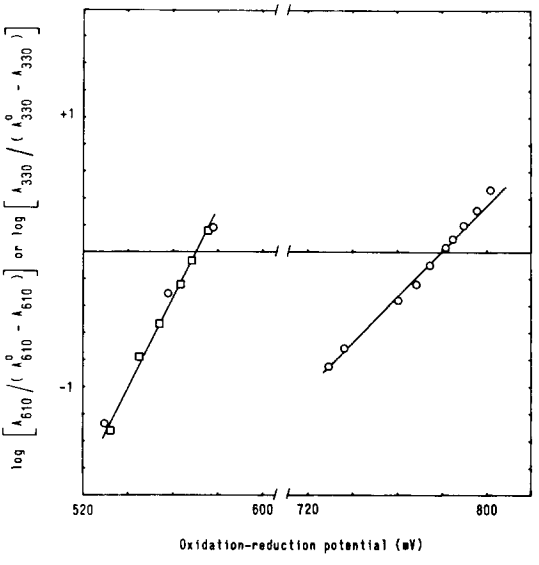

Fig. 6. Oxidation–reduction potentials of Type 1 copper and the two-electron acceptor in fungal laccase B in the presence of excess fluoride. Nernst plot of log $A_{610}/(A°_{610}-A_{610})$ or log $A_{330}/(A°_{330}-A_{330})$ against the potential in mV. The experimental conditions were identical to those in Fig. 5 except 1 mM NaF was added to the enzyme solution. ○, experimental points from the reduction of the enzyme; □, points representing reoxidation of enzyme. Lines with slopes of 1.0 and 2.0 are drawn through the experimental points obtained for the 610-nm and 330-nm chromophores, respectively.

330-nm chromophore started to reduce[3]. The time needed for reaching equilibrium on adding reductant was in the range of 10–40 min. Reoxidation of the two-electron acceptor was accomplished within 10–15 min, while reoxidation of Type 1 showed a complicated pattern. The blue colour first increased rapidly after addition of oxygen-saturated buffer and then it decreased very slowly. These reactions of Type 1 copper in the presence of fluoride have also been observed in stopped-flow studies, under about the same experimental conditions, performed recently in our laboratory (L. E. Andreasson, C. Strömberg and T. Vänngård, unpublished). Due to the uncertainty this slow process has on the determination of the potential during reoxidation of this copper atom, no points from the reoxidation of Type 1 copper are included in the plot. The results obtained in this study and those of other investigators are summarized in Table I.

DISCUSSION

Although all three proteins examined in this study contain one copper atom each with Type 1 character, according to spectroscopic properties[1,2], the oxidation–reduction potential of this copper atom in the different proteins shows a great variation. For example, the difference in potential between the copper atom in stellacyanin and Type 1 in *Rhus* laccase or *Polyporus* laccase is about 200 mV and 600 mV, respectively. The structural differences between the proteins, responsible for this variation in oxidation–reduction potential of this copper atom, is not known. However, there is an obvious difference between stellacyanin and the laccases in that stellacyanin contains only one copper atom, which probably is the only electron-accepting site in this molecule, while the laccases have two more electron acceptors with which their Type 1 copper atom supposedly stands in dynamic equilibrium. This interaction with other electron-accepting sites might affect the oxidation–reduction potential of this copper atom. For example, as shown in Fig. 4 the state of Type 2 copper in *Rhus* laccase is changed in the presence of hexacyanoferrate (II). This change, in turn, affects the oxidation–reduction potentials of Type 1 copper and the two-electron acceptor.

In both laccases, the 330-nm chromophore was found to be associated with a two-electron acceptor. Furthermore, in both laccases, the oxidation–reduction potential of this electron acceptor is found to have the highest potential of the electron acceptors present in the *Rhus* enzyme or as high as Type 1 (in the *Polyporus* enzyme) under native conditions. Also in ceruloplasmin the potential of the 330-nm chromophore is probably higher than the Types 1 and 2 copper atoms, as it reduces first in reductive titrations in acetate buffer at pH 6.0 (ref. 7). In the presence of the inhibitor fluoride the oxidation–reduction potential of the 330-nm chromophore decreases, as shown in this paper for the two laccases, and this seems to be the case also for ceruloplasmin.

It has been suggested that the electrons taken up by oxygen enter this molecule *via* the 330-nm chromophore, which in its turn reoxidizes Type 1 copper, and maybe also Type 2, if this acceptor becomes reduced in the catalytic process[21]. The finding that the 330-nm chromophore has the highest potential among the electron acceptors in *Rhus* laccase and in ceruloplasmin, and about the same potential as Type 1 copper in *Polyporus* laccase, supports this suggestion for the following reasons: First, from a

thermodynamic point of view, electrons from reduced Types 1 and 2 will tend to flow to an electron acceptor which has a more positive oxidation–reduction potential than these electron acceptors. Secondly, the effect of the interaction with the inhibitor fluoride, in all three enzymes, is to lower the oxidation–reduction potential of the 330-nm chromophore relative to Type 1 copper. For *Polyporus* laccase the difference in oxidation–reduction potential amounts to about +200 mV in the fluoride-treated enzyme. Thus, the transfer of electrons from reduced Type 1 copper to the 330-nm chromophore is energetically unfavourable, which might explain the observation that the reoxidation of Type 1 copper in *Polyporus* laccase is very slow in the presence of fluoride (see above and L. E. Andreasson, C. Strömberg and T. Vänngård, unpublished) although the reduction of this copper atom by substrates was not affected by fluoride[22].

The detection of Type 2 copper is only possible with EPR spectroscopy and as the technique is complicated the potential of this copper atom can only be determined with difficulty. This is the reason why the potential of this copper atom has not yet been determined for *Polyporus* laccase and ceruloplasmin. For *Rhus* laccase it could be determined in reductive titrations[4] as its oxidation–reduction potential was not very different from the potential of the other electron acceptors. However, the relative potential of Type 2 compared to the other electron acceptors in *Polyporus* laccase and in ceruloplasmin can be estimated from published reductive titration patterns of the three electron acceptors in these enzymes[5,7]. The results from these titrations and the results from *Rhus* laccase[4] show that Type 2 copper has the lowest oxidation–reduction potential among the electron acceptors in the native enzymes. Also in the fluoride-treated enzymes its potential is lowest, except for *Rhus* laccase where it seems to have about the same potential as the other electron acceptors[4]. For Type 2 copper in *Rhus* laccase this means that its oxidation–reduction potential is increased by about 30 mV, while results from the titration of *Polyporus* laccase indicate that its oxidation–reduction potential is decreased on binding of fluoride to the enzyme[3,5].

Apparently, the state of this copper atom has some effects on the oxidation–reduction potential of the other electron acceptors in all three enzymes. As already mentioned above, hexacyanoferrate(II) seems to interact with Type 2 in *Rhus* laccase in a way that changes the potentials of Type 1 copper and the two-electron acceptor in this enzyme. Further, in the presence of the inhibitor fluoride, which is found to bind very strongly to Type 2 in *Polyporus* laccase[5] and presumably interacts similarly with this copper atom in *Rhus* laccase[2] and ceruloplasmin[6], the oxidation–reduction potential of the two-electron acceptor decreases in both laccases (this paper) and the potential of the 330-nm chromophore in ceruloplasmin changes relative to the potential of Type 1[7]. However, fluoride was present in excess in those experiments and it can not be excluded that this ion interacted with other sites than Type 2 copper in these enzymes.

The disappearance of the Type 2 copper EPR signal in *Rhus* laccase in the presence of hexacyanoferrate(II) is probably due to a reduction of this atom. Although there was about 10 times hexacyanoferrate(II) compared to enzyme there was no sign of reduction of Type 1 copper in about 3 min at room temperature. The EPR spectrum remaining after treatment with hexacyanoferrate(II) is rather similar to a spectrum published by others[23]. In their EPR spectrum there is also a signal apparently generated by small amounts of a radical present. These observations suggest

that something in their enzyme solution reduces Type 2 copper specifically, possibly in a similar way as hexacyanoferrate(II) does. The presence of a radical signal in their EPR spectrum might then come from the oxidation product of this unknown reducing agent.

The oxidation–reduction potential of the 615-nm (Type 1 according to our classification) and 330-nm chromophores in *Rhus* laccase were also determined by others[24]. They found that the 330-nm chromophore titrated as a one-electron acceptor although they suggested that two or three Cu^{2+} were associated with this chromophore. The oxidation–reduction potential of this chromophore was found to be 45 mV higher than the potential of the 615-nm chromophore which, in its turn, was supposed to have an oxidation–reduction potential of 415 mV. The potential of this latter chromophore was determined with use of the hexacyanoferrate(III) and (II) system[18]. As the potential of this system is dependent on the ionic strength of the solution[15] the oxidation–reduction potential of the 615-nm chromophore should be about 432 mV in their system[4]. We have obtained the same value under similar conditions (see above), *i.e.* in the presence of excess hexacyanoferrate(II). In their enzyme preparation various amounts of cuprous copper were also present, which was supposed to belong to inactive enzyme molecules. This cuprous ion could not be oxidized by oxygen but was reoxidized with hydrogen peroxide[24]. The reoxidation of this cuprous copper was associated with a strong increase of absorbance in the 330 nm region. We have tested if our enzyme preparation, used in this study, behaved in the same way when treated with hydrogen peroxide. No increase in the 330-nm optical region was found, however, when equimolar amounts of hydrogen peroxide was added to the enzyme in sodium phosphate buffer (pH 7.5, I 0.2) (B. Reinhammar, unpublished observations).

The finding that both laccases have a two-electron acceptor and two one-electron acceptors, as well as the determination of the oxidation–reduction potentials for these electron acceptors under various conditions, are vital discoveries when a model for the catalytic mechanism is considered. The sequence of the reduction and re-oxidation of the various acceptors which are involved when the electrons flow from substrate to oxygen might be determined in kinetic experiments. For fungal laccase extensive studies have recently been performed with use of stopped-flow technique (L. E. Andreasson, C. Strömberg and T. Vänngård, unpublished) and similar studies on the tree laccase have been initiated in this laboratory.

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