Pulse-radiolysis studies on the interaction of one-electron reduced species with blue oxidases

Reduction of native and Type-2-copper-depleted Vietnamese-lacquer-tree and Japanese-lacquer-tree laccases

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The interactions of one-electron reduced metronidazole ($\text{ArNO}_2^{\cdot-}$) and $\text{O}_2^{\cdot-}$ with native and Type-2-copper-depleted Vietnamese- and Japanese-lacquer-tree laccases were studied in aqueous solution at pH 6.0 and 7.4 by using the technique of pulse radiolysis. On reaction with $\text{ArNO}_2^{\cdot-}$, in the absence of O_2 , the holo- and the Type-2-copper-depleted proteins accept, with reduction of Type 1 copper, 2 and 1 reducing equivalents respectively. On reaction with $\text{O}_2^{\cdot-}$ of both holo- and Type-2-copper-depleted Vietnamese-lacquer-tree laccase, almost complete reduction of Type 1 copper was observed and, after completion of the reaction, some (less than 20%) re-oxidation of Type 1 copper occurs. Reduction of Type 1 copper of the laccases by these one-electron donors occurs via a bimolecular step; however, the rate of reduction of Vietnamese-lacquer-tree laccase is over 10 times that of Japanese-lacquer-tree laccase. It is inferred that electrons enter the protein via Type 1 copper with, in the case of the holoprotein, subsequent rapid intramolecular transfer of 1 reducing equivalent within the protein. Furthermore it is suggested that intramolecular electron transfer to Type 3 copper atoms is slow and, in the case of Type-2-copper-depleted protein, may not occur. This slow process may partially account for the variation of the catalytic activities of 'blue' oxidases.

Blue oxidases are copper-containing enzymes that catalyse the transfer of four electrons from reducing substrates to O_2 molecules with formation of water. The mechanism of the transfer has been extensively studied in laccases, which are the simplest blue oxidases (Reinhammar & Malmström, 1981). They contain four copper centres, the 'blue' paramagnetic Type 1 copper, so called because of a strong charge-transfer absorption at about 600 nm ($\epsilon = 400\text{--}600\text{ mm}^{-1} \cdot \text{cm}^{-1}$), the paramagnetic Type 2 copper, with normal spectroscopic properties, and the Type 3 copper, a pair of magnetically coupled Cu^{2+} ions. In ascorbate oxidase, a more complicated protein that contains eight copper atoms per molecule (50% paramagnetic with a 3:1 ratio of Type 1 to Type 2 copper), the high reaction rate with its specific substrate, ascorbate, has prevented mechanistic studies with the use of conventional techniques for observation of rapid processes, as used in the studies of laccase. However, by employing the

technique of pulse radiolysis it was shown that this protein is reduced by nitroaromatic radical anions in a bimolecular process involving the 'blue' copper centres and a rapid intramolecular transfer of electrons to the other copper centres (O'Neill *et al.*, 1983). With laccase from the Japanese lacquer tree (*Rhus vernicifera*) the reduction by ascorbate or quinol (hydroquinone) was found by using the technique of rapid mix to involve initial transfer of single electrons from the substrate to the Type 1 copper and subsequently to the Type 2 copper (Andréasson & Reinhammar, 1976). Only at this stage, it was proposed, may the two electrons be simultaneously transferred to the Type 3 copper, but at a low rate, which was suggested to be inconsistent with the catalytic process (Andréasson & Reinhammar, 1976).

It was therefore decided to investigate the interaction of laccase with one-electron reduced species generated by pulse radiolysis and to extend this study to laccase from the Vietnamese lacquer tree (*Rhus succedanea*), in an attempt to establish whether differences in the reduction mechanism are to be ascribed to the protein, to the substrate, or

Abbreviation used: $\text{ArNO}_2^{\cdot-}$, radical anion of metronidazole.

to the method of investigation employed. The Vietnamese laccase has, as yet, received little attention, but from available data it is suggested (Brändén & Deinum, 1978) that it has very similar properties to those of the Japanese species.

Materials and methods

The acetone-dried powders from the latex of the Japanese lacquer tree (*Rhus vernicifera*) and of the Vietnamese lacquer tree (*Rhus succedanea*) were purchased from Saito and Co. (Osaka, Japan). The Japanese and Vietnamese proteins were purified by the methods of Reinhammar (1970) and of Brändén & Deinum (1978) respectively. For the Vietnamese laccase, the fraction eluted from the DEAE-Sephadex column with 0.2M-sodium phosphate buffer, pH 5.5, was employed after a further purification step, consisting of a passage of the protein in 20mM-sodium phosphate buffer, (pH 6.0) through a CM-Sephadex C-50 column equilibrated with the same buffer. Some yellow material was retained on top of the column, and the A_{330}/A_{610} ratio for the purified protein was 0.9. The Type-2-copper-depleted Japanese laccase was prepared as previously described (Morpurgo *et al.*, 1980). The same method was successfully used for the preparation of the Type-2-copper-depleted Vietnamese laccase. Optical and e.p.r. spectra were recorded on a Beckman 5230 UV spectrophotometer and on a Varian E-9 spectrometer respectively. Removal of Type 2 copper resulted in a decrease in A_{330} (Morpurgo *et al.*, 1980).

Ascorbate oxidase activity was tested by the decrease of ascorbate absorbance at 305 nm ($\epsilon = 16 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) in 0.1M-sodium phosphate buffer at pH 6.0 and 7.4 and in the presence of 0.5mM-EDTA. Ferrocyanide oxidase activity was monitored at 420 nm ($\epsilon = 100 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) in the same buffers made up to 20mM in K_2SO_4 in order to minimize the effect of ionic-strength changes at different substrate concentrations.

The pulse-radiolysis set-up, irradiation conditions and dosimetry details were as previously described (O'Neill *et al.*, 1983). All solutions were prepared from triple-distilled water, buffered at pH 6.0 or 7.4 with 10mM-sodium phosphate buffer, and contained 0.1mM-EDTA, 0.1M-formate and laccase, the concentration of which was generally expressed in terms of protein concentration. Methods employed for the production of $\text{O}_2^{\cdot -}$ and one-electron reduced nitroaromatics have been described previously (O'Neill *et al.*, 1983).

Metronidazole [2-(2-methyl-5-nitroimidazol-1-yl)ethan-1-ol] was supplied by May and Baker. All other reagents used were of AnalaR grade from BDH Chemicals.

Results

Interaction of $\text{ArNO}_2^{\cdot -}$ with Japanese laccase and Vietnamese laccase

On pulse irradiation of de-aerated aqueous solutions containing 1mM-metronidazole in the absence and in the presence of laccase (2.2–9.0 μM) at pH 7.4, optical-absorption changes with time, as presented in Fig. 1 for the Vietnamese laccase, were measured at 615 nm and at 400 nm, the wavelength at which $\text{ArNO}_2^{\cdot -}$ absorbs. From a comparison of these changes at 400 nm in the presence and in the absence of Japanese laccase or Vietnamese laccase, the number of reducing equivalents accepted by the laccases was determined at both pH 7.4 and 6.0 and is presented in Table 1. Under these conditions, full bleaching of the absorption at 615 nm, due to Type 1 Cu^{2+} , was observed and approx. 2 reducing equivalents/mol of holoprotein are accepted in the absence of O_2 at the pH values investigated. In the case of the Japanese laccase, the time required to fully bleach the protein, as monitored at 615 nm, is about one order of magnitude greater than that determined in the equivalent system containing Vietnamese laccase. With the Japanese laccase large errors are associated with the stoichiometric determination, since the lifetime of $\text{ArNO}_2^{\cdot -}$ (see, e.g., Fig. 1a) is not greatly decreased in the presence of the protein at the concentrations used. Under identical conditions, the number of reducing equivalents accepted by Type-2-copper-depleted Vietnamese laccase on interaction with $\text{ArNO}_2^{\cdot -}$ was determined at 400 nm and is presented in Table 1. Since full bleaching of the optical absorption at 615 nm was observed, it is inferred from the loss of absorption at 615 nm that the 1 electron equivalent accepted by Type-2-copper-depleted Vietnamese laccase may be accounted for in terms of reduction of only the Type 1 copper.

The second-order rate constant for interaction of $\text{ArNO}_2^{\cdot -}$ with both the holo- and Type-2-depleted Vietnamese laccase was determined to be $1.2 (\pm 0.3) \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ from the dependence of the rate of change of optical absorption at 400 nm and 615 nm on $[\text{ArNO}_2^{\cdot -}]$ (15–33 μM) in the presence of 5.3 μM -laccase at pH 7.4. It is estimated that the rate constant for the interaction of $\text{ArNO}_2^{\cdot -}$ with Japanese laccase is less than $2 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$.

After a train of radiation pulses (12 pulses at 50 pulses/s), bleaching of optical absorption at 330 nm is also observed on interaction of $\text{ArNO}_2^{\cdot -}$ with both the holo- and Type-2-copper-depleted protein. The extent of bleaching at 330 nm is only approx. 10% of that observed at 615 nm. Owing to the poor quality of the traces at 330 nm, accurate kinetic analysis was not possible. However, the slight bleaching at 330 nm was observed to occur

Fig. 1. Absorbance changes with time observed at pH 7.4 on pulse irradiation of a metronidazole solution (1 mM) containing formate (0.1 M) and Vietnamese laccase and saturated with N_2O

Traces (a) and (b) were monitored at 400 nm in the absence (a) and in the presence (b) of 5.2 μM Vietnamese laccase. Trace (c) was monitored at 615 nm in the presence of 5.2 μM Vietnamese laccase. The dose/pulse was equivalent to production of approx. 18 μM - $ArNO_2^{\cdot-}$.

Table 1. Numbers of reducing equivalents accepted by Japanese laccase and Vietnamese laccase from $ArNO_2^{\cdot-}$ determined at pH 6.0 and 7.4 on pulse irradiation of laccase solutions (2.2–9.0 μM) containing metronidazole (1 mM) and formate (0.1 M) and saturated with N_2O

Protein	Reducing equivalents	
	pH 7.4	pH 6.0
Vietnamese laccase	1.9 ± 0.1	2.0 ± 0.2
Japanese laccase	2.0 ± 0.5	N.D.*
Type-2-copper-depleted	1.2 ± 0.1	1.0 ± 0.1

* Not determined: natural lifetime of $ArNO_2^{\cdot-}$ at pH 6.0 is less than that at pH 7.4.

on the same time scale as that for the bleaching of optical absorption at 615 nm

Interaction of superoxide with laccase

The interaction of both holo- and Type-2-copper-depleted Vietnamese laccase with $O_2^{\cdot-}$ was studied at pH 7.4 and not pH 6.0 owing to the increased natural lifetime of $O_2^{\cdot-}$ ($t_1 > 1$ s) at pH 7.4. The study was confined mainly to Vietnamese laccase since the reaction of Japanese

laccase with $O_2^{\cdot-}$ is inefficient (Goldberg & Pecht, 1978). Fig. 2 presents the optical absorption changes with time at 615 nm on pulse irradiation, with a train of ten radiation pulses (20 μM - $O_2^{\cdot-}$ /pulse), an oxygenated solution containing 4.7 μM Vietnamese laccase $\Delta A_{615}/A_{615}^{total}$ was determined to represent 82% bleaching of the total bleachable absorption and was found to be independent of $[O_2^{\cdot-}]$ (train of 15 radiation pulses each producing either 20, 31 or 40 μM - $O_2^{\cdot-}$), the concentration of O_2 (aerated or oxygenated) and the concentration of laccase (4.5–10 μM). As shown in Fig. 2, under the conditions used approx. 30 ms (three radiation pulses) is required to establish equilibrium conditions. After completion of the interaction of $O_2^{\cdot-}$ with Vietnamese laccase, approx. 20% restoration of optical absorption at 615 nm was observed within 2 s; however, this was not investigated further owing to the possible involvement of H_2O_2 produced during the irradiation and produced on dismutation of $O_2^{\cdot-}$ when $[O_2^{\cdot-}]$ is in excess of [laccase].

It is estimated from the rate of bleaching of optical absorption at 615 nm that the rate constant for interaction of $O_2^{\cdot-}$ (20–40 μM) with Vietnamese laccase (16–30 μM) at pH 7.4 is approx. 9

$(\pm 2) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. Under these conditions, the concentration of radiolytically generated H_2O_2 is an order of magnitude less than that of the laccase and is therefore not involved in the initial bleaching process observed at 615 nm, in agreement with previous observations on the time scale of the interaction of H_2O_2 with laccase (Farver *et al.*, 1976, 1982).

On interaction of $\text{O}_2^{\cdot-}$ (six radiation pulses each producing $30 \mu\text{M-O}_2^{\cdot-}$) with Type-2-copper-depleted Vietnamese laccase ($4.5 \mu\text{M}$), over 95% bleaching of the total bleachable absorption at 615 nm was observed. In contrast with the observations with the holo-laccase, no restoration of optical absorption is apparent within 2 s after completion of the interactions of Type-2-copper-depleted laccase with $\text{O}_2^{\cdot-}$. The rate of bleaching

Fig. 2. Absorbance changes with time observed at 615 nm on pulse irradiation of a Vietnamese laccase solution ($4.7 \mu\text{M}$) containing formate (0.1 M) and oxygenated at pH 7.4

A train of ten radiation pulses at a repetition rate of 100 pulses/s was delivered to the solution as indicated. Each pulse produced approx. $20 \mu\text{M-O}_2^{\cdot-}$.

of optical absorption at 615 nm was observed to be on the same time scale as that for the bleaching of the holoprotein on interaction with $\text{O}_2^{\cdot-}$. It is estimated that the rate constant for interaction of $\text{O}_2^{\cdot-}$ with Type-2-copper-depleted laccase is similar to that determined for the interaction with the holoprotein.

From observations at 330 nm, it is apparent that interaction of $\text{O}_2^{\cdot-}$ with Vietnamese laccase ($4.1 \mu\text{M}$) does not result in optical-absorption changes related to the protein within the time of observation of 2 s. Indeed, a slight increase in absorption at 330 nm was observed both in the presence and in the absence of the protein with $\text{O}_2^{\cdot-}$ as the redox substrate.

Steady-state kinetics

In Table 2 are reported k_{cat} values for Japanese laccase and Vietnamese laccase and for ascorbate oxidase (L. Avigliano, A. Finazzi Agrò, B. Mondovì & E. Antonini, unpublished work) relative to the oxidation of ascorbate and ferrocyanide. Of the two laccases, the Vietnamese one is more active by at least one order of magnitude. Both types of laccase are considerably less active than ascorbate oxidase when ascorbate is used as substrate, whereas the activities are more similar when ferrocyanide is used as substrate. The activity of Vietnamese laccase, with either ferrocyanide or ascorbate as substrate, is lowered by 0.1 M -formate to 60% of that of the native enzyme at both pH 6.0 and 7.5. Japanese laccase, however, is inhibited to the same extent as Vietnamese laccase at pH 6.0, but it is not inhibited at pH 7.5. Since most of the radiolysis studies were performed with Japanese laccase at pH 7.4, formate inhibition should not interfere. Furthermore, the stoichiometries of reduction of the laccases (see Table 1) are independent of pH and do not, therefore, reflect a difference due to formate inhibition as apparent in the steady-state experiments.

Table 2. Ascorbate oxidase and ferrocyanide oxidase activities of Japanese laccase, Vietnamese laccase and ascorbate oxidase determined at pH 6.0 and 7.5

	Ascorbate		Ferrocyanide	
	k_{cat} (s^{-1})	K_m (M)	k_{cat} (s^{-1})	K_m (M)
pH 6.0				
Japanese laccase	1.5	1.0×10^{-2}	20	5.0×10^{-2}
Vietnamese laccase	30	1.7×10^{-2}	220	2.2×10^{-2}
Ascorbate oxidase*	7500	2.0×10^{-4}	25	3.0×10^{-3}
pH 7.5				
Japanese laccase	8.2	5.9×10^{-2}	2.3	1.0×10^{-2}
Vietnamese laccase	88	6.0×10^{-3}	78	1.1×10^{-2}
Ascorbate oxidase*	Unaffected by pH		1.7	5.0×10^{-3}

* L. Avigliano, A. Finazzi Agrò, B. Mondovì & E. Antonini (unpublished work).

Discussion

From stopped-flow data on the anaerobic reduction of the Japanese laccase it was concluded that reduction occurs via the Type 1 copper (Andréasson & Reinhammar, 1976). Subsequently Type 2 copper is reduced and only at this stage may two electrons be simultaneously transferred from the Type 1 and Type 2 copper atoms to the Type 3 copper, in a relatively slow process (Andréasson & Reinhammar, 1976). The experimental observations in the present study are consistent with this scheme for reduction of both Japanese laccase and Vietnamese laccase, since only 2 reducing equivalents/mol of protein are consumed and complete reduction of the Type 1 copper occurs at pH 6.0 and 7.4 (see the Results section, concerning formate inhibition). It is inferred that transfer of the two electrons to the Type 3 copper does not occur before consumption of the excess $\text{ArNO}_2^{\cdot-}$. Entry of the electrons into the protein could occur via Type 1 copper with a subsequently rapid intramolecular electron transfer to Type 2 copper, or, alternatively, via Type 1 and Type 2 copper atoms that are reduced independently of each other at the same rate. However, stoichiometric reduction of Type 1 copper of the Type-2-copper-depleted Vietnamese laccase, in agreement with previous findings with Japanese laccase (Andréasson & Reinhammar, 1979), is consistent with Type 1 copper being identified as the primary acceptor site for the electrons in the absence of Type 2 copper. Furthermore, since the rate of reduction of the Type-2-copper-depleted protein by $\text{ArNO}_2^{\cdot-}$ is similar to that for reduction of the holoprotein, it is inferred that the initial interaction of $\text{ArNO}_2^{\cdot-}$ with laccase is independent of the presence of Type 2 copper. Subsequent intramolecular transfer of electrons to the Type 3 copper atoms for both the holo- and Type-2-copper-depleted proteins, in agreement with previous findings (Reinhammar & Malmström, 1981), is slow and, in the case of the Type-2-copper-depleted protein, may not occur. This is consistent with the small changes in absorption observed at 330 nm, a wavelength at which Type 2/3 copper atoms absorb in the holoprotein. Since the absorption changes at 330 nm are similar for both proteins, conclusions concerning the oxidation state of the Type 3 copper atoms cannot be drawn. Indeed, reduction of Type-2-copper-depleted laccase has been shown to result in only minor absorbance changes at 330 nm even though the protein accepts approx. 3 reducing equivalents with ascorbate and other reductants (Morpurgo *et al.*, 1980; Frank *et al.*, 1983). Furthermore, intramolecular redistribution of the electrons within the Type-2-copper-depleted protein has been demonstrated not to occur on the time scale of the experiments presented in this

study (Morpurgo *et al.*, 1980; Frank *et al.*, 1983).

In the present study with $\text{ArNO}_2^{\cdot-}$ as reducing agent, no effect of pH (6.0 and 7.4) was apparent with respect to the kinetics and stoichiometry of the reduction process. Previous pH-dependences may, indeed, reflect an increase in reactivity on deprotonation of quinol, the substrate used by Reinhammar & Malmström (1981).

The Type-1-copper absorption of Vietnamese laccase is also bleached efficiently by $\text{O}_2^{\cdot-}$ (approx. 80% at equilibrium), in contrast with the observations with Japanese laccase (Goldberg & Pecht, 1978; Farver & Pecht, 1981), where not more than 3% reduction of the Type 1 copper occurs. Furthermore, a relatively high yield (approx. 60%) of bleaching of Type 1 copper was observed with fungal laccase (Henry *et al.*, 1981; Guissani *et al.*, 1982). These differences in the percentage reduction of Type 1 copper for the different laccases are probably related to the relative efficiency of the competition by the oxidized laccases for $\text{O}_2^{\cdot-}$ with the self-dismutation of $\text{O}_2^{\cdot-}$, especially at below pH 7.

This relative efficiency is demonstrated by the finding that the rate of reaction of $\text{O}_2^{\cdot-}$ with Vietnamese laccase is at least an order of magnitude greater than with Japanese laccase. It is also inferred that, if an equilibrium is established on reaction of $\text{O}_2^{\cdot-}$ with Vietnamese laccase under conditions of $[\text{O}_2^{\cdot-}] \gg [\text{laccase}]$, then the Type-1-copper-reduced protein is favoured. In other words, intramolecular transfer of electrons to Type 3 copper atoms in the holoprotein is slow, in agreement with the system with $\text{ArNO}_2^{\cdot-}$. Similarly, intramolecular electron transfer within the Type-2-copper-depleted protein should be slow, on the basis of the complete reduction of the Type 1 copper. A slow intramolecular transfer of electrons to Type 3 copper atoms in these proteins is consistent with the lack of bleaching of optical absorbance at 330 nm, a band characteristic of the oxidized Type 3 copper site in the holoprotein. It is, as yet, not known whether Type 2 copper is reduced on interaction of the holoprotein with $\text{O}_2^{\cdot-}$. The possibility of inhibition by formation of an O_2 -laccase complex (see, e.g., Goldberg *et al.*, 1980), so that entry of electrons into the protein is retarded, cannot be ruled out.

Partial re-oxidation of the Type 1 copper after completion of the reactions of $\text{O}_2^{\cdot-}$ may involve reactions with O_2 or H_2O_2 (generated during radiolysis). Such interactions may result in the production of peroxy complexes of the laccases, as has previously been shown (Farver *et al.*, 1976, 1982; Frank *et al.*, 1983). These changes were not investigated further owing to lack of sufficient protein.

It is concluded that Type 1 copper acts as the

primary electron acceptor and that subsequent intramolecular electron transfer from Type 1 copper to Type 3 copper is slow, on the basis of the stoichiometry with $\text{ArNO}_2^{\cdot-}$ and the observations at both 615nm and 330nm on interaction with $\text{O}_2^{\cdot-}$ and $\text{ArNO}_2^{\cdot-}$. Furthermore, on reaction with $\text{ArNO}_2^{\cdot-}$, the Type 2 copper in the holo-protein is probably reduced. These observations are in contrast with those obtained with holo ascorbate oxidase where a rapid intramolecular transfer occurs within the protein (O'Neill *et al.*, 1983; P. O'Neill, E. M. Fielden, L. Avigliano, G. Marcozzi, A. Ballini & A. Finazzi Agrò, unpublished work) provided that the Type 2 copper is present when $\text{ArNO}_2^{\cdot-}$ and $\text{O}_2^{\cdot-}$ are produced as the source of reducing equivalents. A slow intramolecular step for electron transfer may account for the lower catalytic efficiencies of these laccases as compared with that of ascorbate oxidase. Indeed, the rate constants for interaction of $\text{ArNO}_2^{\cdot-}$ and $\text{O}_2^{\cdot-}$ with both Vietnamese laccase and ascorbate oxidase (O'Neill *et al.*, 1983; P. O'Neill, E. M. Fielden, L. Avigliano, G. Marcozzi, A. Ballini & A. Finazzi Agrò, unpublished work) are similar. On the basis of the differences of the rate constants for interaction of $\text{O}_2^{\cdot-}$ and $\text{ArNO}_2^{\cdot-}$ with Japanese laccase and Vietnamese laccase, differences in catalytic efficiencies (Table 2) may also depend, in part, on the ability of the proteins to accept reducing equivalents.

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