Anaerobic reactions of *Rhus vernicifera* laccase and its type-2 copper-depleted derivatives with hexacyanoferrate(II)

Takeshi SAKURAI

College of Liberal Arts and Sciences, Kanazawa University, Kanazawa, Ishikawa 920, Japan

Anaerobic reactions of *Rhus vernicifera* laccase and its type-2 copper-depleted derivatives with hexacyanoferrate(II) were investigated by absorption and e.s.r. spectroscopy. When native laccase was treated with excess hexacyanoferrate(II), the type-1 and type-2 coppers were immediately reduced and the e.s.r. signal due to type-3 copper was transiently observed. After incubation, a novel e.s.r. signal ($g_{\parallel} = 2.31$, $g_{\perp} = 2.08$) developed together with the type-1 copper signal. Only the novel e.s.r. signal was left after the sample had been treated with ascorbate. In the corresponding absorption spectrum, a new band was observed at around 490 nm. A similar new e.s.r. signal did not appear for the type-2-copper-depleted (T2D) laccase, in which the type-3 copper had been reduced during the procedure to deplete the type-2 copper. On the other hand, the novel e.s.r. signal emerged when the type-3 copper in T2D laccase had been previously reoxidized with H_2O_2 . The novel e.s.r. signal was not significantly saturated even by 200 mV microwave power at 4 K. Quantitative estimations and a small molecule study for $\text{Cu}^{\text{II}}\text{-Fe}^{\text{II}}(\text{CN})_6$ and $\text{Cu}^{\text{II}}\text{-Fe}^{\text{III}}(\text{CN})_6$ systems suggested that the novel e.s.r. signal corresponds to some sort of adduct involving the type-3 copper and hexacyanoferrate(II).

INTRODUCTION

Laccase (*p*-diphenol: oxygen oxidoreductase, EC 1.10.3.2) is a multicopper oxidase which is widely distributed in higher plants and fungi (Reinhammar, 1984). The most extensive studies have been performed on the enzyme from Japanese and Chinese lacquer latex (*Rhus* species). Laccase oxidizes urusiol, a derivative of catechol contained in the latex, and the resultant quinone polymerizes to form thin film under air (Nakamura, 1976). Molecular oxygen serves as the acceptor of four electrons to form two water molecules.

The active site of multicopper oxidases consists of a set of four copper ions classified according to their e.s.r. features: type 1 ($A_{\parallel} < 0.0095 \text{ cm}^{-1}$), type 2 ($A_{\parallel} > 0.014 \text{ cm}^{-1}$) and type 3 (e.s.r. undetectable) in the resting enzymes. The type-1 site exhibits several charge-transfer bands [$\text{S}^-(\text{Cys}) \rightarrow \text{Cu}^{2+}$, $\text{N}(\text{His imidazole group}) \rightarrow \text{Cu}^{2+}$, $\text{S}(\text{Met}) \rightarrow \text{Cu}^{2+}$], of which the band near 600 nm ($\text{S}^- \rightarrow \text{Cu}^{2+}$) is the strongest one (Dooley *et al.*, 1979). The antiferromagnetically coupled type-3 coppers contribute to another peculiar band at 330 nm together with the type-2 copper (Sakurai *et al.*, 1987; Cole *et al.*, 1990*a,b*). An X-ray-crystallographic study of ascorbate oxidase demonstrated that the binding and reduction site of dioxygen is a trinuclear cluster composed of the type-2 and type-3 coppers (Messerschmidt *et al.*, 1989; Messerschmidt & Huber, 1990).

Fe^{2+} ion is one of the substrates for multicopper oxidases (Morpurgo *et al.*, 1980). The systematic name of ceruloplasmin is $\text{Fe}(\text{II})$: oxygen oxidoreductase and the enzyme is also named ferroxidase. On the other hand, hexacyanoferrate(II) has been used to deplete type-2 copper from laccase (Graziani *et al.*, 1976, 1990; Klemens & McMillin, 1990) and ascorbate oxidase (Avigliano *et al.*, 1979) with the aid of chelating agents such as dimethylglyoxime and EDTA. This selective removal of type-2 copper from laccase and ascorbate oxidase has made a significant contribution to the elucidation of the structure of the active site and the reaction mechanism of these enzymes (Reinhammar & Oda, 1979; Kawahara *et al.*, 1985; Sakurai *et al.*, 1986; Frank & Pecht, 1986; Spira-Solomon & Solomon, 1987; Kau *et al.*, 1987; Cole *et al.*, 1990). The procedure used to deplete type-2 copper has been substantially established, but is strictly empirical.

In the present study, we report the results obtained by anaerobic treatment of native laccase and its T2D derivatives with hexacyanoferrate(II). A novel Cu^{2+} e.s.r. signal, which differs from all types of coppers hitherto known, emerged at the final stage of the reaction. The complex reaction processes and the origin of the new e.s.r. signal are discussed.

EXPERIMENTAL

Laccase was purified from Chinese lacquer latex (*Rhus vernicifera*) supplied by Takano and Co., Kanazawa, Japan, according to the method of Reinhammar (1972) with minor modifications. The type-2 copper-depleted derivative was prepared by the procedure of Graziani *et al.* (1976) with minor modifications. All experiments were performed in 0.1 M potassium phosphate (pH 6.0).

Anaerobic treatment of laccase with hexacyanoferrate(II) and measurements were performed by using laboratory-made quartz-ware attached to a 5 mm e.s.r. tube, 10 mm optical cell and three-way stopcock. Hexacyanoferrate(II) and ascorbate were transferred to the quartz-ware with syringes under purified Ar or N_2 .

E.s.r. spectra at 77 K were measured on a Jeol JES RE1X spectrometer and those at 4 K on a Jeol FE2XG spectrometer attached to a liquid-helium transfer system (Air Products LTD-3-110). Cu-EDTA was used to estimate e.s.r. signal intensity by the usual double-integration method. 1,1-Diphenyl-1-picrylhydrazyl (DPPH) in a quartz capillary tube was used as the external standard to calibrate the estimation error of e.s.r. signal intensities arising from the difference in tuning conditions. Absorption spectra were recorded at room temperature on either a Jasco Ubest-50 spectrometer or an Otsuka Denshi MCPD-1000 spectrometer with diode array detector. Copper contents in native, T2D and $\text{Fe}^{\text{II}}(\text{CN})_6$ -reacted enzymes were determined with a Hitachi Z-6000 polarized Zeeman atomic absorption spectrometer.

RESULTS

Reaction of native laccase

Native laccase was treated with 120 equiv. of hexacyanoferrate(II) under Ar or N_2 and the reaction process was followed

Fig. 1. Absorption spectra of native laccase (—), $\text{Fe}^{\text{II}}(\text{CN})_6$ ($\times 120$)-laccase (---) (3 min), (.....) (20 h) and ascorbate-treated $\text{Fe}^{\text{II}}(\text{CN})_6$ -laccase (-.-.-).

Fig. 2. E.s.r. spectra of native laccase (0.08 mM) (A), $\text{Fe}^{\text{II}}(\text{CN})_6$ ($\times 120$)-laccase (2 min) (B), (1 h) (C) and ascorbate-treated $\text{Fe}^{\text{II}}(\text{CN})_6$ -laccase (D).

Frequency, 9.2 GHz, microwave power, 4 mW, modulation width 0.79 mT (100 kHz), temperature, 77 K.

by absorption (Fig. 1) and e.s.r. spectra (Fig. 2). On manual mixing of hexacyanoferrate(II) and native laccase, the colour of the solution changed from intense blue to pale yellow. The

Fig. 3. Power-saturation curves of the type-1 (○) and type-2 (●) coppers of native laccase and the novel signal (◐) of ascorbate-treated $\text{Fe}^{\text{II}}(\text{CN})_6$ -laccase at 4 K.

absorption spectrum indicated that the type-1 copper was almost completely reduced and a small amount of hexacyanoferrate(II) was oxidized. In the corresponding e.s.r. spectrum (Fig. 2, spectrum B), the signal due to type-2 copper ($g_{\parallel} = 2.24$, $A_{\parallel} = 0.021 \text{ cm}^{-1}$) disappeared on reduction of $\text{Cu}(\text{II})$ and the signal due to type-3 copper ($g_{\parallel} = 2.34$, $A_{\parallel} = 0.0093 \text{ cm}^{-1}$) appeared. The signal is similar to the new $\text{Cu}(\text{II})$ signals found for laccase ($g_{\parallel} = 2.31$, $A_{\parallel} = 0.0084 \text{ cm}^{-1}$) and cytochrome *c* oxidase ($g_{\parallel} = 2.28$, $A_{\parallel} = 0.0108 \text{ cm}^{-1}$) (Reinhammar *et al.*, 1980). The reason why a considerable amount of the type-1 copper signal ($g_{\parallel} = 2.30$, $A_{\parallel} = 0.0043 \text{ cm}^{-1}$) was superimposed on the type-3 copper signal is that freezing induced electron transfer from type-1 copper to type-3 copper. The colour of the solution became bluish-green on freezing, but returned to pale yellow on thawing, probably indicating that back electron transfer from type-3 copper to type-1 copper had occurred. Such temperature-dependent electron shuttling between redox centres has been reported for NO-treated laccase (Martin *et al.*, 1981) and amine oxidase (Dooley *et al.*, 1991). While the solution was allowed to stand at 4 °C for 1 h, its colour gradually became green. In the e.s.r. spectrum (Fig. 2, spectrum C), the type-3 copper signal was not observed any more but the type-1 copper signal and a novel signal were observed. Type-2 copper was in the cuprous form ever since it was initially reduced by hexacyanoferrate(II). At this stage, the total amount of the e.s.r.-detectable species was 1.5 spins per protein molecule. The absorption band due to type-1 copper became strong on incubation of the solution for 1 day at 4 °C. Addition of ascorbate to the sample produced only the novel signal which does not have hyperfine structure in its $g_{\parallel}$ region ($g_{\parallel} = 2.31$, $g_{\perp} = 2.08$), as shown in Fig. 2, spectrum D. The signal intensity was 0.8 spin per protein molecule. The corresponding absorption spectrum exhibited a broad band at around 490 nm. Similar experiments were performed seven times to confirm that the process described above is reproducible.

Power-saturation behaviour of the new e.s.r. signal

In order to obtain information about the new e.s.r. signal, microwave-power-saturation behaviour was studied at 77 and 4 K. Fig. 3 shows that the new signal is only slightly saturated, even at 200 mW, although the half-saturation power ($P_{1/2}$) of type-1 and type-2 coppers in native laccase is about 20 mW (Sakurai *et al.*, 1990). We could observe a similar new e.s.r. signal for ascorbate oxidase ($g_{\parallel} = 2.28$, $g_{\perp} = 2.08$). It also was only slightly saturated by a microwave power of 200 mW at 4 K.

Fig. 4. E.s.r. spectra of T2D laccase (0.08 mM) (previously treated with H_2O_2) (A), $\text{Fe}^{\text{II}}(\text{CN})_6$ ($\times 120$)–T2D laccase (2 min) (B), (1 day) (C) and ascorbate-treated $\text{Fe}^{\text{II}}(\text{CN})_6$ –T2D laccase (D)

Frequency, 9.2 GHz, microwave power, 4 mW, modulation width 0.79 mT (100 kHz), temperature, 77 K.

Fig. 5. E.s.r. spectra of aqueous Cu^{II} (A), $\text{Cu}^{\text{II}}\text{--Fe}^{\text{II}}(\text{CN})_6$ (1:2) (B) and $\text{Cu}^{\text{II}}\text{--Fe}^{\text{III}}(\text{CN})_6$ (1:2) (C)

Frequency 9.2 GHz, microwave power 2 mW, modulation width 0.79 mT (100 kHz), temperature, 77 K.

Reaction of T2D laccase

There has been controversy about the oxidation state of type-3 coppers in T2D laccase (Reinhammar & Oda, 1979; Hahn *et al.*

Fig. 6. Absorption spectrum of $\text{Cu}^{\text{II}}\text{--Fe}^{\text{II}}(\text{CN})_6$ (1:2)

al., 1983; Frank & Pecht, 1986). However, at least the type-3 coppers in T2D laccase and ascorbate oxidase prepared according to Graziani *et al.* (1976) and Avigliano *et al.* (1979) seem undoubtedly to be in the reduced form (Sakurai *et al.*, 1988; Sakurai & Suzuki, 1990; Sakurai, 1991). When hexacyanoferrate (II) was allowed to react with the laccase derivative, no change occurred in the absorption and e.s.r. spectra (not shown). In contrast, the novel e.s.r. signal ($g_{\parallel} = 2.30$, $g_{\perp} = 2.08$) appeared when type-3 copper had been previously reoxidized with H_2O_2 (excess H_2O_2 was dialysed off) (Fig. 4). The sample treated with hexacyanoferrate(II) for 1 day gave 1.23 spins (type-1 copper and new signals) per protein molecule. Only the new e.s.r. signal with an intensity of 0.71 spin per protein molecule was retained after the ascorbate treatment (Fig. 4, spectrum D). However, the absorption band at about 490 nm was not observed, in contrast with the case for native laccase (results not shown).

Model systems

In order to discover the origin of the novel e.s.r. signal, $\text{Cu}^{\text{II}}\text{--Fe}^{\text{II}}(\text{CN})_6$ and $\text{Cu}^{\text{II}}\text{--Fe}^{\text{III}}(\text{CN})_6$ systems were spectroscopically investigated. Whereas aqueous Cu^{2+} ion gives an anisotropic e.s.r. signal, an isotropic signal was obtained when $[\text{Fe}^{\text{II}}(\text{CN})_6]^{4-}$ was added (Fig. 5, spectra A and B). A broad band was observed at around 480 nm in the absorption spectrum (Fig. 6). On the other hand, the $\text{Cu}^{\text{II}}\text{--Fe}^{\text{III}}(\text{CN})_6$ system did not give an e.s.r. signal at all (Fig. 5, spectrum C). However, the reduction with ascorbate afforded spectra similar to those given by the $\text{Cu}^{\text{II}}\text{--Fe}^{\text{II}}(\text{CN})_6$ system (results not shown). These behaviours were not modified in the presence of bovine albumin.

DISCUSSION

Hexacyanoferrate(II) has been widely used as a substrate or a reducing agent for blue copper proteins (Rosen & Pecht, 1976; Jackman *et al.*, 1987) and multicopper oxidases (Morpurgo *et al.*, 1980). In addition, hexacyanoferrate(II) has been utilized to deplete laccase (Graziani *et al.*, 1976) and ascorbate oxidase of type-2 copper (Avigliano *et al.*, 1979). However, it has not been clarified why hexacyanoferrate(II) is very effective and how it operates in the selective removal of type-2 copper. According to the anaerobic kinetic study of laccase using hexacyanoferrate(II), one $[\text{Fe}^{\text{III}}(\text{CN})_6]^{3-}$ ion is rapidly produced in the initial reaction, and a further two $[\text{Fe}^{\text{III}}(\text{CN})_6]^{3-}$ ions are formed in the succeeding reaction (Malmström *et al.*, 1969).

Fig. 2, spectrum B indicates that some electrons furnished by $[\text{Fe}^{\text{II}}(\text{CN})_6]^{4-}$ are transferred to the trinuclear centre through the type-1 copper site. Considering the geometrical arrangement of the three types of copper (Messerschmidt *et al.*, 1989; Messerschmidt & Huber, 1990), an electron is supposed to reach the type-2 copper site via the type-3 copper site (Meyer *et al.*, 1991). The transient appearance of the e.s.r. signal due to type-

3 copper and the gradual reoxidation of type-1 copper after its initial rapid reduction (Figs. 1 and 2) are concerned with the intramolecular electron-transfer process between redox centres.

Superficially, superoxide shows an e.s.r. feature analogous with that of the novel e.s.r. signal. But, whereas the $g_{\parallel}$ value of superoxide has been reported to be about 2.1 (Fee & Valentine, 1977), that of the novel e.s.r. signal is 2.31. Therefore, the novel e.s.r. signal cannot come from $O_2^{\cdot -}$. Since the average g value of the new signal ($g = 2.16$) is much bigger than that of a free electron ($g = 2.0023$), it must come from a metal ion, Cu^{2+} .

The following possibilities for the origin of the new e.s.r. signal were taken into consideration: (1) the modified type-1 copper; (2) certain Fe^{III} species; (3) $Cu^{II}-NC-Fe^{II}(CN)_6$ (Cu originates from type-2 Cu); (4) $Cu^{II}-NC-Fe^{III}(CN)_6$ (Cu originates from type-2 Cu); (5) type-3 copper in an e.s.r.-detectable form.

Because the hyperfine structure is lacking in the $g_{\parallel}$ region (Fig. 2, spectrum D), the new signal might be supposed to be coming from type-1 copper, the original geometry and electronic state of which were profoundly changed by deformation of the protein structure. However, since the spin concentrations of type-1 copper and the new signal are 0.74 and 0.79 per protein molecule respectively (Fig. 2, spectrum C), the number of the total spins exceeds 1 per protein molecule. Therefore possibility (1) can be excluded first.

In spite of numerous reports on the e.s.r. spectra of non-haem Fe^{III} ions in a $S = \frac{1}{2}$ ground state, a signal with axial symmetry ($g_{\parallel} > g_{\perp}$) has been found only for adrenodoxin with the 2Fe-2S cluster (Palmer, 1980). Neither $[Fe^{II}(CN)_6]^{4-}$ nor $[Fe^{III}(CN)_6]^{3-}$ gave e.s.r. spectra similar to Fig. 2, spectrum D. The spin Hamiltonian parameters of the novel e.s.r. signal are typical for Cu^{2+} species, and therefore possibility (2) is very unlikely.

The new e.s.r. signal does not arise from CN^- -bridged binuclear species such as possibilities (3) and (4). When Fe was in the 3+ state, the binuclear model system did not give an e.s.r. signal (Fig. 5, spectrum C) as similarly reported elsewhere (Morpurgo *et al.*, 1985). Probably because of this behaviour, type-2 copper in galactose oxidase was wrongly concluded to become trivalent (Hamilton *et al.*, 1978). On the other hand, when Fe was in the 2+ state, an isotropic e.s.r. signal was obtained because Cu^{2+} and Fe^{2+} interact through a bridged CN^- (Fig. 5, spectrum B). The binuclear model system gave a broad band in the visible region similar to that afforded by the ascorbate-treated $Fe^{II}(CN)_6$ -laccase system (Figs. 1 and 6). However, we concluded that $Cu^{II}-NC-Fe^{II}(CN)_6$ is not the species in question, because T2D laccase whose type-3 copper had been previously reoxidized with H_2O_2 did not give an absorption band, but it did produce the new e.s.r. signal (Fig. 4). Further, the small-molecule system did not show an anisotropic e.s.r. signal but an isotropic one (Fig. 5, spectrum B), although we do not have data for Cu^{2+} ion being firmly bound to proteins. Therefore only possibility (5) can be considered as the candidate that gives rise to the new e.s.r. signal.

The new e.s.r. signal is hardly saturated at all by a high microwave power of 200 mW at 4 K (Fig. 3). Its very sharp feature, as for Cu^{2+} , might be derived from spin exchange between type-3 coppers or between one type-3 copper and $[Fe^{II}(CN)_6]^{4-}$ ion through bridged CN^- . On the other hand, the species that produces the absorption band (Fig. 1) must be $Cu^{II}-NC-Fe^{II}(CN)_6$, the Cu^{2+} of which is not detectable by e.s.r. This can occur if the Cu^{2+} ion is firmly bound to a protein molecule. Moreover, the e.s.r.-silent $Cu^{II}-NC-Fe^{III}(CN)_6$ will also be present, judging from the amount of e.s.r.-detectable species and absorption intensity present. The absorption band observed in the visible region must be the metal-to-metal charge-transfer band between Cu^{2+} and Fe^{2+} . It has been reported that the Fe^{2+} to Cu^{2+} charge-transfer band appears in the near-u.v. to visible region for CN^- -bridged complexes containing certain

Cu^{2+} complexes and hexacyanoferrate(II) (Suzuki & Uehara, 1984). A weak transient feature has been observed at 470 nm ($\epsilon = 700 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) in the reaction of reduced laccase, the type-1 copper of which was substituted by Hg (Cole *et al.*, 1990a,b). However, it is accompanied by an absorption band at 670 nm. Therefore the origin of the novel e.s.r. signal will differ from that of the transient species found by Cole *et al.* (1990a,b).

The complete T2D laccase was obtained by centrifuging the ascorbate-treated $Fe^{II}(CN)_6$ -laccase (results not shown). The supernatant gave an isotropic e.s.r. signal similar to that shown in Fig. 5, spectrum B. This result indicates that type-2 copper can be removed only by $[Fe^{II}(CN)_6]^{4-}$ under anaerobic conditions. The reason chelating agents are used during the selective removal of type-2 copper is to catch the depleted copper. Since a small amount of CN^- ($\times 5$) could not extract type-2 copper from the reduced laccase under anaerobic conditions (results not shown), it is not probable that some free CN^- ions could have extracted the labile type-2 copper in the reduced form. Otherwise, hexacyanoferrate(II) might have approached the trinuclear centre directly. According to X-ray-crystallographic data on ascorbate oxidase (Messerschmidt *et al.* 1989; Messerschmidt & Huber 1990), there seems to be a crevice leading to the type-2 copper. Hexacyanoferrate(II) ion can directly bind to type-3 copper after type-2 copper has been removed. Not only small molecules, such as F^- , CN^- , N_3^- , NO , SCN^- , OCN^- , but also large molecules, such as *NN*-diethyldithiocarbamate, are directly accessible to the trinuclear centre (Holwerda *et al.*, 1982; Morpurgo *et al.*, 1982; Allendorf *et al.*, 1985; Koudelka & Ettinger, 1988). Since the new e.s.r. signal is also produced by ascorbate oxidase, the behaviour observed in the present study is not a special case limited to laccase.

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