Spectroscopic and Kinetic Studies on the Oxygen-centered Radical Formed during the Four-electron Reduction Process of Dioxygen by *Rhus vernicifera* Laccase*

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The oxygen-centered radical bound to the trinuclear copper center was detected as an intermediate during the reoxidation process of the reduced *Rhus vernicifera* laccase with dioxygen and characterized by using absorption, stopped-flow, and electron paramagnetic resonance (EPR) spectroscopies and by super conducting quantum interface devices measurement. The intermediate bands appeared at 370 nm (ε ~ 1000), 420 nm (sh), and 670 nm (weak) within 15 ms, and were observable for ~2 min at pH 7.4 but for less than 5 s at pH 4.2. The first-order rate constant for the decay of the intermediate has been determined by stopped-flow spectroscopy, showing the isotope effect, k_H/D, of 1.4 in D_2O. The intermediate was found to decay mainly from the prototated form by analyzing pH dependences. The enthalpy and entropy of activation suggested that a considerable structure change takes place around the active site during the decay of the intermediate. The EPR spectra at cryogenic temperatures (<27 K) showed two broad signals with g ~ 1.8 and 1.6 depending on pH. We propose an oxygen-centered radical in magnetic interaction with the oxidized type III copper ions as the structure of the three-electron reduced form of dioxygen.

Laccase (diphenol:dioxygen oxidoreductase) as a member of multicopper oxidase is divided into two categories: plant (1–3) and fungal (4, 5) enzymes. The most studied laccase so far is *Rhus vernicifera* laccase (6), which contains four coppers in a single protein molecule. In analogy with the related enzymes such as ascorbate oxidase (7, 8), ceruloplasmin (9, 10), bilirubin oxidase (11–13, 44), and phenoxazinone synthase (14), laccase utilizes dioxygen as the final electron acceptor (15).

Based on the redox, optical, and magnetic properties, the coppers in laccase have been classified into three types: type I, type II, and type III coppers (16, 17). Type I copper (blue copper) gives four charge transfer bands in the visible region, of which the band at 614 nm originating from Cys → Cu^2+ is the strongest (ε = 5800 m^−1 cm^−1), and a very narrow hyperfine splitting (A_I = 4.3 × 10^−3 cm^−1) in the EPR1 spectrum. Type II copper (non-blue copper) is not coordinated by a soft ligand such as Cys and has a broad hyperfine splitting (A_II = 20.6 × 10^−3 cm^−1) in the EPR spectrum. Type III coppers (EPR non-detectable copper) exhibit a 330-nm shoulder band and no EPR signal because of the strong antiferromagnetic interaction through a hydroxide ion.

Spectroscopic studies on laccase, ascorbate oxidase, and ceruloplasmin combined with crystallographic studies (18) have generated a detailed description of the active site concerned with the catalytic function of them. Type I copper is four-coordinated and has the tetrahedrally distorted geometry. Type II copper is three-coordinated by two imidazoles and a water as ligands, but shows EPR spectral features for a tetragonal or slightly tetrahedrally distorted geometry (19, 20). Paired type III coppers with highly tetrahedrally hindered geometry (21) are coordinated by three histidines and a hydroxide or oxide ion as the bridging group. Type II and type III coppers are not bridged but located within 0.4 nm to form the trinuclear copper center (17) and thus bind and reduce dioxygen utilizing the electrons transferred from the type I copper site through the type I-Cys-His-type III pathway.

The reactions concerned with laccase have been studied at the early stage of the studies about multicopper oxidases, and several reaction mechanisms have been proposed (7, 22, 23). One intermediate has been observed in the reaction of the reduced laccase with dioxygen, showing an EPR signal with the g value smaller than 2 at <20 K, which was broadened when ^17O_2 was used (24). The intermediate was supposed to give an absorption band at 364 nm as a three-electron reduced oxyl radical or hydroxyl radical bound to one of type III coppers. Recently, the peroxide intermediate has been defined for the derivative of laccase in which the type I copper site was substituted by the redox-inactive Hg^2+ (T1Hg) (25, 26). This type of intermediate is extremely difficult to detect during the reoxidation process of the native enzyme or may not be the species present in the normal reaction process. One structural model of this intermediate showed the μ-1,1-peroxide ion bridging between one of the type III Cu^2+ and type II Cu^+ (25). Another model to give the μ_3-(η_3)^2-peroxide intermediate was such that one oxygen atom bridges between both type III Cu^2+ and another oxygen atom binds to type II Cu^+ (26). However, since the third electron is apparently furnished from type I copper in the reaction of the native enzyme, the T1Hg derivative may give an artifactual intermediate. Despite these pioneering studies, confusion is present about the structure of the two- and three-electron reduced oxygen intermediates because of deficiency of data. The four-electron reduction process of dioxygen by multicopper oxidase has not been fully established yet and awaits detailed investigation based on current structural information.

Recently, we performed detailed EPR spectroscopy and
copper-depleted laccase; T1Hg, mercury derivative of laccase at the type I copper site; T, tesla.

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1 The abbreviations used are: EPR, electron paramagnetic resonance; SQUID, super conducting quantum interface devices; T2D, type II
SQUID measurements of laccase and ascorbate oxidase from cryogenic temperature to room temperature, and have shown that the magnetic interaction in the trinuclear copper center varies depending on temperature (19, 20). Based on this information about the trinuclear copper center, we investigated the reduction process of dioxygen in order to reveal the function of this unique metal center in multicopper oxidase using UV-visible, stopped-flow, EPR, and SQUID measurements.

EXPERIMENTAL PROCEDURES

Materials—Chinese lacquer latex from R. vernicifera was obtained from Takano and Co., Kanazawa, Japan. Laccase was purified according to the method of Reinhämmer (27) with modification as described earlier (28). Protein concentrations were determined on the basis of the extinction coefficient of 5800 M⁻¹ cm⁻¹ at 614 nm and the absorption ratio of \( A_{614}/A_{315} = 16 \). Reduced laccase was prepared by adding four-electron equivalent of dithionite under argon (>99.995%). (In the case of stopped-flow measurements, a slight excess of dithionite was used. The amount was controlled from the absorption at 315 nm (ε = 8000 M⁻¹ cm⁻¹) (29).)

Phosphate buffer (0.2 M) was used throughout measurements. The sample solutions with different pH were prepared by dialyzing the protein solution against ambient buffers for 6–12 h. As for the studies of the isotope effect, laccase was dialyzed against phosphate buffer in D₂O. Water distilled after deionization was used throughout the present study. All reagents used were of the highest grade commercially available.

Measurements—Absorption spectra were recorded using a Shimadzu MultiSpec-1500 spectrophotometer with a photodiode array detector at room temperature. A laboratory-made quartz cell attached to a three-way stopcock was used for the anaerobic measurements (21). Laccase was fully reduced with a least amount of dithionite and was evacuated using a vacuum line before mixing with an oxygen-containing buffer to start the reoxidation.

Stopped-flow measurements were performed on an Otsuka Densh RA-401A stopped-flow spectrometer (1-cm optical cell, 2 atm) (22). Temperature was controlled by flowing thermostatted water. A home-designed reservoir of the reactants was attached to the spectrometer in order to keep the samples under an anaerobic condition. More than three independent results were averaged and analyzed using Delta Graph (version 4.0.5).

EPR spectra were measured by a Bruker ESP-300E spectrometer attached to an Oxford cryostat at X-band microwave (9.5 GHz). Measurements were performed so that saturation of signal did not take place (19, 20). The samples were quickly frozen by dipping in a liquid nitrogen bath after mixing the fully reduced laccase with air. An EPR tube to which a three-way stopcock was attached on its head was used.

Magnetic susceptibility measurements were carried out on a Quantum Design MPMS-7 SQUID magnetometer at 2 T. A specially designed polytetrafluoroethylene cell with an O-ringed cap was used throughout measurements. Fully reduced laccase was evacuated and dialyzed against buffer under argon and was transferred to the cell under helium in a glove-box. After introducing air into the cell, the sample was soon frozen with liquid nitrogen and evacuated for 30 min. The cell was capped under helium prior to the sample loading into the SQUID magnetometer. A 2-min incubation was used before starting the SQUID scan to allow the temperature to stabilize. Four scans of 48 points have been averaged (19, 20). Measurements were performed between 5 and 50 K and between 5 and 200 K, starting from lower to higher temperature and returning from higher to lower temperature. Only the magnetic susceptibility arising from the specific paramagnetic component in the intermediate could be obtained from the difference susceptibility between the sample frozen soon after starting reoxidation and the fully reoxidized sample.

RESULTS

Reoxidation of the Reduced Laccase with Dioxygen—The reoxidation of the reduced laccase with the oxygen-containing buffer was studied at pH 4–8 using the absorption spectrometer with photodiode array detector. The three-dimensional spectra (data not shown) obtained by acquiring spectra at 1-s intervals showed the bands at 614 nm and 330 nm specific for the oxidized type I copper and coupled type III coppers, respectively, recovered within 2 s, indicating that at least three electrons have been transferred to dioxygen (2). Further, three transient bands were observed at 370 nm, 420 nm (sh), and 670 nm (weak) and decayed according to the first-order process. The similar transient spectrum in the range of 300–620 nm has also been reported by Andreasson et al. (22).

Spectral Features of the Intermediate and Kinetics by Stopped-flow Spectroscopy—Formation and decay of the intermediate to give the transient bands were studied in detail by stopped-flow spectroscopy over the pH range of 4.0–7.4. After mixing the reduced laccase with the oxygen-containing buffer, the absorption bands at 330 and 610 nm were recovered within 15 ms to reach the maximum intensities with the first-order rate constants of \( k_{330} = 253 s^{-1} \) and \( k_{610} = 260 s^{-1} \) (pH 7.4–8 at 14–25 °C), respectively. Simultaneously, those at 370 and 420 nm reached the maximum values and then began to gradually decay according to the first-order process to give the same absorption spectrum with that of the resting enzyme, although the lifetimes of the transient bands were conspicuously different depending on pH (\( k = 0.058 s^{-1} (t_{1/2} = 12 s) \) at pH 7.4 and \( k = 2.1 s^{-1} (t_{1/2} = 0.35 s) \) at pH 4.2 regardless of the enzyme concentrations). The transient spectrum at 200 ms was shown in Fig. 1 together with the spectrum of the fully oxidized laccase at 5 s (pH 4.2). The difference spectrum of them clearly gave a band at 370 nm (ε = 1000 M⁻¹ cm⁻¹) and a shoulder band at 420 nm (ε = 550 M⁻¹ cm⁻¹). The analogues transient spectra were obtained at several pH values between pH 4.2 and 7.4, while the band at 670 nm was not clearly detected by stopped-flow spectroscopy because of its low absorption intensity.

The first-order decay rate of the band at 370 nm was determined at pH 4.2–7.4, showing the acceleration with lowering pH (Fig. 2A). When D₂O was used in the place of H₂O as solvent, the isotope effect of \( k_{D}/k_{H} = 1.4–1.5 \) was observed (Fig. 2, A and B). This strongly suggests that a certain protonation process (31) is concerned with the decay of the intermediate.

The pH dependence of the decay of the intermediate (Fig. 2A) was analyzed as follows. Scheme 1 shows the process in which the proton transfer is coupled with the electron transfer to the intermediate \( E_{\text{red}} \) is the intermediate, \( E_{\text{ox}} \) the fully reoxidized enzyme, and \( n \) the number of protons). The equation for the decay process predicted a linear relationship between log \( k_{\text{obs}} \) and pH, and \( n \) calculated gave an unreasonable value of 0.4 (simulation not shown).
The solid curve based on the equation in the scheme gave the fit shown by the open circle and protonated intermediates (32). The simulation performed and interpreting the pH dependence of the decay process. The equation derived from this scheme was not also suitable for estimating that a novel broad signal appeared at the higher magnetic field (2). It is analogous to that reported by Aasa et al. (2). The parameters obtained were $K_{o, int} = 3.79 \times 10^{-6}$ M, $k_1 = 8.89 \times 10^{-2}$ M$^{-1}$ s$^{-1}$, and $k_2 = 1.06$ s$^{-1}$. The result demonstrated that the intermediate mainly decayed through the step with $k_2$, because it is $\sim 10$-fold higher than $k_1$.

Temperature dependence of the decay of the intermediate was studied between pH 4.4 and 7.4. The thermodynamic parameters obtained are plotted in Fig. 4. The enthalpies of activation have large positive values in the range of 37–58 kJ mol$^{-1}$ and the entropies of activation have large negative values in the range of $-143$ to $-58$ J mol$^{-1}$ K$^{-1}$. As shown in Fig. 4, a peak was obtained at pH 6 for both the enthalpy of activation and the entropy of activation. However, $\Delta G^\ddagger$ at 25 °C linearly decreased with lowering pH as shown in Fig. 4C.

**EPR Spectral Features of the Intermediate**—We obtained the EPR spectra of the intermediate by quickly freezing the sample after mixing the reduced laccase with air. The intermediate exhibited different features from that of the native enzyme at low temperatures (Fig. 5A for pH 6). Although the type I copper signal was fully recovered, the signal due to type II copper was apparently weak, demonstrating that type II copper was reduced in the intermediate (a part of laccase has been fully reoxidized, giving the type II copper EPR signal) (2). It is interesting that a novel broad signal appeared at the higher magnetic field around 370 mT ($g = 1.88$) only below 27 K (at pH 6). This signal is analogous to that reported by Aasa et al. (24).

The broad signal at 370 mT decreased in its intensity with increasing temperature and was not observable at 27 K, but was recovered by lowering temperature. There was no change in the ratio of the intensities of the type I and type II copper signals due to this temperature change. Therefore, the change
in the intensity of the 370-mT signal was due to the change in its relaxation behavior depending on temperature. However, when the temperature was raised to 200 K, the reversibility was no longer observed. The EPR spectrum coming from the fully reoxidized laccase was identical with that of the native enzyme. Another very weak EPR signal coming from type III copper was occasionally observed at 345 mT (21) in addition to the signal at 370 mT (Fig. 5).

As for the signal due to the intermediate, another broad strong signal was observed at 420 mT (g = 1.61) at pH 7.4 and 3 K (Fig. 5B). Intensities of both of the intermediate signals at 370 and 420 mT varied in the similar manner with temperature. The two signals were thought to be arising from the different species depending on pH (E_int and E_intH) as analyzed by the stopped-flow study (see above).

SQUID Measurements of the Intermediates—SQUID measurements of the intermediate were performed between 5 and 200 K. Diamagnetic contributions due to the apoprotein and the cell were easily eliminated by using the same cell (33) (data at pH 6 in Fig. 6A). The magnetic susceptibilities obtained starting from 5 K toward 200 K for the sample frozen soon after mixing the reduced laccase with air (see a in Fig. 6A) did not change linearly with temperature, but rather displayed behavior specific for the systems with relatively weak antiferromagnetic interactions. In contrast, when the scanning was returned from 200 toward 5 K (see b in Fig. 6A), an almost linear correlation of the magnetic susceptibility with temperature was observed (34, 35). On the other hand, when measurements were performed between 5 and 50 K, magnetic susceptibilities did not change depending on direction to scan. The similar behaviors were also observed at pH 7.4.

The difference magnetic susceptibility between a and b in Fig. 6A, \( \Delta x_m \), is shown in Fig. 6B; only the intermediate species contributes to this reference. Taking into consideration that type I and type III coppers have already been oxidized and type II copper was still in the reduced form in the intermediate (see above), the SQUID measurement data were simulated for the models of the three-spin system (Fig. 6C) (35–37). Model 1 is the four-centered system composed of an oxyl and/or hydroxyl radical, two type III coppers and a bridging hydroxide ion (or oxide ion). The magnetic interaction between oxyl and/or hydroxyl radical and type III coppers and that between type III coppers were evaluated as to be \( J_a = 0.32 \text{ cm}^{-1} \) and \( J_b = 2.13 \text{ cm}^{-1} \), respectively, indicating that the radical species is weakly ferromagnetically coupled with type III coppers, which are antiferromagnetically interacted through a hydroxide ion. Alternatively, model 2 with a linear three-spin system is that the oxygen-based radical is antiferromagnetically interacted with only one of type III coppers with \( J_a = 1.95 \text{ cm}^{-1} \). All three models fit Fig. 6B satisfactorily.

**DISCUSSION**

While a variety of spectroscopic techniques have been applied to laccase in order to reveal its structure and properties of the metal binding sites (16, 17) to provide insight into the reaction mechanism (15), the four-electron reduction process of...
dioxygen has been a black box until recently. Some fragments of studies on the reaction mechanism have been performed by using stopped-flow and EPR spectroscopies for the native laccase (22), the derivative in which type I copper was substituted by Hg$^{2+}$ (T1Hg) and that in which type II copper was selectively depleted (T2D). In this study, we characterized the key intermediate species in detail by using stopped-flow, cryogenic EPR, and SQUID measurements, and proposed the mechanism whereby dioxygen is converted into two water molecules.

The transition spectra during the four-electron reduction of dioxygen by laccase showed three bands at 370 nm, 420 nm and 670 nm (Fig. 1). The bands at 370 nm ($\varepsilon = 1000 \text{ M}^{-1} \text{cm}^{-1}$) and 420 nm ($\varepsilon = 3300 \text{ M}^{-1} \text{cm}^{-1}$) decayed following the first-order process regardless of the enzyme concentration over the wide pH range 4.0–7.4, although it was difficult to follow the change of the band at 670 nm. The reactions of T1Hg with dioxygen by Shin et al. (25) showed the three bands at 340 nm ($\varepsilon = 3300 \text{ M}^{-1} \text{cm}^{-1}$), 470 nm ($\varepsilon = 1200 \text{ M}^{-1} \text{cm}^{-1}$), and 670 nm ($\varepsilon = 400 \text{ M}^{-1} \text{cm}^{-1}$) originating from a peroxide intermediate. A hydroperoxide group has been supposed to bridge between type II copper and one of type III copper in the $\mu$-1,1 fashion based on comparison with Cu$^{2+}$-peroxo model spectra. Our intermediate spectrum was different from that of the peroxide intermediate and also from those of the other Cu$^{2+}$ models and oxyhemocyanin. Unfortunately, no data are available for models of the copper-hydroxyl radical and copper-oxyl radical species. Nevertheless, a transient spectrum analogous to that we obtained in this study has been reported in the early kinetics study of laccase at pH 7.4 by Andreasson et al. (22), although they proposed a different reaction mechanism (see below). Another important fact of the transient system is that the absorptions at 614 nm and 330 nm have already been recovered, indicating that type I copper and type III coppers have finished to donating electrons to dioxygen. The appearance of the 330-nm band indicates that type III coppers are bridged by OH$^{-}$ or O$_2^-$.

The pH dependence of the decay process of the intermediate bands has shown increasingly acceleration with decreasing pH (Fig. 2). Another important fact of the transient system is that the absorptions at 614 nm and 330 nm have already been recovered, indicating that type I copper and type III coppers have finished to donating electrons to dioxygen. The appearance of the 330-nm band indicates that type III coppers are bridged by OH$^{-}$ or O$_2^-$.

The pH dependence of the decay process of the intermediate bands has shown increasingly acceleration with decreasing pH (Fig. 2). The apparent isotope effect was observed in deuterium oxide ($k_D/k_H = 1.4–1.5$ irrespective of pH and pD), and it appeared that a certain proton transfer process is coupled with the decay of the intermediate (a certain proton transfer process
and/or hydrogen bonding(s) might be involved in the rate-determining step. Analyses of the kinetics results based on several schemes indicated that there is an equilibrium between the unprotonated form and the protonated form for the intermediate. (This proton transfer is strongly coupled with the electron transfer of the intermediate. Since type I and type III copper have already been oxidized, type II copper is the only candidate as the electron source as evidenced by the EPR spectra in Fig. 5A.) It was found that the prior proton transfer facilitates the electron transfer, although the simultaneous proton and electron transfers also take place. While Scheme 3 was the best among all four schemes to account for the prominent accelerated decay of the intermediate with decreasing pH, the simulation for the neutral pH region higher than pH 5 could be admirably simulated according to Scheme 4 to give a sigmoidal curve. The increasing acceleration of the decay of the intermediate below pH 5 might have been brought about by a certain loosening of the protein structure. The decay rate of the intermediate ($k_{obs} = 2.1 \text{s}^{-1}$, $t_{1/2} = 0.33 \text{s}$ at pH 4 and $k_{obs} = 0.058 \text{s}^{-1}$, $t_{1/2} = 1.2 \text{s}$ at pH 7.4) was much faster than that of the hydroperoxo intermediate in T1Hg ($k_{obs} = 0.60 \text{min}^{-1}$, $t_{1/2} = 1.2 \text{min}$ at pH 4 and $k_{obs} = 0.013 \text{min}^{-1}$, $t_{1/2} = 12 \text{s}$ at pH 7.4) (see Ref. 25). This peculiar difference is considered to be brought about by the fact that the third electron donor is type II copper in the case of T1Hg but is type I copper in the case of the native enzyme.

The enthalpy of activation and the entropy of activation for the decay of the intermediate were considerably large and negative values, respectively, indicating that the decay of the radical intermediate is accompanied by an appreciable structure change around the active site of laccase and/or activation barriers associate with the decay of the intermediate are high. The possible protonation from a certain amino acid residue to the oxyl radical and deformations leading to the resting form could be the causes of these large activation parameters. The copper-copper distances in the trinuclear center in ascorbate oxidase are 0.41–0.51 nm in the reduced form, but decrease to 0.34–0.40 nm in the resting form (18, 23).

The redox potential of type I copper and type II copper in the resting laccase have been determined to be 394 and 365 mV, respectively (1). The experimental fact that the electron transfer from type I copper to oxygen has already finished and the forth electron is transferred from type II copper to the radical intermediate (evidenced by the EPR measurements) is contradictory relative to the driving forces. In addition, the distance between type I copper and the trinuclear copper center is ~1.3 nm. The reason that the electron transfer from type I copper takes place before that from type II copper could be caused by the redox potential of each copper site (38, 39) changes under turnover conditions. To support this, the redox potentials of the trinuclear copper center easily change by acting the exogenous ligands such as N$_3$ and F$^-$ (12) and by the mutations for ligand groups (13, 40, 41). Alternatively, the redox potential of the type II copper in T1Hg might shift toward a more positive potential than that in the native laccase. The fact, the electron transfer from type II copper to the oxyl and hydroxyl radical is relatively slow ($k_{obs} = 0.1–1.6 \text{s}^{-1}$) suggests the possibility that the type II copper might not be oxidized when excess substrates and dioxygen are present. In this case, the role of type II copper is simply to assist the binding of dioxygen and/or to stabilize the intermediate.

In harmony with the appearance and decay of the intermediate in the transient spectra, two broad EPR signals could be detected at $g = 1.83$ and 1.61 at cryogenic temperatures below 27 K. The former is similar to that reported by Andreasson et al. (22), being assigned to be a certain radical species derived from dioxygen. The isotope effect using $^{17}$O$_2$ supported the assignment by Aasa et al. (24). The $g = 1.83$ signal was detected at pH 6, but both signals were apparent at pH 7.4, suggesting that the radical species is in an equilibrium with H$^+$. This is consistent with the kinetic results for Schemes 3 and 4. We tentatively assign the $g = 1.83$ species as being associated with the hydroxyl radical and the $g = 1.61$ species with the oxyl radical. No Cu$^{2+}$ model compound bound by a hydroxyl radical or oxyl radical has been prepared to support the assignment. This EPR-detectable species cannot be an isolated radical but is intimately interacted with the partly oxidized trinuclear copper center. The S = 1/2 or even S = 3/2 state from a three-spin system may be the origin of these signals. Only EPR spectroscopy might have been possible to directly discriminate the hydroxyl radical and oxyl radical, the difference of which is only the protonation.

The magnetic property of the intermediate was also characterized by the magnetic susceptibility measured using a SQUID magnetometer (Fig. 6). Contribution from only the radical-bound trinuclear copper center could be obtained, since type I copper and the already fully reoxidized laccase obeyed the Curie law (19, 20). As for the resting form of laccase, the type I copper site is magnetically isolated, and type II and a pair of type III copper (the antiferromagnetic interaction between them is very strong ($-2J > 400 \text{ cm}^{-1}$) (19, 20) form the trinuclear center with $S = 1/2$. In contrast, the radical-bound trinuclear radical in the intermediate has more than one spin, 1/2 < S < 3/2. The magnetic susceptibility due to the transient species could be simulated by three models, in which the intermediate radical is bound to one or both of type III coppers. It is not certain whether the radical is bound to type II copper, which is apparently EPR-undetectable in the intermediate species (type II copper is in a cuprous state, while the possibility that the oxygen radical and the type II cupric ion interact to give S = 0 and S = 2 states is not completely excluded; in this case, no fourth electron donor is present). It seems to be difficult to discriminate which model is the most probable, although it is certain that the intermediate contains the complex three-spin system. Otherwise, these two or three forms might be in equilibrium in the intermediate, as detected by a small amount of type 3 copper EPR signal at 345 nm in the transient state (this is possible when the interaction in Fig. 6C (3) contributes).

Taking all information described above into consideration, we present the reaction scheme in Fig. 7 for the four-electron reduction process of dioxygen at the trinuclear copper center in laccase. In the intermediate, type I and type III coppers are oxidized and both type III coppers have already been bridged by a hydroxide ion or an oxide ion (23). As for the proton source to form water or hydroxide ion, bulk water or certain amino acid(s) near the active site is the candidate. It has been pointed
out by Karlin et al. (42) that several acidic amino acids are present in the second coordination sphere of the trinuclear copper center in ascorbate oxidase. The sigmoidal simulation curve obtained in Fig. 3B suggests an amino acid having the pK$_a$ value of 5.4 is the proton donor to the oxyl radical intermediate. We concluded that the intermediate radicals are in equilibrium with H$^+$ depending on pH. Although we have not yet determined the two-electron reduced species, we suppose that dioxygen is bound to type III coppers in the $\mu$-$\pi^2$$\cdot$$\pi^2$ fashion as in oxyhemocyanin. By taking this binding form, one of the oxygen atoms is smoothly converted to the bridging hydroxide ion (or oxide ion) and another to the radical species bound to the trinuclear center. The reaction is completed by transferring the final electron to the intermediate radical to form a hydroxide ion or a water molecule bound to type II copper. This non-protein ligand is exchangeable with the bulk water in the resting laccase (43). As for the peroxide-bound form of the resting ascorbate oxidase, the end-on binding fashion was shown by Messerschmidt et al. (23) from the x-ray crystallography. However, in the peroxide-ascorbate oxidase, all coppers are in the oxidized form differing from the turnover condition and it is not certain whether the binding mode of dioxygen in laccase is similar to that of the peroxide-ascorbate oxidase or to that of oxyhemocyanin. Further study is necessary to detect the two-electron reduced form, although it seems to be very difficult because the third electron transfer is very rapid compared with the binding of dioxygen to the trinuclear center and the fourth electron transfer to the radical species.

CONCLUSION

The intermediate radical species was detected during the four-electron reduction process of dioxygen by laccase and characterized on the basis of the spectroscopic and magnetic properties. The intermediate showed different spectral features from those of the peroxide intermediate reported for T1Hg. The hydroxyl and oxyl radicals in the equilibrium depending on pH are bound to the trinuclear copper center, in which type III coppers have already been bridged by a hydroxide ion. Type II copper is still in the reduced form. The intermediate radical magnetically interacts with the nearby type III coppers to give the broad EPR signals with the g values much smaller than 2.0023. The decay process of the radical species due to the electron transfer from type II copper was found to closely couple with the proton transfer. Therefore, the successful detection and the detailed characterization of the O-centered radical was realized because the proton transfer controls the final electron transfer during the four-electron reduction process of dioxygen.

REFERENCES

1. Reimharrmar, B. R. M., and Vanngård, T. L. (1971) Eur. J. Biochem. 18, 463–468
2. Branden, C., and Deinum, J. (1978) Biochim. Biophys. Acta 524, 297–304
3. Bligny, R., and Douce, R. (1988) Biochem. J. 253, 489–496
4. Froehner, S. C., and Eriksson, K.-E. (1974) J. Bacteriol. 120, 458–465
5. Jonsson, L., Sjostrom, K., Hagström, I., and Nyman, P. O. (1995) Biochim. Biophys. Acta 1251, 210–215
6. Reinhammar, B. (1984) in Copper Proteins and Copper Enzymes (Lontie, R., ed) Vol. 3, pp. 1–35, CRC Press, Boca Raton, FL
7. Mondevi, B., and Avigiano, L. (1984) in Copper Proteins and Copper Enzymes (Lontie, R., ed) Vol. 3, p. 161, CRC Press, Boca Raton, FL
8. Messerschmidt, A., Ladenson, R., Huber, B., Bolognesi, M., Avigiano, L., Petruzzelli, R., Rossa, A., and Finazzi-Agro, A. (1992) J. Biol. Chem. 267, 179–205
9. Ryden, L. (1984) in Copper Proteins and Copper Enzymes (Lontie, R., ed) Vol. 3, p. 37, CRC Press, Boca Raton, FL
10. Calabrese, L., Carbonaro, M., and Musci, G. (1988) J. Biol. Chem. 263, 6480–6483
11. Hiromi, K., Yamaguchi, Y., Sugiyama, Y., Iwamoto, H., and Hirose, J. (1992) Biochim. Biophys. Acta 1125, 1349–1355
12. Hirose, J., Inoue, K., Sakuragi, H., Kikkawa, M., Minakami, M., Morikawa, T., Iwamoto, H., and Hirose, K. (1998) Inorg. Chem. Acta 273, 204–212
13. Shimizu, A., Kwon, J. H., Sasaki, Y., Yamaguchi, S., and Samejima, T. (1999) Biochemistry 38, 3034–3042
14. Freeman, J. C., Nayar, P. G., Begley, T. P., and Vilafranca, J. (1993) Biochemistry 32, 4826–4830
15. Malkin, R., and Malmström, B. G. (1970) Adv. Enzymol. Relat. Areas Mol. Biol. 33, 177–244
16. Rontie, R., and Douce, R. (1983) Biochem. J. 214, 489–496
17. Sakurai, T., and Suzuki, S. (1997) in Multi-Copper Oxidases (Messerschmidt, A., ed) pp. 225–250, World Science, Singapore, Republic of Singapore
18. Messerschmidt, A., Rossi, A., Ladenson, R., Huber, B., Bolognesi, M., Gatti, G., Marchesini, A., Petruzzelli, R., and Finazzi-Agro, A. (1989) J. Biol. Chem. 264, 513–529
19. Huang, H., Sakurai, T., Monjushiro, H., and Takeda, S. (1998) Biochim. Biophys. Acta 1384, 160–170
20. Huang, H., Sakurai, T., Mariano, S., Marchesini, A., and Suzuki, S. (1999) J. Inorg. Biochem. 75, 19–25
21. Sakurai, T., and Takahashi, J. (1995) Biochim. Biophys. Acta 1248, 143–148
22. Andréasson, L.-E., Brandén, B., and Reimharrmar, B. (1976) Biochim. Biophys. Acta 438, 370–379
23. Messerschmidt, A., Luecke, H., and Huber, R. (1993) J. Mol. Biol. 230, 997–1014
24. Aasa, R., Branden, B., Deinum, J., Malmström, B. G., Reimharrmar, B., and Vanngård, T. (1976) Biochem. Biophys. Res. Commun. 70, 1204–1209
25. Shin, W., Sundaram, U. M., Cole, J. L., Zhang, H. H., Hedman, B., Hodgson, K. O., and Solomon, E. I. (1996) J. Am. Chem. Soc. 118, 3202–3215
26. Sundaram, U. M., Zhang, H. H., Hedman, B., Hodgson, K. O., and Solomon, E. I. (1997) J. Am. Chem. Soc. 119, 7788–7797
27. Reimharrmar, B. (1976) Biochim. Biophys. Acta 205, 35–47
28. Sakurai, T. (1992) Biochemistry 31, 9844–9847
29. Dixon, M. (1971) Biochim. Biophys. Acta 236, 241–258
30. Atkins, P. W. (ed) (1990) Physical Chemistry, 4th Ed., pp. 855–856, Oxford University Press, Oxford
31. Reimharrmar, B. (1985) Chem. Scripta 25, 172–175
32. Forsheit, A. (1985) Enzyme Structure and Mechanism, 2nd Ed., pp. 155–173, W. H. Freeman and Co., New York
33. Sakurai, T., Suzuki, S., and Chikira, M. (1990) J. Biochem. (Tokyo) 107, 37–42
34. Earnshaw, A. (1968) Introduction to Magnetochrome, Academic Press, New York
35. Drago, R. S. (1977) Physical Method in Chemistry, W. B. Saunders, Philadelphia
36. Bleesey, B., and Bowers, K. D. (1953) Proc. R. Soc. London 214, 451
37. Boudreaux, E. A., and Mulay, L. M. (eds) (1976) Theory and Applications of Molecular Paramagnetism, John Wiley & Sons, New York
38. Spira-Solomon, D. J., and Solomon, E. I. (1987) J. Am. Chem. Soc. 109, 1723–1726
39. Langen, R., Jensen, G. M., Jacob, U., Stephens, P. J., and Warshel, A. (1992) J. Biol. Chem. 269, 25674–25677
40. Malmström, B. G. (1994) Eur. J. Biochem. 223, 711–718
41. Ducros, V., Brzozowski, A. M., Wilson, K. S., Brown, S. H., Østergaard, P., Schneider, P., Yaver, D. S., Pedersen, A., and Davies, G. J. (1998) Nat. Struct. Biol. 5, 310–316
42. Karlin, K. D., Zhu, Z.-Y., and Karlin, S. (1998) J. Biol. Inorg. Chem. 3, 172–187
43. Goldberg, M., Vuk-Pavlovic, S., and Pecht, I. (1980) Biochemistry 19, 5181–5189
44. Shimizu, A., Sakurai, T., Kwon, J. H., Nakao, A., Satoh, T., Sakurai, N., Sakurai, T., Yamaguchi, S., and Samejima, T. (1999) J. Biochem. 123, 662–668