A novel mixed valence form of Rhus vernicifera laccase and its reaction with dioxygen to give a peroxide intermediate bound to the trinuclear center

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A Novel Mixed Valence Form of *Rhus vernicifera* Laccase and Its Reaction with Dioxygen to Give a Peroxide Intermediate Bound to the Trinuclear Center[1]

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***Rhus vernicifera* laccase, in a novel mixed valence state [T1oxT23red: type 1 Cu as Cu(II), and type 2 and 3 Cus as Cu(I)], was formed by reacting Cu(I) on the type 2 Cu-depleted laccase [T1oxT3red: type 1 Cu as Cu(II) and type 3 Cus as Cu(I)] under argon. Contrary to T1oxT3red, T1oxT23red was highly reactive with dioxygen, and gave the three transient bands at 340, 475, and 680 nm due to the two-electron reduced form of dioxygen [charge transfer bands from peroxide to Cu(II)]. The first order decays were highly dependent on pH, which led to the successful detection of the intermediate for *ca.* 2 h at pH 7.5. Another mixed valence derivative, T12oxT3red [type 1 and type 2 Cus as Cu(II), and type 3 Cus as Cu(I)] prepared through the action of Cu(II) on T1oxT3red was not reactive with dioxygen, but showed high enzyme activity as to the oxidation of *N,N*-dimethyl-*p*-phenylenediamine. The whole reaction mechanism of the reduction of dioxygen by laccase was proposed based on the present results together with data for the former detection and characterization of the three-electron reduced form of dioxygen [Huang, H. *et al.* (1999) *J. Biol. Chem.* 274, 46, 32718–32724].**

Key words: dioxygen reduction, laccase, multicopper oxidase, trinuclear Cu center.

Multicopper oxidase belongs to the class of enzyme containing four or more Cu centers in a protein molecule (*1*). The minimum functional unit of multicopper oxidase comprises a set of one type 1 Cu, one type 2 Cu, and a pair of type 3 Cus. The type 1 Cu functions as the electron mediator from a substrate to the trinuclear center composed of the type 2 Cu and type 3 Cus, where dioxygen is bound and reduced to two water molecules. All these copper sites are indispensable for the four-electron reduction of dioxygen without the release of activated oxygen species as intermediates.

When lacquer tree laccase, the best studied multicopper oxidase, was reduced fully and reacted with dioxygen, the three-electron reduced form of dioxygen appeared within 15 ms with a life-time of s to min order depending on the pH (*2, 3*). We spectroscopically and magnetically characterized this species as the oxygen-centered racial bound to the trinclear copper center, finding that it decayed on accepting the final electron from type 2 Cu under the control of proton transfer gating (*4*). In order to detect the preceding intermediate, such as the two-electron reduced form of dioxygen, we investigated the earlier reaction process of the reduced laccase with dioxygen by means of stopped-flow spectroscopy (*2*). However, it was impossible to detect the two-electron reduced form of dioxygen because the diffusion of dioxygen towards the active site was too slow (ms order) compared with the rate of the electron transfer from type 1 Cu to the putative peroxide intermediate (the two-electron reduced form) (μs or less order). The content of the two-electron reduced species has been estimated to be a few percent and only the formation of the metastable three-electron reduced form of dioxygen (the radical intermediate) could be observed insofar as the reactions of the native enzyme were studied (*3*).

The type 2 Cu in laccase has been selectively depleted through the action of potassium hexacyanoferrate(II) (K_4-[$Fe(CN)_6$]) on the resting enzyme under anaerobic conditions, followed by the action of dimethylglyoxime and EDTA (*5*). In the type 2 Cu-depelted laccase the type 1 Cu is cupric and the coupled type 3 Cus are cuprous (T1oxT3red, which has been simply abbreviated as T2D hitherto). Contrary to in the case of hemocyanin, the oxygen carrier of arthropods and molluscs containing the coupled Cu site, T1oxT3red is inert toward dioxygen in spite of the reduction of its coupled type 3 Cus. This fact prompted us to incorporate a Cu ion (Cu^+ or Cu^{2+}) into the vacant type 2 Cu site (T1oxT23red and T12oxT3red) in order to reveal the crucial role of the type 2 Cu in the binding and reduction of dioxygen. We performed the reactions of T1oxT23red and T12oxT3red with dioxygen, detecting the two-electron reduced form of dioxygen. Based on the results we proposed the reaction mechanism by which dioxygen is converted to two water molecules in the normal reaction pathway of laccase.

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Abbreviations: T1oxT23red, type 1 Cu as Cu(II), and type 2 and 3 Cus as Cu(I); T1oxT3red, type 2 Cu-depleted laccase in which type 1 Cu is Cu(II) and type 3 Cus are reduced; T12oxT3red, type 1 and 2 Cus as Cu(II), and type 3 Cus as Cu(I); EPR, electron paramagnetic resonance; CD, circular dichroism; T, tesla (1 T = 10,000 G); T1Hg, mercury derivative of laccase at the type 1 Cu site; FTIR, Fourier-transform infrared; DPPH, diphenylpicrylhydrazyl.

MATERIALS AND METHODS

Materials—Laccase was purified from Chinese lacquer latex (A_{280}/A_{614} < 16), and the enzyme assay was performed for the oxidation of *N,N*-dimethyl-*p*-phenylenediamine as reported previously (*6*). The selective depletion of type 2 Cu was performed as reported with minor modification (*5*). CuCl was synthesized according to the literature and stored under Ar until use (*7*). T1oxT3red and a stoichiometric amount of CuCl were reacted for 6 h under Ar in order to incorporate a cupric ion into the type 2 Cu site. Since the solubility of CuCl is only 6 mg in 100 ml water and it is very sensitive to dioxygen, CuCl was dissolved in the least amount of water under Ar (>99.9995% purity) just before use and a fixed volume of the CuCl solution was introduced into the reaction vessel using a gas-tight syringe. The reaction vessel was protected from light to avoid fading of the blue colour (photoreduction of type 1 Cu takes place under light when type 2 Cu has been depleted). The action of CuCl on T1oxT3red was also directly observed in an optical quartz cell with a three-way stop-cock at its head. The reaction of T1oxT23red with dioxygen was started by directly introducing air into the cell using a syringe. $CuCl_2$ was also reacted with T1oxT3red in place of CuCl for the prolonged incubation of 2 days under Ar.

Measurements—Absorption spectra were measured with a JASCO U-best 50 spectrometer or a Shimadzu Multi-Spec-1500 spectrometer with a diode array detector. The EPR measurements were performed with a JEOL RE1X spectrometer at 77 K or a JEOL FE3X spectrometer at below 20 K. Samples were transferred from the reaction vessel to a quartz cell with to a three-way stopcock at its head using a syringe under Ar. Signal intensities were determined by the double integration method using diphenylpicrylhydrazyl (DPPH) as the standard. Circular dichroism (CD) spectra were obtained with a JASCO J-500C spectropolarimeter.

RESULTS AND DISCUSSION

T1oxT3red—In the type 2 Cu-depleted laccase formed using hexacyanoferrate(II), EDTA and dimethylglyoxime, the type 1 Cu is cupric and the type 3 Cus are cuprous (T1oxT3red) (*5*). This T1oxT3red does not react with dioxygen in spite of the reduction of the coupled type 3 Cus and accordingly shows no enzyme activity practically. T1oxT3red affords the charge transfer band due to the oxidized type 1 Cu at 614 nm, but not the band at 330 nm due to the oxidized type 3 Cus with a bridging hydroxy group [Cu(II)-OH-Cu(II)] differing from the native enzyme (line a in Fig. 1A). No signal other than that of the type 1 Cu was observed in the EPR spectrum (Fig. 2A).

T1oxT23red—A stoichiometric amount of CuCl was reacted with T1oxT3red under Ar at least for 6 h in order to ensure the formation of the novel mixed valent laccase, T1oxT23red. The resulting absorption spectrum is shown as line b in Fig. 1A. The absorption at 614 nm remained unchanged, ensuring that the type 1 Cu had been kept in the cupric form by rigorously cutting out light. However, the absorption intensity at 330 nm increased to almost half of that of the resting laccase. The corresponding EPR spectrum showed the full type 1 Cu signal and the 0.5 type 2 Cu signal (Fig. 2B). This indicates that a mixture of T1ox T23red and T12oxT3red was obtained after incubation of CuCl on T1oxT3red for 6h (simply abbreviated as T1ox-T23red). Unfortunately, it was impossible to use the highly

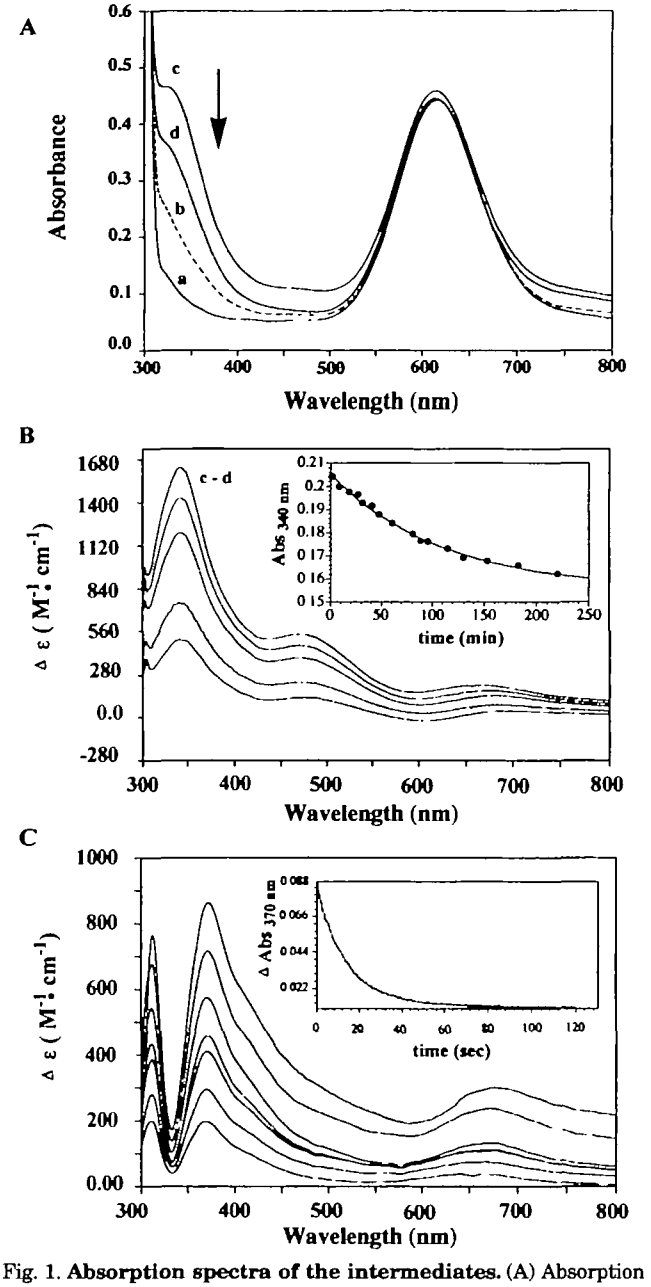

Fig. 1. **Absorption spectra of the intermediates.** (A) Absorption spectra of T1oxT3red (a), T1oxT23red (b), (b) soon after reaction with dioxygen (*ca.* 1 s) (c), and the completely oxidized laccase (d). Protein concentration, 78 μM; phosphate buffer, 0.2 M, pH 7.5. (B) Difference absorption spectra between the transient spectra and that of the fully oxidized laccase. Line c–d is as the difference spectrum of c minus d in Fig. 1A. The others are the transient spectra at arbitrary times. The inset shows the exponential decay of the band at 340 nm. (C) The absorption spectra of the radical intermediate (the oxyl and hydroxyl radicals bound to the trinuclear center) as the three-electron reduced form of dioxygen by laccase (*4*). The inset shows the exponential decay of the band at 370 nm. Protein concentration, 100 μM; phosphate buffer, 0.2 M, pH 7.4. The transient spectrum was obtained at 1 s after the reaction had started.

concentrated T1oxT3red to minimize the oxidation of the reacted Cu(I) ion due to its limited solubility in water. In addition, CuCl gradually disproportionates to $CuCl_2$ and Cu in water, although this did not seem to be serious (*vide infra*).

In spite of the presence of the oxidized type 2 Cu at a considerably high level, the laccase derivative recovered after the reaction with dioxygen showed almost full activity (98%) for the conventional assay of *N,N*-dimethyl-*p*-phenylediamine (Table I). This controversial fact indicates that practically all exogenous Cu ions reached the type 2 Cu site regardless of their final oxidation state, but that only the Cu(I) form had the ability to react with dioxygen. In line with this, when $CuCl_2$ was reacted with T1oxT3red under anaerobic conditions, the resulting derivative of laccase, T12oxT3red, was completely inactive toward dioxygen but showed high enzyme activity as to the oxidation of *N,N*-dimethyl-*p*-phenylenediamine (94% of the native enzyme).

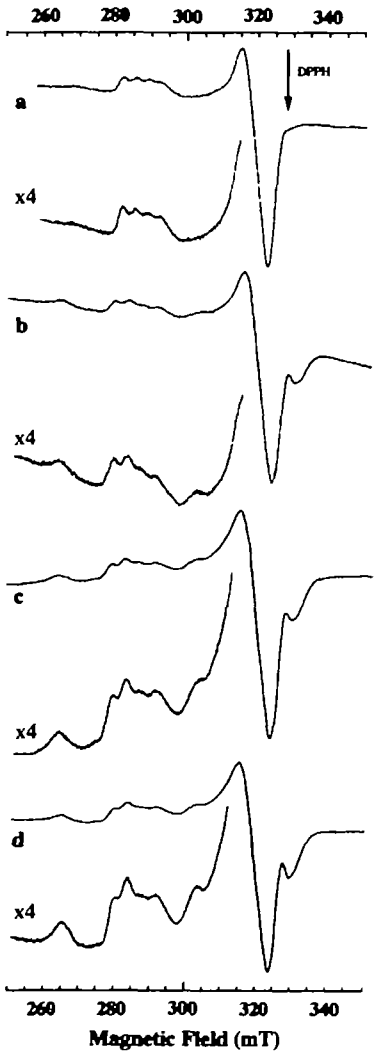

Fig. 2. **EPR spectra of T1oxT3red (A), T1oxT23red (B), T1oxT23red reacted with air at 10 min (C), and T1oxT23red reacted with air at 140 min (D).** The protein concentration was 47 μM in 0.2 M Tris-H_2SO_4 buffer, pH 7.4. Modulation, 100 kHz; frequency, 9.21 GHz; sweep time, 4 min; time constant, 0.03 s; amplitude, 1 mT; temperature, 77 K.

This indicates that T12oxT3red became reactive toward dioxygen after all Cus had been reduced with the exogenous reducing reagent. Furthermore, Co(II)-reacted T1oxT3red did not show the enzyme activity (*8*), and accordingly it appears that a cuprous ion should be present at the type 2 Cu site for the binding and reduction of dioxygen.

Kinetic and Spectroscopic Studies of T1oxT23red Reacted with Dioxygen—Soon after the reaction of T1oxT23red with air the absorption spectral features changed to give the strong band at 340 nm (ε_{340} ~ 6,300) (line c in Fig. 1A). The intensity of this band decreased gradually (slightly shifted from 340 to 330 nm with its decay), but the decrease stopped at the intensity the resting enzyme showed (ε_{330} ~ 4,700) (line d in Fig. 1A). The difference spectrum between the spectrum obtained soon after the reaction with air and that obtained after the decrease of the 330–340 nm band had stopped (c–d in Fig. 1B) showed three bands at 340 (ε_{340} ~ 1,600), 475 and *ca.* 680 nm (the molar extinction coefficient of the 340 nm band would be *ca.* 3,200 $M^{-1}\cdot cm^{-1}$, if we could start from the pure T1oxT23red). The former two bands decayed at the same rate, although the latter one was too weak to follow its decay and a red shift from 680 to 690 nm took place (*vide infra*).

Similar transient spectra have been reported for the reaction of laccase in which the type 1 Cu was changed to the redox-inactive Hg (T1Hg) (*9*), and also for the NO-reacted laccase in the presence of dioxygen (*10*). However, discussion of the structure of the intermediates has been confusing. A peroxide intermediate corresponding to the two-electron reduced form has been proposed from the former and a radical intermediate corresponding to a three-electron reduced form from the latter. We recently reported the absorption and EPR spectra of the oxyl and hydroxyl radicals reflected the three-electron reduced form of the native laccase (*4*). Its absorption spectrum (Fig. 1C) is unequivocally different from that of the intermediate given by laccase in which only the trinuclear center has been reduced (Fig. 1B). Apparently, the type 2 Cu had been reduced in the species to give the transient spectrum, but it was oxidized gradually, this being accompanied by the decay of the 340 nm band. In the EPR spectra in Fig. 2, the type 2 Cu signal increased to give the full intensity with the decay of the intermediate (B → C → D). It is supposed that the superficial red-shift of the transient band at *ca.* 680 nm to *ca.* 690 nm with the decay of the intermediate was due to that the d–d transition of type 2 Cu(II) developed concomitantly with the electron transfer from type 2 Cu (I) to the peroxide intermediate.

The 340 nm band decayed exponentially when the contribution of the fully oxidized enzyme was subtracted (Fig. 1B, inset). Therefore, it appears that a hydroxide ion has already bridged the type 3 Cus [Cu(II)-OH-Cu(II)] in the

TABLE I. **The relative enzyme activity as to oxidation of *N,N*-dimethyl-*p*-phenylenediamine and the reactivity towards dioxygen of the laccase derivatives before and after the reaction with dioxygen.** Relative activity is based on the activity of the native enzyme.

Laccase derivative	Enzyme activity (%)	Reactivity toward dioxygen when just prepared
T1oxT3red	0.5	no
T1oxT23red	98	yes
T12ox T3red	94	no

peroxide-bound form, contributing to the fixed absorption of ε_{330} ~ 4,700. This bridging group is supposed to be derived from a water molecule or a hydroxide ion which had been present in or near the active site. X-ray crystallography of ascorbate oxidase has shown that some water molecules are present around the active site (*11*).

The presence of the reduced type 2 Cu is indispensable for the binding of dioxygen. Therefore, the peroxide ion, as the two-electron reduced species of dioxygen, may interact with the type 2 Cu and one of the type 3 Cus in the μ-1,1 bridging mode, as indicated by a Hg derivative study (*9*) (*vide infra*, shown in the reaction scheme in Fig. 4). This binding mode is quite different from the μ-$\eta^2\eta^2$ and μ-1,2 bridgings between the coupled Cu(II) site in oxyhemocyanin (*12*) and many model compounds (*13*). According to the results of X-ray crystallography of ascorbate oxidase doped with peroxide (*14*), a hydroperoxide ion is terminally coordinated to one of the type 3 Cus, indicating that the end-on binding of a hydroperoxide ion is possible in the case of multicopper oxidases. As for the μ-1,1 binding of peroxide to the coupled Cu(II) center, an acylperoxo group coordination has been found in a small molecule study (*15*). Otherwise, a novel binding mode, in which a peroxide ion is vertically positioned at the center of the trigonal plane formed by the type 2 Cu (cuprous) and type 3 Cus (cupric), should not be excluded at the present stage.

The third electron donor is not the type 2 Cu but the type 1 Cu in the reaction of the native laccase (*4*), and accordingly the present successful detection of the peroxide intermediate was due to that the laccase derivative containing the oxidized type 1 Cu was prepared. The population of the peroxide intermediate has been estimated to be only a few percent in the reaction of the native laccase (*3*) since the diffusion of dioxygen to the trinuclear center (ms order) is rate-determining and the third electron transfer from the type 2 Cu to the putative peroxide intermediate takes place very rapidly (ms order or faster).

The decay of the present peroxide intermediate was apparently faster than that reported for the reaction of the Hg derivative, which has given a similar intermediate (rates greater than twice) (*9*). In addition, the pH dependency was quite different: the decays of the present intermediate continuously accelerated with decreasing pH, as shown in Fig. 3, but the decays of the intermediate in the case of the Hg derivative gave a sigmoidal curve. This difference might have been brought about by a certain difference in the protein conformation around the trinuclear center due to positioning of the different metal ion at the type 1 Cu center. Otherwise, the difference in the experimental procedures might have been the cause: we changed the pH of the enzyme derivative by dialysis to ensure the conformational change of the protein molecule, but in the Hg-derivative study aliquots of the reduced sample at pH 7.4 were injected into an acidic buffer pre-equilibrated with dioxygen and the actual pH was determined after the reactions.

We tried to characterize the present intermediate by means of resonance Raman spectroscopy. However, the fluorescence was too strong. FTIR spectral measurement was also unsuccessful because of the very low solubility of CuCl in water, although we concentrated the sample during the incubation of CuCl using a home-made gas-tight centrifuge tube. According to the results of CD spectroscopy for the reaction of T1oxT23red with dioxygen, the feature of the region, 320-400 nm, changed to give a new negative-signed band at 320 nm and a positive-signed band at 360 nm (not shown) (a similar spectrum has been reported for the reac-

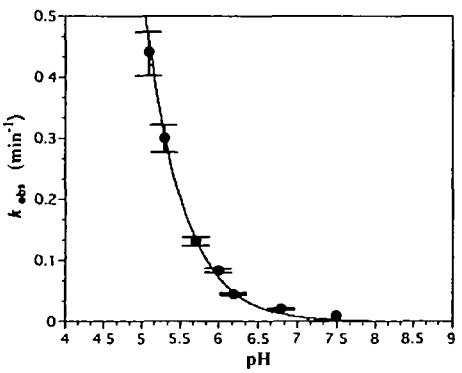

Fig. 3. **The first order decay rate of the intermediate band at 340 nm as a function of pH.** The protein concentrations were in the range of 31–80 μM in phosphate or Tris- H_2SO_4 buffer, 0.2 M. At least three independent results were averaged.

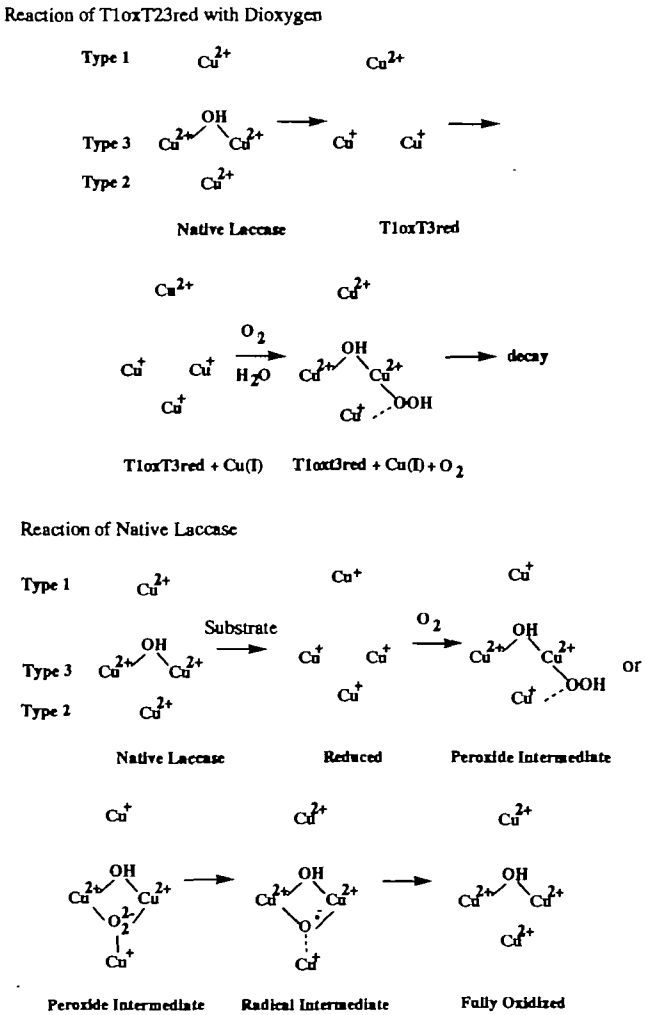

Fig. 4. **Proposed reaction mechanisms of T1oxT23red and native laccase with dioxygen.**

tion of the Hg derivative (*9*)). However, this change was due to the intermediate itself because the life-times of these bands were much longer than that in the case of the two-electron reduced species of dioxygen. Therefore, the CD spectrum shows that laccase under turnover conditions, is in a slightly different state from that of the resting enzyme.

Four-Electron Reduction of Dioxygen by Laccase—Finally, the reaction mechanism by which dioxygen is reduced to two water molecules was schematically depicted by taking the present results, and those for the formation and decay of the three-electron reduced form of dioxygen into consideration (Fig. 4) (*3, 4*). The presence of the reduced type 2 Cu was found to be indispensable for the binding and reduction of dioxygen. The third electron transfer does not take place from this nearby type 2 Cu but from the remote type 1 Cu. This will be because the type 2 Cu has the highest redox potential among the four Cu ions in laccase [only the redox potential in the resting laccase has been reported (*16*)]. This originates in the unique structure of the type 2 Cu, which is only two-coordinated in the reduced form and three-coordinated even in the oxidized form. The heterolytic cleavage between the O-O bond will not take place in the two-electron reduced form, although it may take place in the cases of the dioxygen-activation processes of the singly Cu-containing oxidases and of the four-electron reduction of dioxygen by cytochrome *c* oxidase (*17*). In the reaction process of the latter terminal oxidase containing the coupled heme-Cu site, oxoheme species have been detected. When dioxygen accepts two-electrons in the antibonding orbital, the bond between two oxygen atoms becomes single. Once another electron is supplied to the vacant antibonding orbital, the bond-order becomes 0.5, the O-O bond being cleaved formally to give O^{2-} (H_2O) and $O^{-\cdot}$. (oxyl and/or hydroxyl radical), as we have recently characterized in detail (*4*). It is considered that a similar reaction pathway is followed by all multicopper oxidases such as ceruloplasmin, ascorbate oxidase, bilirubin oxidase, *etc.* The supply of protons would play a key role in the four-electron reduction of dioxygen to two water molecules, as evidenced by the increasing acceleration of the decay of the intermediate with decreasing pH (Fig. 3).

REFERENCES

1. Messerschmidt, A., ed. (1997) *Multicopper Oxidases*, World Scientific, Singapore
2. Andreasson, L.-E., Branden, R., and Reinhammar, B. (1976) Kinetic studies of *Rhus vernicifera* laccase. Evidence for multielectron transfer and an oxygen intermedaite in the reoxidation reaction. *Biochim. Biophys. Acta* **438**, 370–379
3. Zoppellaro, G., Huang, H., and Sakurai, T. (2000) Kinetic studies on the fully reduced laccase with dioxygen. *Inorg. Rec. Mech.* **2**, 79–94
4. Huang, H., Zoppellaro G., and Sakurai, T. (1999) Spectroscopic and kinetic studies on the oxygen-centered radical formed during the four-electron reduction process of dioxygen by *Rhus vernicifera* laccase. *J. Biol. Chem.* **274**, 32718–32724
5. (a) Graziani, M. T., Morpurgo, L., Rotilio, P., and Mondovi, B. (1976) Selective removal of type 2 copper from *Rhus vernucifera* laccase. FEBS Lett., 70, 87–90; (b) Sakurai, T. and Takahashi, J. (1995) EPR Spectra of type 3 copper centers in *Rhus vernicifera* laccase and *Cucumis sativus* ascorbate oxidase. *Biochim. Biophys. Acta* **1248**, 143–148
6. Reinhammar, B. (1970) Purification and properties of laccase and stellacyanin from *Rhus verninicifera Biochim. Biophys. Acta* **205**, 35–47
7. Kelle, R.L. and Wycoff, H.D. (1945) Copper(I) chloride. *Inorg. Syn.* **2**, 1–4
8. Sakurai, T., Suzuki S., and Fujita, M. (1988) Visible and magnetic circular dichroism studies on cobalt(II)-substituted *Rhus vernififera* laccase. *Inorg. Chim. Acta* **152**, 139–143
9. Shin, W., Sundaram, U.M., Cole, L., Zhang, H.H., Fedman, B., Hodgson K.O., and Solomon, E.I. (1996) Chemical and spectoscopic definition of the peroxide-level intermediate in the multicopper oxidases: Relevance to the catalytic mechanism of dioxygen reduction to water. *J. Am. Chem. Soc.* **118**, 3202–3215
10. Torres, J. and Wilson, M.T. (1999) The reactions of copper proteins with nitric oxide. *Biochim. Biophys. Acta* **1411**, 310–322
11. Messerschmidt, A., Ladensteinm R., Huber, R., Bolognesi, M., Avigliano, L., Petruzzeli, R., Rossi R., and Finazzi-Agro, A. (1992) Refined crystal structure of ascorbate oxidase at 1.9 Å resolution. Dioxygen copper reactivity: X-ray structure and characterization of (acylperoxo)dicopper complex. *J. Mol. Biol.* **224**, 179–205
12. Magnus, K.A., Hazes, B., Ton-That, H., Bonaventura, C., Bonaventura, J., and Hol, W.G.J. (1994) Crystallographic analysis of oxygenated and deoxygenated states of arthropod hemocyanin shows unusual differences. *Proteins Str. Gen.* **19**, 302–309
13. Karlin, K.D., Kaderli, S., and Zuberbuhler, A.D. (1997) Kinetics and thermodynamics of copper(I)/dioxygen interaction. *Acc. Chem. Res.* **30**, 139–147 and papers cited therein
14. Messerschmidt, A., Luecke, H., and Huber, R. (1993) X-ray structures and mechanistic imlications of three functional derivatives of ascorbate oxidase from zucchini. *J. Mol. Biol.* **230**, 997–1014
15. Ghosh, P., Tyklar, Z., Karlin, K.D., Jocobson, R., and Zubieta, J. (1987) Dioxygen-copper reactivity: X-ray structure and characterization of an (acylperoxo)dicopper complex. *J. Am. Chem. Soc.* **109**, 6889–6891
16. Reinhammar, B.R.M. (1972) Oxidation-reduction properties of the electron acceptors in laccase and stellacyanin. *Biochim. Biophys. Acta* **275**, 246–259
17. Kitagawa, T. and Ogura, T. (1998) Time-resolved resonance raman investigation of oxygen reduction mechanism of bovine cytochrome *c* oxidase. *J. Bioeng. Biomem.* **30**, 71–79