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pH and Microwave Power Effects on the Electron Spin Resonance Spectra of *Rhus vernicifera* Laccase and *Cucumis sativus* Ascorbate Oxidase¹

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The present study shows that the electron spin resonance (ESR) spectral features of *Rhus* laccase depend considerably on the pH value of the enzyme solution and the irradiated microwave power. Because of the local protein structure change, the type 1 copper is appreciably autoreduced at alkaline pH as monitored both by the ESR and absorption spectroscopies. In addition, the ESR signal of the type 2 copper, especially its $g_{\perp}$ region, becomes prominent at alkaline pH. Protein dissociation from a water or an imidazole group coordinated to the type 2 copper is supposed to be responsible for this behavior. Besides above pH effects, the $g_{\perp}$ component of the type 2 copper ESR signal is obscured with rising microwave power level. The power saturation behavior of native laccase and its derivatives reveals that the type 2 copper is more easily saturated than the type 1 copper. *Cucumis* ascorbate oxidase also exhibits similar behavior upon pH variation and microwave power saturation.

Laccase (benzenediol: oxygen oxidoreductase) [EC 1.10.3.2], ascorbate oxidase (L-ascorbate: oxygen oxidoreductase) [EC 1.10.3.3], and ceruloplasmin (Fe(II): oxygen oxidoreductase) [EC 1.16.3.1] are multicopper oxidases containing three distinct types of copper centers. Type 1 copper, or so-called blue copper, exhibits an intense visible absorption at around 600 nm, attributable to the $S^-(\text{Cys}) \rightarrow \text{Cu}^{2+}$ charge transfer band, and yields an ESR spectrum with a very narrow hyperfine splitting. The ESR spectrum of the type 2 copper is characteristic of the usual tetragonal copper complex. In the visible spectrum the d-d transition is observable only for the derivatives of multicopper oxidases (1, 2). The type 3 copper site accepts two-electrons and functions as the binding and reaction center for O_2 . Type 3 coppers are ESR silent even in the oxidized form, because the two copper(II) ions are very strongly antiferromagnetically coupled. This type of copper does not contribute to the visible absorption either, but does to the shoulder at around 330 nm. However, a highly rhombic tetragonal Cu ESR signal has been reported for a semi-reduced form of the type 3 copper site (3). Although laccase, ascorbate oxidase, and ceruloplasmin respectively contain 4, 8, and 5 plus some extra copper ions, it was recently established that every active site is composed of one type 1, one type 2, and two type 3 coppers (henceforth denoted by "1-1-2") (ascorbate oxidase is a dimeric enzyme containing two active sites) (4-6).

We have wondered why the hitherto reported ESR spectra of native laccase are quite diverse in spite of the fixed copper content (7-9). In addition, the ESR spectrum of zucchini (summer squash) ascorbate oxidase has been

simulated not with 2-2-4 coppers but with 3-1-4 coppers (10, 11). In order to solve these fundamental problems on ESR spectra we made detailed ESR studies on laccase and ascorbate oxidase in the range of pH 5.1 to 9.5. Ceruloplasmin was excluded from the present study because of its extra type 1 copper which does not participate in the enzyme reaction (5, 6).

MATERIALS AND METHODS

Purification of Laccase and Ascorbate Oxidase—Laccase was prepared from Chinese lacquer latex (*Rhus vernicifera*) supplied by Takano and Co., Kanazawa, according to the method of Reinhammar (12). $A_{280}/A_{615} = 17$ was satisfactory, ensuring the purity of the enzyme. Ascorbate oxidase was purified from cucumber peelings by the usual method (13). The final absorption ratio, A_{280}/A_{610} was 24. Potassium phosphate buffer (pH 6) was used throughout the preparation and storage of both enzymes.

Preparation of Samples for Spectral Measurements—Native enzymes at different pHs were prepared by dialyzing the enzymes in pH 6 buffer against an appropriate pH potassium phosphate or Tris-HCl buffer for 1 day. Prolonged incubation was not done to avoid fatal denaturation. The laccase derivative, in which the type 1 copper site was occupied by Hg(II), the type 2 copper site by Cu(II), and the type 3 copper sites by 2 Cu(II)s [in the ESR detectable met form ($\text{Cu}^{2+} \text{Cu}^{2+}$)] or by 2 Co(II)s, were prepared according to our previous method (2). Protein concentration was determined based on the absorbance at 280 nm. The absorption coefficients of both enzymes did not change significantly over the pH range in this study. All reagent-grade chemicals were used without further purification.

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Spectral Measurements—ESR spectra at 77 K were measured on a JEOL JES-FE1X spectrometer and those at lower temperatures on a JEOL JES-FE2XG spectrometer. Temperatures below 77 K were obtained with a liquid-helium transfer system (Air Products LTD-3-110). The magnetic field was calibrated using an Echo Electronics EFM-2500 gaussmeter. The microwave frequency was calibrated on a Takedariken TR5212 microwave counter. Cu-EDTA was used to estimate the ESR signal intensities by the usual double integration method. Lithium *N,N,N',N'*-tetracyanoquinodimethane (Li-TCNQ) ($g = 2.0025$) was used to calibrate the estimation error of the ESR signal intensities arising from the difference in tuning conditions. CD spectra were measured on a JASCO J-500A spectropolarimeter.

Computer Simulation of ESR Spectra—Computer simulations of the ESR spectra of native laccase and ascorbate oxidase were performed using a program developed by M.C. As the initial parameters for the type 1 and type 2 coppers, those of the selectively type 2 copper-depleted enzymes (13, 14) and those of the Hg(II)-substituted derivatives at the type 1 copper sites (2) were employed, respectively. Afterwards, spin Hamiltonian parameters were slightly modified during the iteration processes. The effect of Cu isotopes (^{63}Cu and ^{65}Cu) and superhyperfine splitting due to N donors, which are not usually taken into consideration, were also included for the simulations.

RESULTS

Effect of pH on the Absorption and ESR Spectra of Laccase—ESR spectra of native laccase were measured between pH 5.1 and 9.5. The spectra obtained at 0.78 mW

Fig. 1. ESR spectra of laccase at 77 K and 9.21 GHz (ca. 0.22 mM protein, 0.1 M potassium phosphate buffers at pH 5.1 and 6.0, 0.1 M Tris-HCl buffers at pH 8.0, 8.9, and 9.5). The microwave power was 0.78 mW, the modulation frequency was 100 kHz, and the field modulation was 8 G.

are shown in Fig. 1. The reason why such a low microwave power was employed is given later. The type 2 copper signal ($g_{\parallel} = 2.237$, $g_{\perp} = 2.053$, $A_{\parallel} = 198$ G, $A_{\perp} = 20$ G), especially its $g_{\perp}$ region, became conspicuous at higher pH. The spectral features at alkaline pH (9.5) were rather similar to those of the Hg-substituted laccase derivative at its type 1 copper binding site (2, 15). On the other hand, the $g_{\perp}$ component of the type 2 copper signal became weak at lower pH, while the type 1 copper signal ($g_{\parallel} = 2.298$, $g_{\perp} = 2.055$, $A_{\parallel} = 45$ G, $A_{\perp} = 20$ G) became predominant.

The concomitant changes of the absorption maxima near 610 nm (blue band) are shown in Fig. 2 together with the values of the total amount of ESR-detectable Cu(II). The blue color due to the cupric form of the type 1 copper remained between pH 5 to 6, but began to fade with rising pH. Moreover, the amount of ESR-detectable Cu(II) began to decrease from 2 to a lower value, indicating that significant autoreduction of the type 1 copper occurred at alkaline pHs. The type 1 copper must not have changed to type 2 copper because the blue color and the type 1 ESR signal were restored, although not completely, by treating the laccase in a high pH buffer solution with a slight excess of H_2O_2 (unpublished data). The absorbance at about 610 nm and the total amount of the ESR-detectable Cu^{2+} changed in a similar manner on pH variation. The discrepancy between the decrease of the blue color (64% at pH 9.5) and that of the ESR-detectable Cu(II) (56% at pH 9.5) may be explained by a minor change of the absorptivity of the charge transfer band ($\text{Cys} \rightarrow \text{Cu}^{2+}$). A slight but prominent blue shift from 615 to 606 nm of the $\text{Cys} \rightarrow \text{Cu}^{2+}$ charge transfer band was observed in the absorption spectra as the pH was raised (Fig. 2). CD spectra (300–800 nm, not shown) suggested that a minor modification occurs in the protein structure at alkaline pH. In addition, the pH value change on freezing may also contribute to the discrepancy described above (see "DISCUSSION").

When the pH values of laccase in various pH buffers were returned to 6, the most frequently used pH for research on this enzyme, ESR spectra similar to those before pH changes were restored. However, since a portion of the type

Fig. 2. The amount of ESR detectable Cu(II) per protein molecule and molar absorptivity of the blue band of laccase at different pHs. The values in the figure show the absorption maxima of the blue band.

1 copper in the reduced form had been lost from protein molecule, the type 1 copper ESR signal was decreased partly (spectra not shown). In the respective absorption spectra, the absorption maximum position of the blue band returned from 608 nm (pH 8.9) or 606 nm (pH 9.5) to 615

Fig. 3. ESR spectra of laccase (ca. 0.2 mM) at 77 K (A: pH 6.0, 0.025 mW; B: pH 6.0, 200 mW; C: pH 8.0, 0.05 mW; D: pH 8.0, 200 mW). The microwave frequency was 9.24 GHz, the modulation frequency was 100 kHz, and the modulation amplitude was 8 G.

Fig. 4. Power saturation curves of the native laccase (A), T1HgT2CuT3Cu (ESR-detectable met form) laccase (B), and T1HgT2CuT3Co laccase (C) at 10 K. The concentration of each sample was ca. 0.2 mM per protein molecule, and the pH was 8.0. The intersection of the asymptotic slopes at lower and higher power defines the half power saturation. S and P denote signal intensity and microwave power (mW), respectively.

nm. The absorption intensities also appreciably recovered.

Power Saturation Behavior of Laccase—Saturation of a paramagnetic system with incident microwave power has been widely applied in ESR studies to elucidate both electronic and structural properties of spin systems (16). However, the technique has never been applied to laccase, although the ESR spectra of the enzyme have been repeatedly measured. We investigated the ESR spectra of laccase in several buffers with the irradiation of different microwave power levels at 10, 60, and 77 K. Generally, the saturation of paramagnetic species occurs easily at low

Fig. 5. ESR spectra of laccase derivatives measured at 10 K with low and high microwave powers (A: T1HgT2CuT3Co, 0.025 mW; B: T1HgT2CuT3Co, 200 mW; C: T1HgT2CuT3Cu, 0.025 mW; D: T1HgT2CuT3Cu, 200 mW). The other conditions for measurements were similar to those shown in Fig. 4.

Fig. 6. Experimental (A) and simulated (one type 1 Cu and one type 2 Cu) (B) ESR spectra of laccase at 9.21 GHz and 77 K. The broken and dotted lines represent the type 2 copper ($g_{\parallel}=2.237$, $g_{\perp}=2.053$, $A_{\parallel}=198$ G, $A_{\perp}=20$ G) and the type 1 copper ($g_{\parallel}=2.298$, $g_{\perp}=2.055$, $A_{\parallel}=45$ G, $A_{\perp}=20$ G), respectively.

Fig. 7. ESR spectra of ascorbate oxidase (ca. 0.03 mM) at 77 K in different pH buffers [pH 6.0 (potassium phosphate buffer) and pH 8.0, 8.9, and 9.5 (Tris-HCl buffer)]. The microwave power was 3 mW, the microwave frequency was 9.20 GHz, the modulation frequency was 100 kHz and the modulation amplitude was 8.0 G.

Fig. 8. The amount of ESR-detectable Cu(II) and the molar absorptivity of ascorbate oxidase at different pHs.

temperature near 4 K, and accordingly the saturations of both the type 1 and type 2 coppers became more prominent at 10 K than at higher temperatures. ESR spectra of laccase at pH 6 and pH 8 measured at very low and high microwave power levels are illustrated in Fig. 3. Now the diversity of the laccase ESR spectra reported hitherto is found to come from the differences in the irradiated microwave power and in the pH of the buffer solution. Saturation behavior at pH 8 and 10 K is shown in Fig. 4A. Since the signal shape of the type 2 copper, especially its $g_{\perp}$ region, varies considerably at a high microwave power, signal intensity was followed at the $g_{\parallel}$ region (2,946 G for the type 1 copper, 2,684 G for the type 2 copper). The ESR signal of the type 2 copper in laccase was saturated at a lower microwave power than that of the type 1 copper. The half power saturation ($P_{1/2}$) for the type 1 and type 2 coppers were 15 and 4 mW at 10 K and pH 6.0, respectively. The difference of the environment

Fig. 9. The power saturation curves of the type 1 and type 2 coppers of ascorbate oxidase (pH 6.0) at 10 K.

Fig. 10. Experimental (A) and simulated (B for three type 1 coppers and one type 2 copper, C for two type 1 coppers and two type 2 coppers) spectra of ascorbate oxidase at 9.21 GHz and 77 K. Spin Hamiltonian parameters for the type 2 copper are $g_{\parallel}=2.240$, $g_{\perp}=2.050$, $A_{\parallel}=198$ G, $A_{\perp}=20$ G and those for the type 1 copper are $g_{\parallel}=2.230$, $g_{\perp}=2.050$, $A_{\parallel}=62$ G, $A_{\perp}=18$ G.

around each type of copper site will dominate the difference of the saturation behavior. ESR spectra of two laccase derivatives, whose type 1 copper site was substituted by Hg(II) and the type 3 copper sites by 2 Co(II) or 2 Cu(II) are shown in Fig. 5. In the latter derivative, the 2 coppers incorporated into the type 3 copper sites were fully ESR detectable, affording an ESR signal very similar to that of the type 2 copper (2). These type 3 coppers in the ESR-detectable met form ($\text{Cu}^{2+} \text{Cu}^{2+}$) were saturated ($P_{1/2}=2$ mW) somewhat more easily than the type 2 copper in the native enzyme ($P_{1/2}=4$ mW) (Fig. 4, A, B, and C).

Simulation of Laccase ESR Spectra—The results of our

computer simulation of the laccase ESR spectrum at pH 6 are presented in Fig. 6.

Effect of pH on the Absorption and ESR Spectra of Ascorbate Oxidase—ESR spectra of ascorbate oxidase at different pHs are shown in Fig. 7. The total number of ESR-detectable Cu^{2+} s was 4 (50% of total Cu) per protein molecule at pH 6. With increasing pH value, the type 2 copper gradually became prominent, as in the case of laccase (see Fig. 1). Quantitative evaluations of the amount of ESR detectable Cu(II) and the absorbance of the blue band due to the type 1 copper (Fig. 8) indicate that autoreduction of the type 1 coppers occurs, although the extent is not as marked as in laccase.

Power Saturation Behavior of Ascorbate Oxidase—The power saturation behavior of ascorbate oxidase is shown in Fig. 9. The signal intensities of the type 1 and type 2 coppers were followed for the g_z components at 2,970 and 2,640 G, respectively. The reason why the perpendicular components was not followed was to avoid the signal shape change displayed in laccase (Fig. 3). The half saturation powers of both types of copper are about 10 mW at 10 K.

Simulation of ESR Spectrum of Ascorbate Oxidase—Figure 10 illustrates the simulated ESR spectra of ascorbate oxidase. Recently, the numbers of type 1 and type 2 coppers in ascorbate oxidase have both been established to be 2 (4). However, as reported in the simulation study for squash ascorbate oxidase (11), the present cucumber ascorbate oxidase, whose type 1 coppers were not autoreduced, could be better simulated as 3 type 1 coppers and 1 type 2 copper.

DISCUSSION

The pH change of several buffer solutions on freezing has been pointed out by Oori and Morita (17). It is ideal to observe the ESR spectra as well as the absorption spectra at room temperature; however, the measurements were performed below 77 K because ESR sensitivity is significantly lowered at room temperature. The effect of freezing does not seem to be serious because the pH dependences of the absorption and ESR spectra correlated fairly well.

The profound effect of pH on the absorption, CD and ESR spectra has been overlooked or ignored. Most studies on *Rhus* laccase have been performed at pH 6.0, 7.0, and 7.4. The ESR spectrum at pH > 8 is available only in the early study by Malmström *et al.* (pH 10.3) (18). They reported that the ESR parameters of the type 1 copper were simply modified. But we repeatedly observed that the type 1 copper is autoreduced as the pH of the buffer solutions increases (Figs. 1 and 2). Of the blue copper proteins containing only type 1 copper, plastocyanin has been reported to autoreduce at pH 10.2 in parallel with the exposure of a hydrophobic site to the solvent (19). In contrast, Cu(II) of stellacyanin, which is contained in the latex of lacquer tree, was bound to the polypeptide chain at an extremely high pH, giving a complex similar to Cu(II)-biuret (20). Umecyanin has been reported to alter its color almost reversibly, when the pH is extremely high (21). The absorption and ESR spectra of plantacyanin (22) and a stellacyanin-like protein from spinach (23) have also been reported to change at high pH. After all, many blue copper proteins not including plastocyanin, do not prefer the reduced form in alkaline pH buffers in spite of their positive

redox potentials (+180–+370 mV). Since the type 1 copper of laccase, whose redox potential is +470 mV at pH ca. 4 and +380 mV at pH ca. 9 (24), is closely related to other types of coppers (25), a geometrical change leading to autoreduction may occur without difficulty.

The only computer simulation of a laccase ESR spectrum, whose procedure was well documented, was for the spectrum obtained at pH 7.5 (8). In the spectrum the type 2 copper signal is quite conspicuous, because the type 1 copper had been partly reduced. On the other hand, the quantitative studies by the conventional double integration method have often reached the conclusion that the type 1 and type 2 coppers of laccase are almost fully ESR detectable within the experimental error (14, 18, 20, 26, 27). The same conclusion was also obtained by the present study for laccase at pH 6. A fairly good computer simulation of the ESR spectrum at pH 6 could be realized by reducing the type 2 copper content from 1 to a lower value (data not shown). However, it is inconsistent with the fact that the type 2 copper is almost fully ESR detectable. The question may therefore be raised whether the total amount of the ESR detectable Cu(II) had been overestimated. The use of an ESR signal exhibiting a different shape as a standard should be avoided if possible, since it is difficult to retain an identical tuning condition for samples to give a different dielectric loss. The power saturation behavior of Cu-EDTA (data not shown) ensured, however, that at least saturation is not fatal when less than 5 mW is used for the measurements at 77 K. Figure 6 is the final simulated spectrum in which the contributions from ^{63}Cu and ^{65}Cu and the superhyperfine splitting due to the imidazole N ligands were included for equal content of type 1 and type 2 coppers.

The peculiar sharpening of the perpendicular region of the type 2 copper at alkaline pH (Fig. 1) seems to arise from structural changes about this copper. One possibility is deprotonation from the water molecule(s) coordinated to the type 2 copper. The coordination of one or two water molecules has been established through anion binding studies (28, 29). The pK_a values associated with a water molecule and a protein hydroxyl group coordinated to the type 2 copper of laccase have been estimated to be 6.2 and 8.6, respectively (30). Deprotonation from the coordinated imidazole group of a histidine residue is another possibility.

The case of ascorbate oxidase is very similar. As the pH value of the buffer solution increases, autoreduction of the type 1 copper occurs, although its extent is not so large as that of laccase. The rigidity of the local protein structure will determine the sensitivity of type 1 copper to high pH. The reversible fading of the blue color of ascorbate oxidase at pH as high as 10.5 was only described in a review (31): no original data has been furnished hitherto. The numbers of type 1 and type 2 coppers of ascorbate oxidase have both been revealed to be two (4), although three type 1 coppers and one type 2 copper were favorable for the computer simulation (Fig. 10). If the computer simulation reached a satisfactory solution for two type 1 coppers and 0.7 type 2 copper (3 : 1 ratio) (this happens when a portion of the type 2 copper is in the reduced form), the total amount of the ESR-detectable Cu(II) should not have been 4 but 2.7. However, the value estimated by the conventional method using Cu-EDTA was very near to 4, as shown in Fig. 8. It is not certain at present whether the estimation error of the

ESR-detectable amount of Cu(II) or the limitation of the computer simulation is dominant.

The deprotonation process from the water molecule coordinated to the type 2 copper of ascorbate oxidase may be involved in the peculiar sharpening of the perpendicular region of the type 2 copper signal at alkaline pH (Fig. 7). Moreover, the possible deprotonation from the coordinated imidazole group(s) or the shape change of the type 2 copper ESR signal can not be excluded at present.

In Nakamura's pioneering work (7), the ESR spectrum of native laccase lacking the type 2 copper signal was reported. Further, chicken ceruloplasmin isolated promptly by a single-step chromatography did not give the type 2 copper ESR signal (32). However, X-ray crystallography of ascorbate oxidase from zucchini by Messerschmidt *et al.* (33), indicated that type 2 copper is the intrinsic cofactor for multicopper oxidases. A portion of the type 2 copper must be in the cuprous form, occasionally leading to its ESR signal being missing or very limited in the resting multicopper oxidases. Otherwise, it is also plausible that the signal which has been believed to be the type 2 copper is that of the type 3 copper in the ESR-detectable met form. In any case, special attention should be paid in obtaining ESR spectrum, as the present study shows.

The type 2 copper has been supposed to be involved in the O₂ reduction process (34-36). If the dissociation of the proton from the water molecule coordinated to the type 2 copper is concerned with the present ESR signal modification, it may be supposed not the type 2 copper itself but the coordinated water operates to weaken the ⁻O-O⁻ bond through a hydrogen bond and/or to supply H⁺ to the intermediate(s).

In conclusion, the results described herein from the ESR, absorption and CD spectra of laccase and ascorbate oxidase gave significant information about the pH effects leading to the partial autoreduction of the type 1 copper. The profound effect of the microwave power level on the ESR spectra of laccase and ascorbate oxidase was also investigated.

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