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ROOM TEMPERATURE ESR SPECTRA OF *Rhus vernicifera* LACCASE AND DERIVATIVES

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Although both the type 1 and type 2 coppers of *Rhus vernicifera* laccase are fully ESR detectable at 77 K, only 30 % of the type 2 copper are in the cupric form at room temperature. The residual 70% of the type 2 copper was easily transformed into the ESR detectable form by irradiating the resting enzyme with microwave of 200 mW. The enzyme activity did not change by the irradiation with high-powered microwave, indicating that the type 2 copper can be in both the ESR detectable and ESR undetectable forms in solution. The room temperature ESR spectra of the type 2 copper-depleted laccase and of the azide-bound type 3 copper signals were also measured at room temperature and compared with those at 77 K. © 1995 Academic Press, Inc.

Tree laccase is an enzyme to oxidize phenolic lipids such as urusiol in lacquer latex (1). Laccase contains three types of coppers, the type 1 copper (blue copper), the type 2 copper (non-blue copper), and a pair of the type 3 coppers (ESR non-detectable coppers) in the active site (2). These three types of coppers have been classified based on their peculiar spectroscopic, magnetic, and redox properties. The magnetic property is based on the ESR spectrum, in which the type 1 and type 2 coppers are detectable at lowered temperatures such as 77 K and 4 K.

According to the crystal structure of ascorbate oxidase (3) containing the active site analogous to that in laccase, the type 2 copper and a pair of type 3 coppers form a trinuclear site, in which an OH^- group bridges two type 3 coppers, making them ESR-undetectable. On the other hand, no bridging group exists between the type 2 copper and type 3 coppers. Therefore, the type 2 copper is magnetically isolated from the type 3 coppers.

It has been very rare that ESR spectra of biological radicals are measured at room temperature because of sensitivity and line width (4). However, measurements of other spectroscopies are frequently measured at room temperature. Therefore, we have measured ESR spectra of native laccase and derivatives in order to compare them with other spectroscopic data obtained at the same condition. We unexpectedly obtained the well-resolved spectra at room temperature and 70% of the type 2 copper in resting

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laccase appeared to be ESR undetectable in contrast to low temperature data. In addition, ESR spectra of the type 1 and type 3 coppers were also measured in comparison with those at lowered temperatures, showing that freezing affect both the electronic and magnetic states of these copper ions.

MATERIALS AND METHODS

Materials Chinese lacquer latex with the highest quality was obtained from Takano and Co. Kanazawa. Laccase was purified from acetone powder according to Reinhammar(5). Purity was checked by electrophoresis and HPLC. The type 2 copper was selectively depleted from resting laccase according to literature (6). The enzyme activity was determined according to literature using *N*,*N*-dimethyl-*p*-phenylenediamine (5). Potassium phosphate buffer (0.2 M, pH 6.0) was used throughout measurements.

Measurements The X-band ESR spectra were measured at room temperature and at 77 K on a JEOL RE-1X spectrometer. A flat cell for aqueous solution was used for measurements at room temperature. The amount of the ESR detectable Cu^{2+} was determined by the double integration method using Cu-EDTA as a standard by calibrating the difference in tuning conditions with an external marker. Cu-EDTA was sufficient as the standard in spite of the difference in line shapes as far as the signals are not saturated (7). The absorption spectra were measured on a JASCO Ubest-50 spectrometer.

RESULTS AND DISCUSSION

The room temperature ESR spectrum of resting laccase is shown in Fig. 1A together with that at 77 K (Fig. 1B). It is apparent that the hyperfine splitting of the type 1 copper becomes large at room temperature ($g_{\parallel}$ = 2.20 and $A_{\parallel}$ = 5.4 mT (5.5 x 10^{-3} cm^{-1}, $g_{\perp}$ = 2.06) compared with that at 77 K ($g_{\parallel}$ = 2.30, $A_{\parallel}$ = 4.5 mT (4.3 x 10^{-3} cm^{-1}, $g_{\perp}$ = 2.06) (7). The type 1 copper was fully ESR detectable regardless of temperature. More attracting for the spectrum at room temperature is that the type 2 copper signal is very weak and is not well resolved.

The total amount of the ESR detectable Cu^{2+} was reproducibly only 1.3 Cu^{2+} per protein molecule. In contrast, the value at 77 K was exactly 2.0 (Fig. 1B), indicating that at room temperature 70% of the type 2 copper are in the reduced form or in an ESR undetectable form due to a magnetic interaction in the trinuclear site comprised of a type 2 copper and the coupled type 3 coppers (8).

The room temperature ESR spectrum of Fig. 1A was obtained with a relatively low microwave power of 12.5 mW. However, the type 2 copper signal became prominent during the measurement with microwave of 200 mW as shown in Fig. 1C. The total amount of the ESR detectable Cu^{2+} increased to exactly 2.0 and did not decrease any more even by lowering the power of microwave irradiated. The spin Hamiltonian

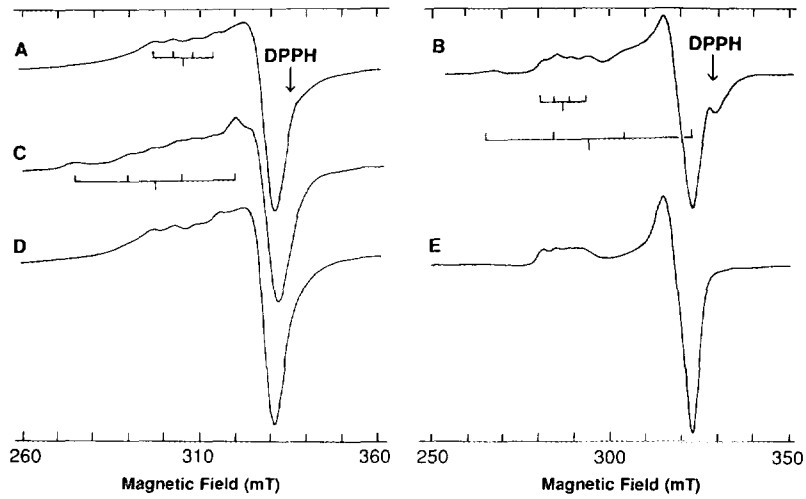

Figure 1. **ESR spectra of native laccase and the type 2 copper depleted derivatives at Room temperature (left) and at 77 K (right).** ESR spectra of native laccase at room temperature (A) and at 77 K (B). The ESR spectrum of (A) after measured with 200 mW microwave and successively measured with 12.5 mW microwave (C). ESR spectra of type 2 copper -depleted laccase at room temperature (D) and at 77 K (E). The total amount of the ESR detectable Cu is 1.3 in A, 2.0 in B, 2.0 in C, 1.0 in D, and 1.0 in E. The spin Hamiltonian parameters are shown in the text. Measurement condition at room temperature: frequency, 9.44 GHz; microwave power, 12.5 mW; modulation, 100 kHz, 1mT; Amp. 100-200; time constant 0.3 s; sweep time, 16 or 32 min.; protein concentration, 1mM. Measurement condition at 77 K: frequency, 9.22 GHz; microwave power, 6.25 mW; modulation, 100 kHz, 1mT; Amp. 100-200; time constant 0.1 s; sweep time, 8 min.; protein concentration, 0.1 mM.

parameters of the type 2 copper appear to prominently differ at room temperature ($g_{\parallel}$ = 2.24 and $A_{\parallel}$ = 14.5 mT (15.1 x 10^{-3} cm^{-1}), $g_{\perp}$ = 2.06) and at 77 K ($g_{\parallel}$ = 2.24 and $A_{\parallel}$ = 19.8 mT (20.6 x 10^{-3} cm^{-1}), $g_{\perp}$ = 2.05). The spectrum obtained by irradiating high-powered microwave is basically same with that reported by Mourpurgo et al (9). The reason why they did not report the spectrum as that of Fig. 1A will be that they performed measurements only with 200 mW microwave. The concomitant absorption spectrum did not change by irradiating high-powered microwave except a slight decrease of the 600 nm band. The oxidase activity was scarcely reduced even after the irradiation of high-powered microwave. The microwave oven effect appears to exert a slight deformation on the laccase molecule (not a fatal deformation for the laccase molecule) to change the ESR undetectable type 2 copper to be ESR detectable.

Since only 30% of the type 2 copper are ESR detectable in resting laccase, freezing is supposed to induce a change in the protein structure, making the type 2 copper to exclusively favor the cupric form. An alternative idea is that the manner of the magnetic interaction within the trinuclear site changes depending on temperature. McMillin et al. (10) proposed that the OH^- group as a ligand to the type 2 copper interacts with the type 3 coppers. However, the relevant OH^- group extends outward

the trinuclear center according to the crystal structure of ascorbate oxidase (3). The type 2 copper site is T-shaped, being more favorable for the cuprous state, and the type 3 copper site is tetrahedrally hindered, being adaptable for both cupric and cuprous states. Therefore, the favorable oxidation state of the type 2 copper is supposed to easily change depending on temperature. This is not the effect of pH change on freezing. Although pH of potassium phosphate buffer changes ca. 1pH unit by freezing, the ESR spectrum of laccase is hardly affected at the present experimental condition (7).

By the selective depletion of the type 2 copper (the total Cu contained in laccase changed from 4 to 3) the feature of the room temperature spectrum does not differ much from that of native laccase (Fig. 1D). This is quite natural because the amount of the ESR detectable type 2 copper in native laccase is only 30% at room temperature. Contrary to this, spectra at 77 K before and after the type 2 copper depletion apparently differ (Figs. 1B and 1E).

The type 3 copper in resting laccase is ESR undetectable because of the strong antiferromagnetic interaction between them (Cu^{2+} - OH^- - Cu^{2+}) (8). However, the type 3 copper becomes to be ESR detectable in the course of the reaction (11) or by acting small anionic inhibitors such as N_3^-, SCN^-, OCN^-, and F^- (12,13). Two azide ions biphasicly bind to one of type 3 coppers in laccase, giving charge transfer bands at ca. 405 and ca. 490 nm. The binding constant is estimated to be 8×10^4 M^{-1} for the high affinity N_3^- and 70 M^{-1} for the low affinity N_3^- from the absorption changes at room temperature. These values are comparable with those reported hitherto (14,15). Recently, we reported two ESR signals of the type 3 copper species derived by acting N_3^- on laccase at 77 K ($g_{\parallel} = 2.27$, $A_{\parallel} = 9.7 \times 10^{-3}$ cm^{-1}, $g_{\perp} = 2.06$ and $g_{\parallel} = 2.25$, $A_{\parallel} = 14.6 \times 10^{-3}$ cm^{-1}, $g_{\perp} = 2.06$) (12). The ESR spectra of these type 3 copper species at room temperature are shown in Fig. 2 together with those at 77 K. Content of the former species with narrower hyperfine splitting is highest in the presence of 40 times of azide on laccase. However, since signals due to the type 1 copper and the latter species, in which 2 N_3^- bind to the type 3 copper, are superimposed, the spectrum was considerably complex (not shown). On the other hand, the signal corresponding to the latter species was obtained for N_3^- (x600) - laccase ($g_{\parallel} = 2.26$ and $A_{\parallel} = 14.5 \times 10^{-3}$ cm^{-1}, $g_{\perp} = 2.06$) (Fig. 2B). The spectrum in which the former species is predominant (Fig. 2A) was obtained by measuring the spectrum of Fig. 2B with 200 mW microwave ($gz = 2.27$, $Az = 8.2 \times 10^{-3}$ cm^{-1}, $gy = 2.07$, $gx = 2.03$, and $Ax = 4.7 \times 10^{-3}$ cm^{-1}) at room temperature because the signal intensity of the type 1 copper was drastically reduced during the measurement with 200 mW microwave. Color of the solution changed from greenish blue to brown due to the charge transfers from N_3^- to Cu^{2+} in a few minutes. Considering from the biphasic binding mode of azide on laccase, one azide ions is considered to bind to the former species (14,15), and two azide ions to the latter species. Alternative possibility for the former species is that no azide anion is bound to the type 3 copper. The spectral feature of this species, the very narrow hyperfine splitting and the rhombic character, seems to be considerably similar to that

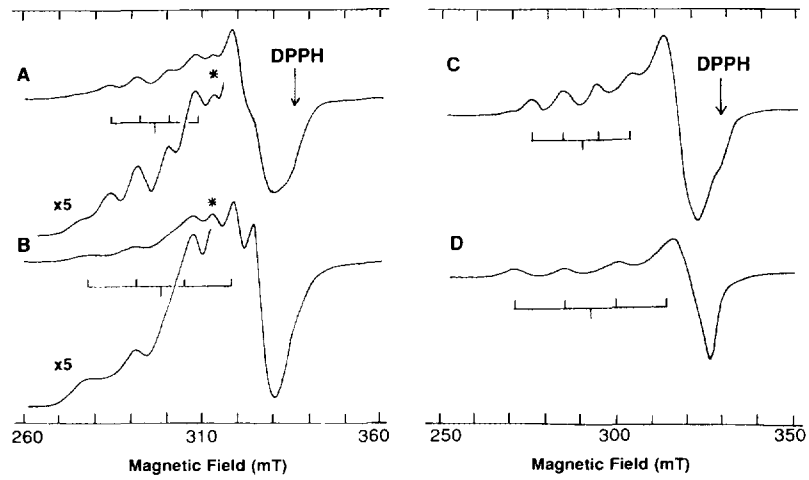

Figure 2. **ESR spectra of N_3^--laccase at room temperature (left) and at 77 K (right).** (A): the ESR spectrum of N_3^- (x300)-laccase obtained by irradiating (B) with 200 mW microwave at room temperature. (B): the ESR spectrum of N_3^- (x600)-laccase at room temperature. (C): the ESR spectrum measured at 77 K by anaerobically reacting N_3^- (x300)-laccase with a slight excess of catechol to reduce the type 1 copper. (D): the ESR spectrum measured at 77 K by anaerobically reacting N_3^- (x1000)-laccase with a slight excess of catechol to reduce the type 1 copper. The type 1 copper signal (*) and 2 N_3^--type 3 copper signal is slightly superimposed on the main signal in (A). The hyperfine splitting of the z component of the main species in each spectrum is indicated. The total amount of the ESR detectable Cu is 1.3 in A, 2.0 in B, 2.0 in C, 1.0 in D, and 1.0 in E. The spin Hamiltonian parameters are shown in the text. Measurement conditions are same with those in Fig. 1.

of the type 3 copper species detected during the enzyme reaction of laccase and to the CuB in cytochrome oxidase (11).

The present findings that the type 2 copper in resting laccase is mainly in the reduced form or in an ESR undetectable form in the trinuclear site and is changed to be fully ESR detectable by irradiating with high-powered microwave at room temperature are also observed in ascorbate oxidase (16 and unpublished data). Considering that ceruloplasmin promptly isolated by the affinity chromatography lacks the type 2 copper signal (17), the type 2 copper in multicopper oxidases appears to be in both the cupric and cuprous forms at room temperature, while the cupric form is favored in a frozen solution. Otherwise, the type 2 copper in the three spin system is magnetically isolated when frozen.

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