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THE PHENOLOXIDASES OF THE ASCOMYCETE *PODOSPORA ANSERINA* XI. THE STATE OF COPPER OF LACCASES I, II AND III

H. P. MOLITORIS and B. REINHAMMAR

Lehrstuhl für Allgemeine Botanik, Ruhr-Universität, Bochum (G.F.R.) and Department of Biochemistry, University of Göteborg and Chalmers Institute of Technology, Göteborg (Sweden)

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SUMMARY

1. Laccases I, II and III were (EC 1.14.18.1) prepared from the mycelium of the ascomycete *Podospira anserina*. The tetrameric laccase I (mol. wt 340 000, 16 copper atoms) and the monomeric laccases II and III (mol. wt 80 000, 4 copper atoms) have been studied by optical absorption-, circular dichroism-(CD) and electron paramagnetic resonance spectroscopy (EPR).

2. The visible and near ultraviolet difference absorption spectrum, which is apparently identical for all three laccases, shows two maxima at 330 and 610 nm and a shoulder at about 725 nm. The molar extinction coefficients of these bands are 4 times larger for the tetrameric laccase I compared to the monomeric laccases II and III which show values similar to other blue copper-containing oxidases.

3. CD spectra between 300 and 730 nm of the three laccases are similar and contain at least 5-bands in the oxidized enzyme. If the enzyme is reduced, only a band at 307 nm remains. The molar ellipticity values of these bands are 4 times larger for laccase I than the corresponding bands of laccases II and III. It is inferred that the reducible bands are associated with the Type 1 Cu^{2+} .

4. In all three laccases the EPR-detectable copper accounts for only about 50% of the total copper content. The 9-GHz and 35-GHz spectra, which are identical for all three laccases, consist of two components of equal intensity. One component shows a rather small copper hyperfine coupling and a small deviation from axial symmetry. It is suggested that this copper is associated with the blue chromophore in analogy to Type 1 Cu^{2+} in other blue copper proteins. The other component has a broader hyperfine coupling similar to Type 2 Cu^{2+} as found in other copper proteins. The assumption that the experimental spectra result from a superposition of the spectra of equal amounts of Type 1 and Type 2 Cu^{2+} has been verified by computer simulation.

5. It is suggested that the copper ions which are not detected by EPR are connected to the absorption band at 330 nm and that these ions are also essential for the function of these laccases.

INTRODUCTION

Laccases (EC 1.14.18.1) are copper-containing glycoproteins which have been prepared from different sources such as the lacquer trees *Rhus vernicifera* [1–5], *Rhus*

sucedanea [2] and the fungi *Agaricus bisporus* [6] and *Polyporus versicolor* [7]. These laccases have been investigated to establish the amount, state and function of their prosthetic copper ions which are essential for the catalytic activity. Different numbers and valence states of the copper ions have been reported for these laccases and there are even contradictory observations for laccase from the same source (for a recent review see [8]).

Recently three other laccases have been prepared from the mycelium of the ascomycete *Podospora anserina*, tentatively designated laccase I, II and III [9, 10]. Laccases II and III each contain four copper ions per molecule and have molecular weights of about 80 000. This molecular weight is in the range found for most of the other laccases investigated. Laccase I however, has a much higher molecular weight of about 340 000, has 4 times the specific activity of laccase II and III and contains 16 copper ions per molecule. It was recently shown by electron microscopy [11] that laccase I represents a tetramer of subunits which are similar in dimension and shape to the laccases II and III which are monomers. Laccase I therefore possibly represents an aggregate of the smaller laccases. In this respect, laccase I is thus quite different from the other known laccases which all seem to be monomeric enzymes with a much smaller molecular weight. Other catalytic, chemical and physical properties of laccase I are, however, similar to those of laccases II and III [9, 10].

Investigations of other laccases have revealed that probably all their copper ions are involved in the catalytic electron transport of these enzymes [12]. The state of copper in these enzymes is in principle similar with respect to the stoichiometry and properties of the various types of copper ions. They show, however, some differences, for instance in their spectroscopic properties, which indicates a different coordination of their copper ions [13–15].

In the present study we have therefore centered our interest on the investigation of the state of copper in the three *P. anserina* laccases. We thus would like to establish whether these laccases are identical and furthermore whether they show differences from the other laccases with respect to the state of copper. As will be shown in this communication the state of copper seems to be identical in all three *Podospora* laccases. Furthermore, they exhibit similar properties to the other laccases with respect to the stoichiometric and spectroscopic properties of the different types of copper.

MATERIAL AND METHODS

Proteins and chemicals

Laccases I, II and III of the ascomycete *P. anserina* were prepared and analyzed for protein concentration, copper content, activity, ultracentrifugation data and electrophoretic behaviour as described by Molitoris and Esser [9] and Esser and Minuth [10]. In addition the laccases were chromatographed once more on a Sephadex G-200 column. The resulting laccases were homogeneous according to ultracentrifugation tests and electrophoresis [9, 10] with the exception of laccase II which shows microheterogeneity [16].

Commercial reagent grade chemicals were used throughout and deionized water was used for making the solutions. For EPR spectroscopy quartz-distilled water solutions were used.

Laccase I was dissolved in either 20 mM ammonium acetate buffer (pH 7.0) or

100 mM sodium phosphate buffer (pH 6.0) which contained 50% glycerol to improve protein stability. Laccases II and III were dissolved in 20 mM ammonium acetate buffer (pH 7.0).

Spectral measurements

Optical absorption spectra were obtained at 25 °C with a Zeiss RPQ 20A recording spectrophotometer with a 1 cm light path. For the difference spectra native oxidized laccase was measured against the same concentration of enzyme which was reduced under anaerobic conditions by 8 mM ascorbate.

CD spectra were recorded with the use of a Cary 6002 spectropolarimeter with a 1-cm light path. Slit widths were programmed to maintain a constant bandwidth of about 4 nm. Measurements were made at 25 °C. Spectra of all three laccases in their oxidized and ascorbate-reduced states were recorded at least twice and the reported values represent the mean values. Blank readings were made with the same buffers as used to dissolve the proteins.

EPR spectra at about 9 GHz were obtained either with a Varian E-4 spectrometer equipped with an E-257 variable temperature accessory at 104 °K or with a Varian E-3 spectrometer at 77 °K. Spectra at about 35 GHz were recorded with a Varian V-4503 spectrometer at about 90 °K.

Total EPR intensities at about 9 GHz were obtained by double integration of the spectra. A water solution of 1 mM Cu^{2+} , 2M NaClO_4 and 10 mM HCl was used as standard.

Simulated EPR spectra were produced with an IBM 360/65 computer on the assumption of Gaussian line shape. The program included rhombic symmetry of the g and A tensors and the hyperfine coupling was treated to first order.

RESULTS

Absorption spectra

In Fig. 1a difference absorption spectrum in the visible and near ultraviolet region of laccase I is shown. All three laccases give apparently identical spectra. There are two maxima, one at 610 nm and the other at 330 nm and a shoulder at about 725 nm. Molar extinction coefficients of the difference maxima and the shoulder are given in Table I.

The amount of copper and the molar extinction coefficients of the laccases at 725, 610 and 330 nm remain constant even after prolonged dialysis or repeated gel chromatography, demonstrating strong binding of the copper ions in the protein molecule. The absorption bands mentioned above disappear when substrates are mixed with the enzyme under anaerobic conditions and reappear when O_2 is added. Deoxygenation of the enzyme solution does not change the absorption spectrum (methods used according to [17]). The absorption bands disappear irreversibly when the enzyme is treated with β -mercaptoethanol, sodium dodecylsulphate, urea and guanidinium·HCl (Molitoris, H.P., unpublished).

CD spectra

All three laccases show almost identical CD spectra. In Fig. 2 a spectrum observed between 300 and 730 nm of oxidized laccase II or III is shown. The only differ-

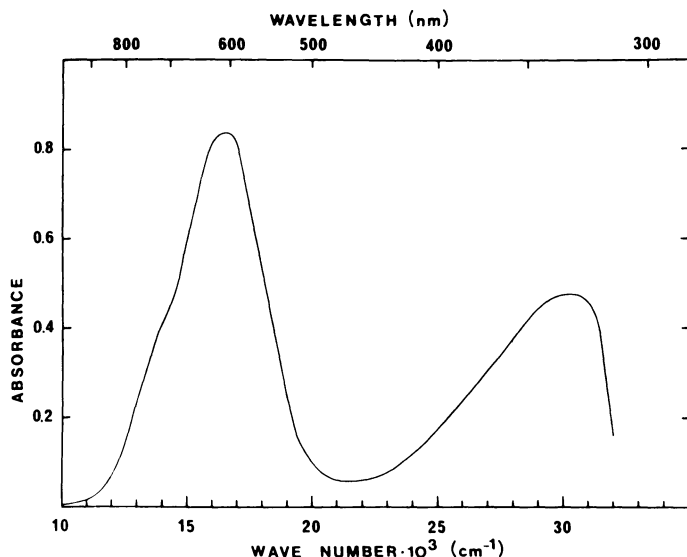


Fig. 1. Difference absorption spectrum of laccase I in the visible and near ultraviolet. Oxidized laccase ($5.5 \cdot 10^{-5}$ M dissolved in 0.1 M sodium phosphate buffer (pH 6.0) which contained 50% glycerol) was measured against the same concentration of laccase reduced under anaerobic conditions by 8 mM ascorbate.

ence between laccase I and laccases II and III are the molar ellipticity values which are 4 times larger for laccase I compared to the other laccases (see Table II). Upon reduction with excess ascorbate all CD bands except the band at 307 nm disappear in all three laccases. Table II shows the band positions and the estimated amplitudes of the CD spectra. The positions and amplitudes were obtained by fitting the experimental spectra to a number of Gaussian components with the help of a computer (dashed lines in the figure). In addition to the bands listed in the table there is a weak ellipticity in the 330–400-nm region.

TABLE I

OPTICAL SPECTRUM OF *PODOSPORA ANSERINA* LACCASES I, II AND III

The molar extinction coefficients ϵ_M are based on protein concentration as determined by copper analysis assuming 16 Cu^{2+} in laccase I and in laccase II and III, 4 Cu^{2+} each in laccases II and laccase III. The laccase I value is a mean from three preparations. The laccase II and III values are from single preparations.

Wave number (cm^{-1})	Wavelength (nm)	ϵ_M	
		Laccase I	Laccases II or III
13 800	725	About 6 000*	About 1500*
16 400	610	14 900	3700
30 300	330	9 500	2300

* Shoulder.

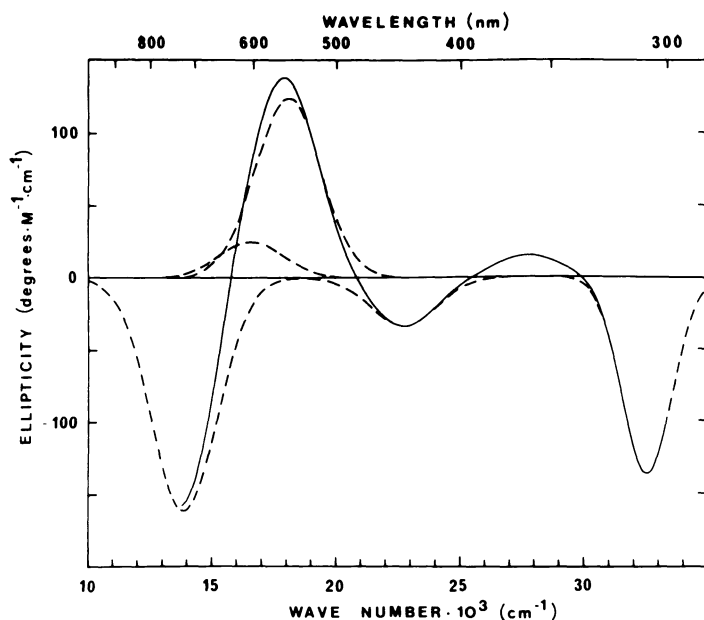


Fig. 2. CD spectrum of laccase II or III in the visible and near ultraviolet. The unbroken line represents the experimental spectrum of 240 mM laccase in the oxidized state; dashed lines represent simulated Gaussian components which were used in the simulation of the experimental curve. The enzyme was dissolved in 20 mM ammonium acetate buffer (pH 7.0).

TABLE II

BAND POSITIONS AND ESTIMATED AMPLITUDES USED IN THE SIMULATION OF CIRCULAR DICHROISM SPECTRA OF LACCASES II AND III

Positions are given as wave numbers (cm^{-1}) and amplitudes as molar ellipticities ($\text{degrees} \cdot \text{M}^{-1} \cdot \text{cm}^{-1}$). For the simulation of laccase I spectra the amplitude values used were four times larger than the values in this table.

Band position	Amplitude
13 800	-165
16 500	+25
18 000	+125
22 700	-35
32 600	-140

EPR spectra

A 9-GHz EPR spectrum of laccase I is shown in Fig. 3. Laccases II and III give almost identical spectra. Three samples from independent preparations of laccase I and one sample each from laccase II and III were integrated. The resulting intensity corresponded to $49 \pm 3\%$ of the total copper content as determined by copper analysis.

The shape of the spectrum indicates that it is composed of two signals, one with a rather narrow copper hyperfine coupling and the other with a wider hyperfine

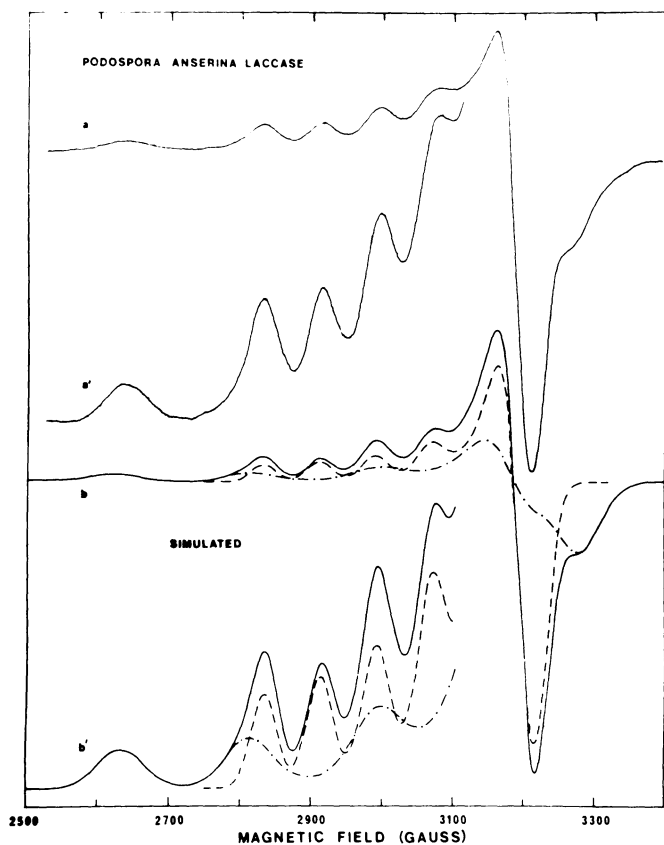


Fig. 3. Experimental (a) and simulated (b) EPR spectra of laccase I at about 9 GHz. Spectrum a was recorded at 77 °K with a sample of 0.9 mM enzyme in 0.1 M sodium phosphate buffer (pH 6.0) which contained 50% glycerol. Spectrum b was simulated with parameters according to Table III. The signals from Type 1 (---) and Type 2 (-·-·-) Cu^{2+} were given line widths of 35 and 65 gauss, respectively, and — is their sum. Parts of the spectra are also shown with 10 times higher gain (a' and b'). The microwave frequency was 9.12 GHz.

TABLE III

EPR PARAMETERS USED IN THE SIMULATION OF EPR SPECTRA OF LACCASES I, II AND III

Absolute values of the hyperfine coupling constants are given in units of 10^{-3} cm^{-1} .

Type of copper	g_x	g_{\perp}	g_y	g_z	A_x	A_{\perp}	A_y	A_z	A_{\parallel}
Type 1	2.034		2.050	2.209	0.5		0.5	8.0	
Type 2		2.046		2.246		1.0			17.6

splitting. That this analysis is correct is supported by the shape of the 35 GHz spectrum (Fig. 4) which again is similar for all three laccases. The intensity of the component with the wider hyperfine coupling, as determined by integrations of the low-field line [18] in the 9 GHz spectra of the five samples of the three laccases, was $51 \pm 2\%$ of the total EPR intensity.

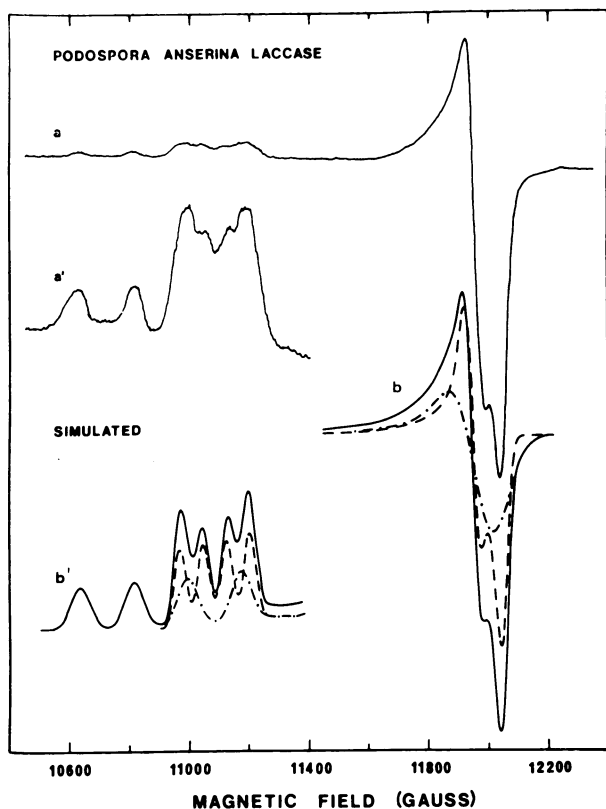


Fig. 4. Experimental (a and a') and simulated (b and b') EPR spectra at about 35 GHz of the same sample of laccase I as in Fig. 3. The protein concentration was about 5 mM. Spectrum a was recorded at about 90 °K. The simulated spectrum was obtained as in Fig. 3b but with line widths for Type 1 (—) Cu^{2+} of 40 gauss and for Type 2 (- - -) Cu^{2+} of 65 gauss in the g_{\parallel} region and 125 gauss in the g_{\perp} region. Parts of the spectra are also shown with 10 times higher gain (a' and b'). The microwave frequency was 34.28 GHz.

Therefore, for the simulations of the 9-GHz and 35-GHz spectra, the two copper species giving rise to the two components in the EPR spectra were chosen to have the same concentration.

It is also obvious from the appearance of the 35-GHz spectra that the coordination of the copper ion with the narrow hyperfine splitting departs from axial symmetry.

The simulated spectra shown in Figs 3 and 4 agree very well with the experimental spectra and Table III gives the parameters which were used in the simulations.

DISCUSSION

The visible and near ultraviolet absorption properties of the laccases from *P. anserina* are identical to each other and are also similar to those of other blue copper-containing oxidases [8, 19]. First, there is a strong absorption band at 610 nm in the oxidized enzyme with a difference molar extinction coefficient of 3700 for laccases II

and III. The value for laccase I (14 900) is much higher than reported for other blue copper proteins. However, these contain only one or two Type I copper ions which have been found to be responsible for the strong blue colour of these proteins. Although it has not yet been proven that the copper ion with the narrow hyperfine splitting in the g_{\parallel} region in the *Podospora* laccases is connected with the blue chromophore, there are good reasons to assume this since e.g. preliminary experiments have shown that after incubation of laccase I at elevated temperature or after reduction by ascorbic acid under anaerobic conditions both the absorption at 610 nm and the EPR signal with the narrow hyperfine coupling are reduced. This assumption would also be in analogy to the situation found in the other blue copper enzymes. Furthermore, the relation between A_{\parallel} and g_{\parallel} for this copper ion is in the same range as for Type I copper in several other copper proteins (cf. Fig. 9-5 in [8]). Consequently, this copper ion in the *Podospora* laccases seems to have all the characteristic properties of a Type I copper and the molar extinction coefficient at 610 nm per Type I copper in all three laccases then becomes close to values reported for other blue copper proteins [8, 19].

Second, the absorption band at 330 nm is also found in other blue copper-containing oxidases. It has been suggested that this band is associated with the EPR non-detectable copper ions in Ceruloplasmin [20], *Polyporus versicolor* laccase [21] and *Rhus vernicifera* laccase [22]. For the latter two proteins this chromophore was found to be a cooperative two-electron acceptor [22, 23] and it was therefore proposed that the EPR non-detectable copper ions are spin-paired $\text{Cu}^{2+} \cdot \text{Cu}^{2+}$ which would accept electrons in pairs. The molar extinction coefficient of this band in laccase I is also about 4 times as high as the corresponding values for laccases II and III or the other laccases [8, 19]. This fact, together with the finding that laccase I contains 8 EPR non-detectable copper ions while laccases II and III or other laccases have only two each, suggests that there are four such chromophoric units in laccase I, possibly one in each subunit. There is thus a possibility of similar two-electron acceptors in each subunit of laccase I and in the monomeric laccases II and III.

CD spectra of the three *Podospora* laccases contain at least 4 bands which disappear when the enzymes are reduced. The oxidized spectrum shows many similarities to CD spectra of other blue copper proteins [23]. The number of bands and their positions are similar, particularly the negative band at about 450 nm and the weak positive band in the region between 330 nm to 400 nm which are found in all of these proteins. For stellacyanin and *P. versicolor* laccase it was shown that all these bands disappear when only the Type I Cu^{2+} of these proteins was reduced [15]. We therefore suggest that the reducible CD bands in the *Podospora* laccases are also correlated with the Type I Cu^{2+} alone. The molar ellipticity values for laccase I are 4 times those of laccases II and III which may be then explained by the fact that it contains 4 times as many Type I Cu^{2+} as the laccases II and III. The 9- and 35-GHz EPR spectra of all three *Podospora* laccases are apparently identical. This finding, together with the similarities in optical properties mentioned above, suggests that the coordination of the copper ion is similar in all three enzymes.

The EPR spectra of the *Podospora* laccases are in principle similar to those of other laccases [8]. They are, for example, also composed of two signals originating from equal amounts of two copper ions in different environments. One signal exhibits a very small A_{\parallel} and departure from axial symmetry in agreement with values reported for Type I copper in other proteins. The other signal shows A_{\parallel} similar to values found

for Type 2 copper in other copper proteins and small copper complexes. This finding and the fact that only 50% of the copper ions are detected by EPR shows that the state of copper in the *Podospora* laccases is in principle similar to that reported for other laccases [13, 14]. Therefore, the view that the laccases generally contain all these forms of copper in the above reported proportion is further supported. The exceptions to this rule, as reported by other investigators, are therefore probably invalid as discussed elsewhere [14].

The question whether the subunits of laccase I contain the three forms of copper in the same stoichiometry as found in laccases II and III is not yet answered. However, several results taken together suggest that this is probably the case. For instance, as reported here, all three laccases exhibit the same spectroscopic properties and the same stoichiometry of the different types of copper. Furthermore, the four subunits of laccase I are very similar in shape and dimensions to laccases II and III [11], whereas molecular weight and specific activity of the tetrameric compound, laccase I, are 4 times that of laccases II and III, respectively [9, 10].

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