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THE STATE OF COPPER IN STELLACYANIN AND LACCASE FROM THE LACQUER TREE *RHUS VERNICIFERA*

BO G. MALMSTRÖM, BENGT REINHAMMAR AND TORE VÄNNGÅRD

Department of Biochemistry, University of Göteborg and Chalmers Institute of Technology, Göteborg (Sweden)

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SUMMARY

1. The blue protein (stellacyanin) and the laccase (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2) obtained from the latex of the Japanese lacquer tree *Rhus vernicifera* have been studied by electron paramagnetic resonance (EPR) and optical spectroscopy.

2. For stellacyanin the EPR parameters have been obtained with results differing from earlier published values. Above pH 9 the spectral properties of this protein are slightly changed, but in the whole pH range 1–11.5 the Cu^{2+} has Type 1 character, *i.e.* it has a strong blue color and a narrow copper hyperfine structure. These unusual features disappear concomitantly and irreversibly at still higher pH.

3. The total EPR intensity of the laccase accounts for 50% of the copper content only. The spectrum consists of two components of equal intensity. It is inferred that each molecule contains four copper ions, one Type 1 Cu^{2+}, which in reduction experiments is selectively reduced, one "non-blue" Type 2 Cu^{2+} with a normal EPR spectrum, and two EPR-nondetectable copper ions.

4. As with fungal laccase and ceruloplasmin the Type 2 Cu^{2+} is a preferred binding site of inhibiting anions. At pH 10–12 the Type 1 Cu^{2+} has slightly different spectral properties, but it still has Type 1 character.

5. It is suggested that the two copper ions in Rhus laccase not seen in EPR are associated with the optical absorption band at 330 nm and that these ions are essential for the function of the enzyme.

INTRODUCTION

For work in this laboratory on the copper proteins fungal laccase and ceruloplasmin, it has been useful to divide those copper ions that can be detected by electron paramagnetic resonance (EPR) into two classes[1], the Type 1 and Type 2 Cu^{2+}. The Type 1 Cu^{2+} gives rise to the strong blue color of these proteins and has a small copper hyperfine splitting in its EPR spectrum, whereas the Type 2 Cu^{2+} has no particularly strong absorption band in the visible region and a more normal EPR spectrum.

For the classification into Types 1 and 2 Cu^{2+} to be generally applicable one

requires that a strong optical absorption around 600 nm of a Cu^{2+} is always accompanied by a narrow hyperfine splitting in EPR and *vice versa*. However, from the work by PEISACH *et al.*[2] on the blue protein, stellacyanin, obtained from the lacquer tree *Rhus vernicifera* grown in Japan, it would appear that at pH 9–11 the single Cu^{2+} in this molecule has a strong optical absorption but a more normal EPR spectrum. NAKAMURA *et al.*[3] conclude that all copper ions in the laccase prepared from the same source have a high absorbance at 615 nm, although they suggest, from EPR experiments, that only part of the copper is divalent. Both these observations would make our classification less general.

The laccase from the fungus *Polyporus versicolor* contains one each[1] of Type 1 and Type 2 Cu^{2+} and, in addition, two diamagnetic copper ions[4,5]. For the laccase from *Rhus vernicifera* the number and valence states of the copper ions have been the subject of contradictory reports. NAKAMURA finds four copper ions in the molecule[6] with a varying fraction detected by EPR[3], whereas OMURA[7] reports five to six copper ions per molecule. BLUMBERG *et al.*[8] suggest, on the basis of experiments showing that four out of a total of six copper ions give an EPR signal, that the only similarity between the tree and fungal laccases may be their substrate specificity.

In a reinvestigation of the properties of the two copper proteins obtained from *R. vernicifera* we have found the classification into Type 1 and Type 2 Cu^{2+} to be entirely valid at all pH values. The EPR spectrum of stellacyanin and the information on the copper coordination available from this has been analyzed. Also, we have found evidence for a close correspondence between the number and properties of the copper ions in the tree and fungal laccases.

MATERIALS AND METHODS

Proteins and chemicals

Stellacyanin and laccase were prepared by the method described in the preceding paper[9] from acetone powder obtained from the lacquer tree *R. vernicifera* and purchased from Saito and Co., Ltd., Tokyo, Japan.

Deionized water was used for making solutions, and reagent grade chemicals were used without further purification.

Copper analyses were performed as described earlier[9].

EPR and other spectral measurements

EPR spectra were recorded in a Varian E-3 spectrometer at 77°K and about 9 GHz and in a Varian V-4503 spectrometer at about 90°K and 35 GHz.

Total EPR intensities at 9 GHz were obtained by double integration of the spectra using a 1 mM water solution of Cu^{2+} containing 2 M $NaClO_4$ and 0.01 M HCl as a standard. Simulated EPR spectra were produced with an IBM 360/50 computer on the assumption of Gaussian line shape. For stellacyanin the program included rhombic symmetry of the g- and A-tensors and treated the hyperfine structure to first order. For laccase, axial symmetry was assumed and the hyperfine coupling was taken to second order.

Optical spectra were obtained at 25° with a Zeiss M4Q II recording spectrophotometer.

pH titrations

Both proteins were titrated at 25° using an ABU 1 Auto-Burette connected to a TTT1 Titrator (Radiometer, Copenhagen, Denmark). The starting protein solutions were at about pH 7 in water and were titrated with 0.2 M NaOH or HCl. Samples were withdrawn at intervals of about one pH unit except at the extremes of the pH range used, where the interval was about 0.5 pH unit. For pH values above 7, both optical and 9- and 35-GHz EPR spectra were recorded with the EPR samples frozen at the time of the recording of the optical spectra. At lower pH values EPR spectra only were taken. About 10 min were required for the establishment of a new pH value including sample withdrawal and recording of the optical spectrum.

RESULTS

Spectral properties of stellacyanin at neutral pH

The position and extinction coefficients of the peaks in the optical spectrum are given in Table I. Fig. 1(a) shows the 9-GHz EPR spectrum of stellacyanin. As this protein has one Cu^{2+} per molecule only[10,8], all copper should be seen in EPR. The integrated intensity of the spectrum in Fig. 1(a) does correspond to 100 ± 5 % of the copper determined by chemical analysis, which indicates that the procedures used for EPR integrations and copper analyses are correct.

TABLE I

OPTICAL SPECTRA AT NEUTRAL pH OF STELLACYANIN AND LACCASE FROM *R. vernicifera*

The extinction coefficients are based on protein concentration and are given for the proteins both *vs.* buffer and *vs.* ascorbate-reduced protein. They represent mean values of five preparations.

Protein	*Wave number* (cm^{-1})	*Wavelength* (*nm*)	ε_M	
			Oxidized	*Oxidized–reduced*
Stellacyanin	11800	850	790	790
	16600	604	4080	4080
	22200	450	960	880
	35700	280	23200	—
Laccase	12500	800	~1100*	~1100*
	16300	614	5700	5500
	30000	333	~4500*	2800
	35700	280	93500	—

* Shoulder.

The 9-GHz EPR spectrum in Fig. 1(b) is simulated assuming one type of copper ion only with the parameters given in Table II. The agreement with the experimental spectrum is satisfactory. The assignment of the peaks in the high-field region was confirmed by running a spectrum at 35 GHz. This shows a well-resolved copper hyperfine splitting around g_x with the expected separation and no splitting around g_y. The line width is quite anisotropic but about the same as at 9 GHz, in contrast to what has been reported earlier[2].

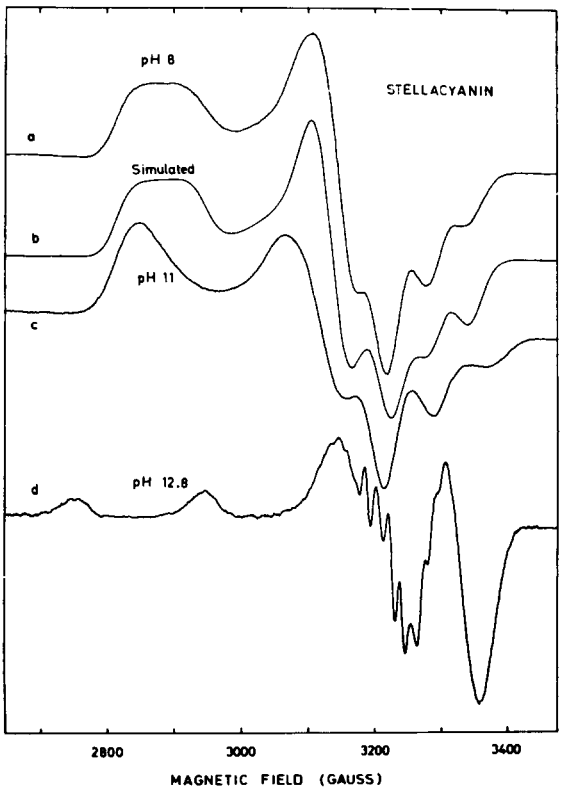

Fig. 1. EPR spectra at 77°K and about 9 GHz of stellacyanin (*R. vernicifera* blue protein). In (a) (c) and (d) are experimental spectra of samples at pH 8, 11 and 12.8, respectively, obtained in a titration with 0.2 M NaOH of an unbuffered solution 2 mM in protein at pH 7. The samples were run in tubes with 1-mm inner diameter for simultaneous examination at 35 GHz (spectra not shown). Spectrum b was simulated to fit Spectrum a with parameters given in Table II and a line width of 40 gauss. The parameters of Spectra c and d are also given in Table II. The microwave frequency was 9.213 GHz.

TABLE II

EPR PARAMETERS OF STELLACYANIN AND LACCASE FROM *R. vernicifera*

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

Protein	*pH*	g_x	g_y	g_z	A_x	A_y	A_z
		$g_\perp$		$g_{\parallel}$	$A_\perp$		$A_{\parallel}$
Stellacyanin	1–9	2.025	2.077	2.287	5.7	2.9*	3.5*
	10–11.5	2.025	2.089	2.312	7.3	—	<1.7*
	12.8	2.04		2.16	—		19.6
Laccase	12.8	2.04		2.19	—		20.0
Type 1 Cu^{2+}	3–8.5	2.047		2.298	1.7*		4.3
	10.3–12	2.04		2.238	—		7.1
Type 2 Cu^{2+}	3–11	2.053		2.237	1.8*		20.0

* Structure not resolved.

Effect of changes in pH on stellacyanin

As judged from the EPR spectrum the copper coordination is stable at low pH values down to about 1. This agrees with the observations by PEISACH *et al.*[2]. Also similar to their results, when the pH is raised above 9 the 16600- and 22200-cm^{-1} transitions shift to higher energy with no great change in their absorbance. Concomitantly, the EPR spectrum is modified and a new but rather similar spectrum is fully developed at pH 11 as shown in Fig. 1(c) (see Table II). This protein can be brought back to neutral pH with complete restoration of the optical and EPR spectra. At still higher pH a very different EPR spectrum appears, reaching the same intensity as the other spectrum at about pH 12. At pH 12.8 all Cu^{2+} is converted to this third form (Fig. 1(d); for parameters, see Table II), and the color of the protein is now violet with lower extinction coefficients than at lower pH. As observed by PEISACH *et al.*[2], from these high pH values, at which the spectral properties are similar to those of Cu^{2+} bound to larger peptides[11,12], the original properties of stellacyanin cannot be restored by lowering of the pH.

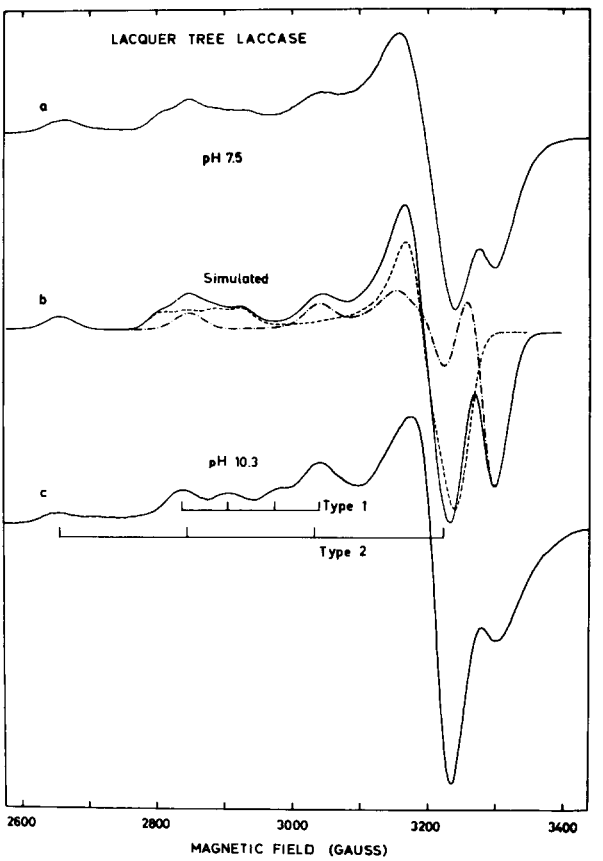

Fig. 2. EPR spectra at 77°K and about 9 GHz of *R. vernicifera* laccase. Spectrum a was obtained from a 0.3 mM protein solution in 0.1 M phosphate buffer (pH 7.5). The simulated spectrum in (b) consists of two components with parameters according to Table II. The signals from the Type 1 (- - - -) and Type 2 (— . —) Cu^{2+} were given the line widths 35 and 42 gauss, respectively, and —— is their sum. The sample for Spectrum c had pH 10.3 and was prepared in a titration of a solution about 0.3 mM in protein. The microwave frequency was 9.21 GHz.

The spectral properties of laccase at neutral pH

Table I gives the positions and extinction coefficients of the peaks in the optical spectrum of the protein *vs.* buffer and *vs.* ascorbate-reduced protein.

The 9-GHz EPR spectrum is shown in Fig. 2(a). A total of six spectra obtained from three different preparations were integrated and the resulting intensity corresponded to 50 ± 2 % of the copper concentration determined by chemical analysis.

It is clear from the appearance of the spectrum in Fig. 2(a) that this consists of two components, one with a very narrow, essentially unresolved copper hyperfine structure around $g_{\|}$ and one with a wider hyperfine splitting. The simulated spectrum in Fig. 2(b) shows that this analysis is correct, and Table II gives the EPR parameters of the two components. Their presence is also obvious in the experimental 35-GHz spectrum (Fig. 3, a and a'), which agrees well with the simulated one (Fig. 3, b and b'), obtained with the same parameters as in Fig. 2(b). However, the peak at about 11500 gauss in the experimental high-frequency spectrum cannot be accounted for by the simulations and presumably it is due to some impurity. Also, in the same spectrum the simulated peak around $g_{\perp}$ is narrower than the experimental. This difference might be caused by a departure from axial symmetry of the Type 1 Cu^{2+} similar to that found for fungal laccase[1].

For the simulations the copper species giving rise to the two components in the EPR spectra were chosen to have the same concentration. The intensity of the component with the wide hyperfine splitting was 53 ± 4 % of the total intensity as determined by integrations of its low-field line[13] in five different 9-GHz spectra.

When 5 mM quinol was added to 0.3 mM laccase in phosphate buffer (pH 6.0),

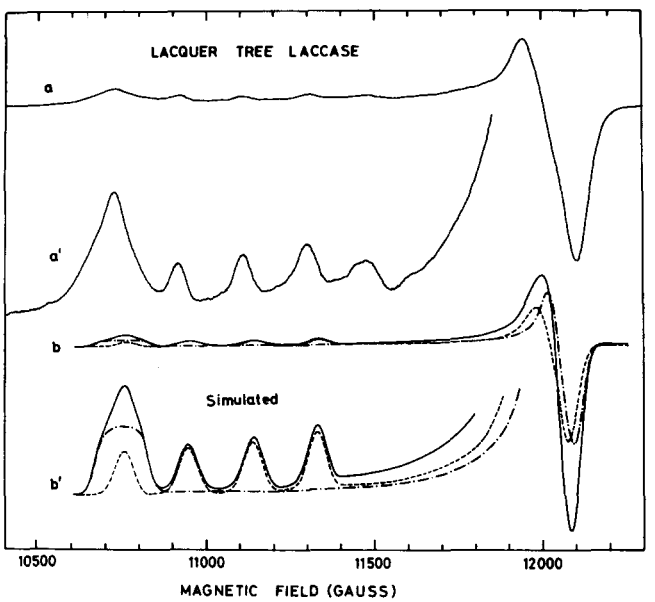

Fig. 3. Experimental (a) and simulated (b) EPR spectra of *R. vernicifera* laccase at about 35 GHz. Spectrum a was recorded at about 90°K with a sample of 1.3 mM laccase in 0.1 M phosphate buffer (pH 7.5). Spectrum b was simulated as in Fig. 2 (b) but with line widths for the Type 1 (— . —) and Type 2 (- - - -) Cu^{2+} of 43 and 55 gauss, respectively. Parts of the spectra are also shown with 10 times higher gain (a' and b'). The microwave frequency was 34.56 GHz.

the blue color disappeared. One sample was frozen 15 sec after mixing and its EPR spectrum showed none of the component with the narrow hyperfine splitting. Only the component with the wide hyperfine splitting remained, but reduced in intensity.

Addition of about 0.1 M of the inhibiting[14] anions, azide and fluoride, causes changes in the EPR signal with the wide hyperfine splitting but has no observable effect on the other component. However, addition of fluoride does not produce the well-resolved fluorine splitting observed with fungal laccase[15], although the general shifts in the peaks are the same.

Effect of changes of pH on laccase

Down to pH 3 there does not seem to be any change in the optical and EPR spectra of laccase. At still lower pH irreversible changes occur, but the solution is slightly blue even at pH 1.

When the pH is increased above 8.5, there is a shift in the main peak in the visible spectrum to higher energy as in stellacyanin. Thus, at pH 11 the laccase and stellacyanin absorption maxima have been shifted by 21 and 16 nm, respectively. Accompanying this shift is a change in the EPR spectrum of the component with the narrow hyperfine splitting which is complete at pH 10.3. Fig. 2(c) shows that the new spectrum has a somewhat wider splitting around g_{II}, but this is still much smaller than that of the other component (*cf.* Table II). On a return of pH to lower values, the original spectral properties are restored. The new EPR spectrum persists to about pH 12, above which complicated spectra are obtained. At pH 12.8 the protein is violet and the EPR spectrum does not show the presence of two components any longer but is related to that of stellacyanin at high pH.

DISCUSSION

The type and coordination of Cu^{2+} in stellacyanin

The optical spectrum given in Table I is similar to the spectra published by OMURA[10] and by PEISACH *et al.*[2]. The main difference is that the latter authors seem to have a much larger background absorption in their ascorbate-reduced protein. For example, this accounts for more than 50 % of the total absorption at 22200 cm^{-1} in their spectrum.

The EPR spectrum at neutral pH in Fig. 1(a) has the same appearance as the spectra given in many other publications[8,16–18,2]. A simulated spectrum has been shown by BLUMBERG[18] in which the assignment of the peaks at the high-field side of the spectrum is incorrect. The large hyperfine splitting is associated with the intermediate g value rather than with the lowest one (*cf.* Table IV of ref. 2 and Table II in this work) which is inconsistent with the 35-GHz spectrum (see RESULTS). The correct assignment of the features of the EPR spectrum was of importance in electron nuclear double resonance studies on stellacyanin that recently have been performed[19].

The results obtained on the EPR properties of stellacyanin at high pH differ from those reported by PEISACH *et al.*[2]. They find that between pH 8.3 and 11.5 the EPR spectrum becomes axial, apparently with a rather large copper hyperfine splitting, but that the strong blue color is retained. Above this pH range the strong color disappears with no great alteration in the EPR spectrum. They interpret the changes as due to successive relaxation of constraints on the ligand field. Our results

show that the reversible changes that occur in the pH interval 9–11 are small not only in the optical but also in the EPR spectrum, which has the same rhombic character and narrow hyperfine splitting as at lower pH (see Fig. 1 and Table II). Above pH 11 both the blue color and the unusual features of the EPR spectrum disappear together. Thus, in our nomenclature, the Cu^{2+} of stellacyanin is essentially of Type 1 character in the whole pH range 1–11.5, and it offers no exception to the general observation that a strong blue color and a narrow hyperfine splitting in EPR are closely connected. The use of very strong base, 6 M NaOH, for the increase of pH in the work by PEISACH *et al.*[2] might have caused a partial denaturation of the protein, which could explain the discrepancies between their results and ours.

The EPR spectrum of stellacyanin at highest pH (Fig. 1, d) shows superhyperfine splitting probably due to four nitrogen ligands as observed by PEISACH *et al.*[2]. On the basis of their mechanism discussed above for how this state was reached, they concluded that the four strong ligands of Cu^{2+} must be nitrogen atoms at neutral pH also. Obviously, this argument is no longer valid as replacement of ligand atoms can easily occur on irreversible changes produced by an increase in pH. However, eletron nuclear double resonance studies[19] indicate that at least one nitrogen atom coordinates to Cu^{2+} at neutral pH.

Forms of copper in R. vernicifera laccase

Our result that 50 % of the copper in laccase is seen in EPR disagrees with most other reports. NAKAMURA *et al.*[3] found 50–90 % of the copper in the divalent state as estimated by EPR and chemical methods. The fraction depended on the district in Japan from which the latex originated. We have no such detailed information available on the source of our starting material. However, we believe that our preparations are homogeneous, particularly as only one band is found in isoelectric-focusing electrophoresis[9]. As further evidence for the presence of a varying fraction of divalent copper, NAKAMURA AND OGURA[17] presented data indicating that the steady-state level of the absorption at 615 nm in aerobic kinetic experiments was directly related to the amount of EPR-detectable copper in the preparation. In preliminary experiments[20] we find no such relation for our protein. Unfortunately, we can provide no consistent explanation for the results obtained by NAKAMURA *et al.*[3,16,17]. In particular, their finding that the blue color was proportional to the total amount of copper, although only part of it was detected by EPR, and their conclusion that Cu^{2+} and Cu^{+} would have the same high absorbance seem quite unreasonable. Our results also differ from those obtained by BLUMBERG *et al.*[8], who detect 70 % of the copper by EPR. However, an incomplete separation[2] of laccase and stellacyanin would increase the percentage of EPR-detectable copper.

The EPR spectra of laccase presented in this work (Figs. 2 and 3) consist of two components (see RESULTS). For example, the peak at highest field in the 9-GHz spectrum (Fig. 2, a), also clearly seen in the spectrum published by BLUMBERG *et al.*[8], is due to an 'overshoot' line of the component with the wide hyperfine splitting. Earlier, this peak was associated with a low g value of a single type of Cu^{2+} responsible for the whole spectrum[8] or with a free radical[17]. In later studies by PEISACH AND BLUMBERG[21] they also find two components in the EPR spectrum.

The reduction experiment indicates that the blue color is associated with the EPR component with the narrow hyperfine splitting and that this copper ion has

Type 1 character. Similarly, the copper component with the broad splitting has no strong absorption band in the visible region and is a Type 2 Cu^{2+}. Thus, the nomenclature introduced for ceruloplasmin[13,22] and fungal laccase[1,15] is applicable to the tree laccase as well. Recent anaerobic redox titrations[20] conclusively show that the Type 1 Cu^{2+} alone is responsible for the blue color of the protein.

At pH 10.3–12 the Type 1 Cu^{2+} has slightly altered spectral properties but it still has Type 1 character. At higher pH the unique features of both the optical and EPR spectra disappear. The Type 1 Cu^{2+} of fungal laccase behaves quite differently in that it becomes reduced at high pH[23]. This might be related to the much higher standard oxidation–reduction potential of the Type 1 Cu^{2+} in this protein[24] as compared with that of the corresponding ion in the tree enzyme[6].

The finding that inhibiting anions bind preferentially to the Type 2 Cu^{2+} in tree laccase is in complete agreement with the results obtained for fungal laccase[15] as well as ceruloplasmin[22] and illustrates the similarity between these proteins.

Judging from the Type 2 Cu^{2+} low-field line in EPR spectra published by NAKAMURA *et al.*[3,17], these authors have a varying intensity ratio of the two types of Cu^{2+} in *R. vernicifera* laccase. In all our preparations, we have equal intensity of the two components, each one corresponding to 25 % of the total copper content. This shows that the number of copper atoms per molecule must be a multiple of four. As the copper content has been determined[9] to be 0.23 %, the molecular weight must be a multiple of 110000. The values measured by sedimentation methods are 120000[6] and 141000[7]. Thus, the only reasonable number of copper atoms per molecule is four, and as in fungal laccase[1], there is one each of Types 1 and 2 Cu^{2+}. Consequently, the extinction coefficient in Table I of the 16600-cm^{-1} band, which is due to the Type 1 Cu^{2+}, is based on the concentration of this ion. Calculated in this manner it becomes rather similar to the extinction coefficients of other 'blue' Cu^{2+} ions in proteins[25]. In fact, it is higher than that of stellacyanin (see Table I, *cf.* ref. 2).

In addition to the Type 1 and Type 2 Cu^{2+}, *R. vernicifera* laccase contains two copper ions that are not seen in EPR. This is true for the fungal laccase also, and there the two ions are thought to exist in a spin-paired Cu^{2+}–Cu^{2+} couple, associated with a two-electron acceptor[4]. In its oxidized state this has a strong absorption band centered at 30000 cm^{-1} with an extinction coefficient *vs.* reduced protein of about 3 $mM^{-1} \cdot cm^{-1}$ (ref. 5). Such a band is also observed for the *R. vernicifera* laccase (Table I and ref. 17), and as discussed in a recent review[25], we suggest that this band is associated with a structural unit similar to that in the fungal enzyme. Recent titration experiments[20] on the *R. vernicifera* laccase have indeed shown that the 30000-cm^{-1} band is not due to the same electron acceptor as the 16600-cm^{-1} band.

In fungal laccase the copper ions not detected by EPR are diamagnetic even at room temperature[26]. The magnetic susceptibility of *R. vernicifera* laccase as measured by NAKAMURA[27] would indicate that all copper in this protein contributes to the paramagnetism. However, as almost all copper in the particular preparation studied by him was seen in EPR[3], his data have no relevance for the magnetic properties of the EPR-nondetectable copper in our preparations of the same protein.

In summary, contrary to earlier suggestions[8], *R. vernicifera* laccase shows many similarities to the fungal enzyme not only in its enzymic activity but also in the state of its copper components. Therefore, it would seem quite probable that the function of the copper ions in the two proteins is the same. In particular, kinetic data[28,5]

indicate that the EPR-nondetectable Cu^{2+} which is associated with the 330-nm band[5] plays an important role in the mechanism of the fungal enzyme. NAKAMURA AND OGURA[17] also suggest that the presence of such an optical band is necessary for activity, and therefore the statement by the same authors[3,17] that the EPR-nondetectable copper ions are not involved in the active center of the tree enzyme must be questioned. On the other hand, differences do exist between the two proteins, particularly interesting ones in their oxidation–reduction behavior. This will be discussed in forthcoming publications.

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