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Pulsed Electron Paramagnetic Resonance Studies of Types I and II Copper of *Rhus vernicifera* Laccase and Porcine Ceruloplasmin†

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ABSTRACT: Electron spin-echo decay envelopes for types I and II copper of *Rhus vernicifera* laccase and for type II copper of porcine ceruloplasmin have been studied. Nuclear modulation patterns show that imidazole is a ligand for all of them. The linear electric field effect (LEFE) in EPR was studied for type I copper in a laccase preparation from which type II had been removed. The symmetry of the site is near tetrahedral and the magnitude of the LEFE is correlated with the intensity of blue color.

It has recently been shown that pulsed EPR[1] techniques provide a useful means for assigning ligands (Peisach and Mims, 1973; Mims and Peisach, 1976a) and for investigating the symmetry of metal sites (Mims and Peisach, 1974, 1976b; Peisach and Mims, submitted) in proteins. In one kind of experiment (Mims and Peisach, 1976a) a recording is made of the decay envelope for electron spin-echo signals. Periodicities in the envelope indicate the presence of electron nuclear coupling. This effect is termed the "nuclear modulation effect". A second type of experiment, also involving the generation of electron spin-echoes, is concerned with the measurement of the g shifts induced by the application of an external electric field (Peisach and Mims, 1973; Mims and Peisach, 1974, 1976b). These "linear electric field effect" experiments yield information about the odd symmetry part of the ligand field

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[1] Abbreviations used are: EPR, electron paramagnetic resonance; LEFE, linear electric field effect.

which cannot be obtained by standard EPR methods.

Both techniques have been applied to the dark-blue copper proteins. It has been shown by means of the nuclear modulation effect that one of the ligands in stellacyanin is a histidyl imidazole (Mims and Peisach, 1976a). In addition, electric field effect measurements have demonstrated that the crystal field in both azurin and stellacyanin is approximately tetrahedral (Peisach and Mims, submitted). An anomalous feature in the LEFE data for the blue proteins, not found in tetrahedral models, suggests, however, that one ligand is distinct from the rest in its bonding properties. From the work of the Rome group (Finazzi-Agro et al., 1970; Rotilio et al., 1970; Morpurgo et al., 1972; Graziani et al., 1974) and of McMillin et al. (1974a,b) it appears likely that this odd ligand is cysteinyl sulfur.

In the present paper, we report an extension of this work to the proteins laccase and ceruloplasmin. Laccase and ceruloplasmin each contain two paramagnetic copper centers, one with a small value of $A_{\parallel}$ and an intense optical absorption, as in the blue proteins mentioned above, and the other with optical and EPR properties characteristic of simple copper–peptide complexes (Malkin and Malmström, 1970; Peisach and Blumberg, 1974). The first is usually designated as type I copper and the second as type II (Malmström et al., 1968).

One difficulty which arises when studying laccase is that the observed behavior tends to be due to both copper sites at once. This difficulty has, however, been largely eliminated by the work of Graziani et al. (1976), who have prepared a decuprolaccase from *Rhus vernicifera* in which type II copper has been reversibly removed from the molecule. As the optical and EPR properties of the remaining type I copper centers are essentially unaltered when the type II copper is removed, it is reasonable to suppose that the nuclear-modulation effect and the LEFE curves obtained with this material will be the same as those which we should obtain if the dark-blue copper contributions could be resolved in hololaccase. Pulsed EPR data for the type I center in laccase obtained in this way is reported under Materials and Methods.

For the type II center in laccase, the nuclear-modulation effect can be studied by setting the magnetic field at a position in the resonance spectrum where the type I center no longer contributes. In the same way, the nuclear-modulation effect was also observed for the type II copper centers in ceruloplasmin.

The envelope modulation experiments on laccase indicated that there is an imidazole ligand bound to both types I and II copper. Since this assignment of the observed modulation pattern to imidazole (Mims and Peisach, 1976a) was based previously on a comparison with only one model (i.e., Cu^{2+}–bovine serum albumin in which the copper is ligated to a single imidazole and other nitrogenous ligands (Peters and Blumenstock, 1967)), it was felt that a second model, consisting preferably of a simple chemical substance, would be desirable. We have therefore prepared a complex with Cu^{2+}–diethylenetriamine and imidazole. Envelope modulation experiments on this compound confirmed our earlier conclusions.

Materials and Methods

Porcine ceruloplasmin was prepared from defibrinated blood according to the method of Levine and Peisach (1963). Laccase was prepared from *Rhus vernicifera* acetone powder (Saito and Co., Tokyo) according to the method of Reinhammer (1970). Both proteins were passed through Chelex resin before use.

Decuprolaccase was prepared according to the published procedure of Graziani et al. (1976). More than 90% of the type II copper was removed from the holoprotein as judged from the EPR spectrum. The total copper content was assayed by atomic absorption spectroscopy.

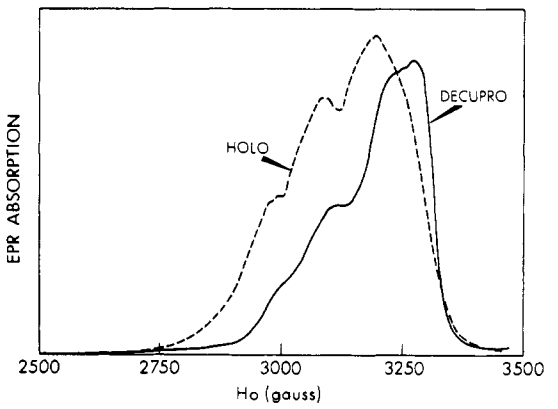
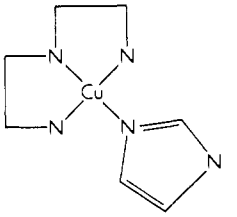


FIGURE 1: Echo signal amplitude as a function of magnetic field H_0 for holo (---) *Rhus vernicifera* laccase (frequency = 9243 MHz) and (—) decuprolaccase from which the type II copper has been removed (frequency = 9358 MHz). The time τ between the echo generating pulses is 1.0 μs. The spectrum is substantially the same as that which one would obtain by integrating a typical field-modulated EPR absorption spectrum. The inflections in the curves are partly due to the nuclear hyperfine lines of the copper and partly due to nuclear modulation effects caused by ^{14}N coupling. The maximum in the curve occurs near the $g_{\perp}$ setting.

The model compound Cu^{2+}–diethylenetriamine–imidazole was prepared by the optical titration of 10 mM copper diethylenetriamine at pH 8.3 with imidazole. The concentration of Cu^{2+} was 10.0 mM, while the diethylenetriamine was 10.5 mM. After each addition of imidazole, the pH was adjusted to 8.3 with a small volume of concentrated NaOH.



Pulsed EPR experiments were performed at 4.2 K or lower, on an X-band instrument described by Mims (1974) with a cavity described by Mims and Peisach (1976a). Nuclear modulation studies were performed by examining the electron spin-echo amplitude as a function of τ, the period between the microwave pulses. Recordings of the echo amplitude function were obtained as described in a previous paper (Mims and Peisach, 1976a). For this study, microwave pulses were about 20 ns long, microwave power levels were about 200 W, the integration time constant of the boxcar circuitry was 0.5 μs, and the time required to trace the echo envelope was 400 s. The LEFE was studied using the signal half-fall procedure (Peisach and Mims, 1973). Concentration of proteins used varied from 3 to 5 mM as judged from published extinction coefficients near 600 nm (Nakamura and Ogura, 1966; Blumberg et al., 1963).

Results and Discussion

Nuclear Modulation Effect. Figure 1 shows the electron spin-echo amplitude as a function of magnetic field for holo- and for decuprolaccase. These curves are approximately the same as those which one might obtain by integrating a typical field-modulated EPR absorption spectrum and serve to define the limits within which magnetic resonance phenomena occur. It can be seen that the signal from the holoprotein, which

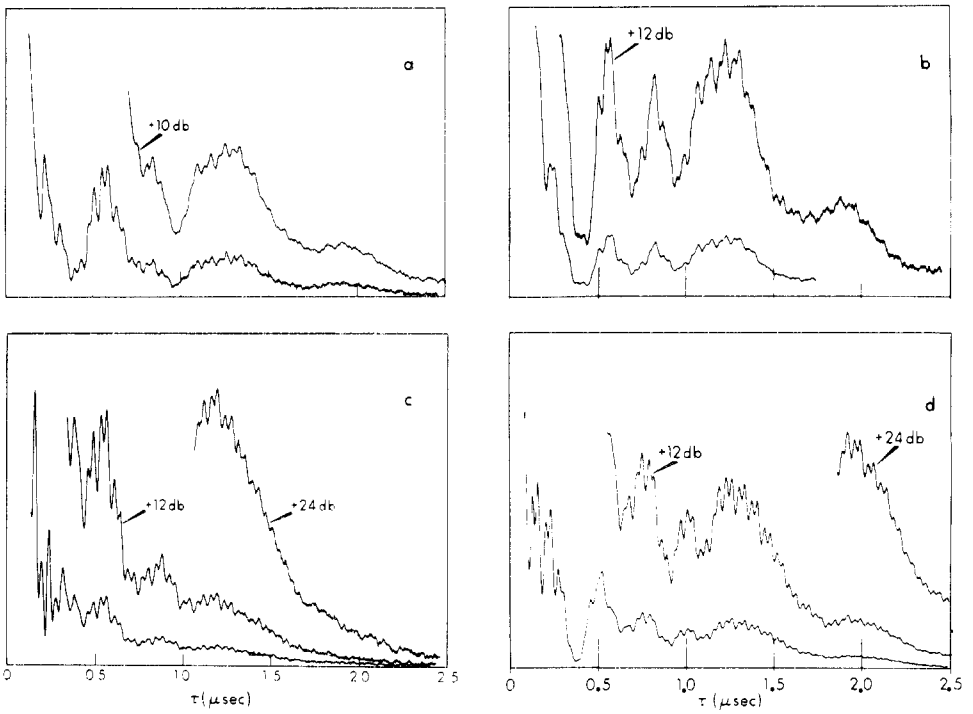


FIGURE 2: X-Y recordings of the nuclear modulation effect for (a) type II copper of holo laccase, (b) type II copper of porcine ceruloplasmin, (c) type I copper of decuprolaccase, and (d) Cu^{2+}-diethylenetriamine-imidazole complex. In a, 5 mM laccase in 0.05 M phosphate buffer, pH 7.0, was examined at the low-field end of the EPR spectrum. (H_0 = 2818 G, frequency = 9243 MHz, see Figure 1). In b, 3 mM ceruloplasmin, in 0.05 M acetate, pH 5.7, containing 1% NaCl, was studied at the low-field end of the EPR spectrum (H_0 = 2888 G, frequency = 9564 MHz). The signal here is due to type II copper. In c, 3 mM decuprolaccase in 0.05 M phosphate, pH 7.0, was examined near $g_\perp$ (H_0 = 2970 G, frequency = 9358 MHz, see Figure 1). In d, a mixture containing 10 mM cupric acetate, 10.1 mM diethylenetriamine, 16 mM imidazole in 1:1 glycerol-water, pH 8.3, was studied (see Figure 4). The modulation spectrum was taken near $g_\perp$ (H_0 = 3195 G, frequency = 9251 MHz). The pattern ascribed to ^{14}N is observed over the complete range of EPR absorptions in both c and d. The corresponding superhyperfine frequencies are determined mainly by the ^{14}N quadrupole coupling (Edmonds and Summers, 1973).

contains both types I and II copper (Malmström et al., 1970), is spread over a larger range than the signal from the decupro protein, which contains only type I copper (Graziani et al., 1976). By selecting field settings at the low end of the spectrum for the holoprotein one should therefore be able to observe envelope modulation patterns which are predominantly due to the type II copper site. A typical curve is shown in Figure 2a. Two distinct components can be identified. The high-frequency component is due to protons, ubiquitous in all biological material, and the low-frequency component to ^{14}N. Detailed studies of the modulation patterns arising from coupling to protons have been presented elsewhere (Mims and Davis, 1976; Mims et al., 1977). These are of little interest to us here, however, because our main concern is with the low-frequency nitrogen component, which, following the arguments used in the case of stellacyanin (Mims and Peisach, 1976), we assign to imidazole. The modulation envelopes obtained for the type I copper site in decuprolaccase (Figure 2c) are similar and also indicate the presence of an imidazole ligand.

The primary reason for this interpretation of the nuclear modulation envelope shown in Figure 2a,c is the close similarity between these patterns and the patterns obtained in materials where a single imidazole ligand is known to be bound to copper, as in the case of Cu^{2+}-bovine serum albumin (Mims and Peisach, 1976a). Data for an additional model is presented in Figure 2d where we show the echo envelope for the complex prepared by titrating imidazole with Cu^{2+}-diethylenetriamine at pH 8.3.[2] This pattern is not associated with the coordinated amino nitrogen groups as one can see by comparing Figure 2d

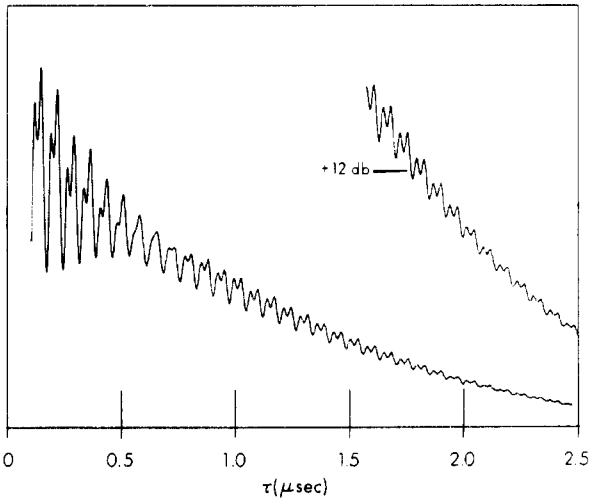


FIGURE 3: X-Y recording of the nuclear modulation effect for Cu^{2+}-diethylenetriamine (H_0 = 3231 G, frequency = 9280 MHz). The pattern is due solely to electron-nuclear interaction with protons. Coupling with the ^{14}N nuclei of the amine groups makes no contribution to the modulation effect.

with the curve obtained for Cu^{2+}-diethylenetriamine alone (Figure 3). It is also not attributable to peptidic nitrogen coordination of copper as can be found in proteins (see Mims and Peisach, 1976, Figure 7).

This points out an important aspect of the nuclear modulation effect as applied to strongly coupled ^{14}N. If the electron-nuclear coupling term of the spin Hamiltonian largely excedes the Zeeman term and the quadrupole term, no contribution from ^{14}N in the modulation envelope is seen. Thus,

[2] By studying the nuclear modulation of ^{14}N at different Zeeman fields, small shifts in pattern are observed (compare Figures 2a and 2d).

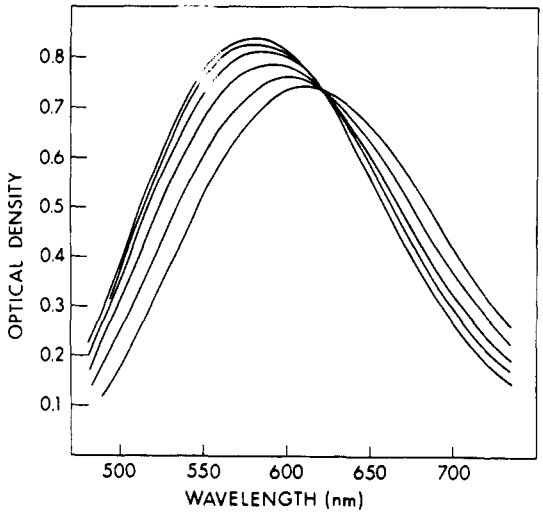


FIGURE 4: Optical titration of 10 mM Cu^{2+}–diethylenetriamine at pH 8.3 with imidazole. Cu^{2+}–diethylenetriamine absorbs at 600 nm. Imidazole addition shifts the absorption maximum to 580 nm. The nanomolar concentrations of imidazole employed in the titration were 0, 3.3, 6.6, 10.0, 13.3, and 16.6. The spectrum for the solution containing 20 mM imidazole was virtually superimposable on the spectrum for the solution containing 16.6 mM imidazole. At a higher concentration of imidazole, the isosbestic points at 620 nm are shifted to longer wavelengths, suggestive of the formation of a copper complex containing more than a single imidazole ligand.

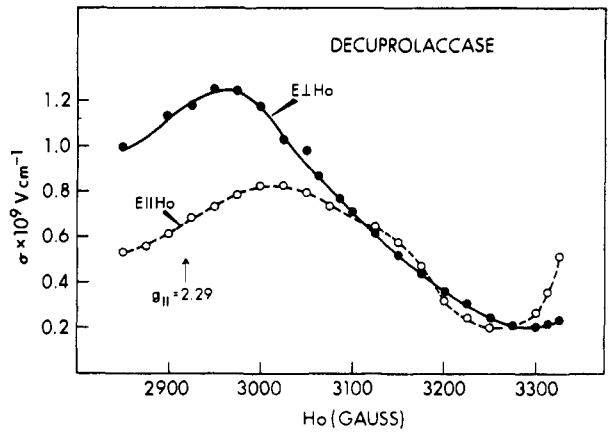


FIGURE 5: Linear electric-field effect curves for the type I copper in decuprolaccase. Data were obtained with E_0, the electric field, aligned parallel to $E_{\parallel}H_0$ or perpendicular to $E_{\perp}H_0$, the applied magnetic field, H_0 (frequency = 9358 MHz). The rise at the low-field end of the $E_{\perp}H_0$ curve is characteristic of type I copper.

directly coordinated nitrogen does not contribute to the modulation pattern.[3]

In Figure 2b, we show the modulation envelope for the type II copper of ceruloplasmin. Here too, both a component assigned to protons and one assigned to ^{14}N of imidazole can be observed.

In principle it should be possible to estimate the number of imidazole ligands present in a given complex by comparing the depths of the characteristic modulation patterns. This can be inferred from the product theorem governing the modulation effect (Rowan et al., 1965, eq 19; Mims, 1972, eq 42) which states that the pattern due to several coordinating nuclei is the product of the patterns due to individual nuclei. It is, of course, important when making such comparisons to be quite certain that the material used as a standard is not a mixture and that it does not contain copper complexes without an imidazole ligand, since these would reduce the apparent depth of ^{14}N modulation. We have therefore taken care to establish that the Cu^{2+}–diethylenetriamine–imidazole complex is a pure species by examining the isosbestic character of the optical titration curves (see Figure 4). The near congruence of the pattern intensities in Figure 2c,d suggests that the same number of imidazole groups is involved in decuprolaccase and in the model. Stellacyanin (see Figure 8 in Mims and Peisach, 1976) would also appear to be coordinated by only one imidazole group. This is in disagreement with the conclusion, based on NMR studies of azurin, that the sites in dark-blue copper proteins are doubly coordinated by imidazole (Markley et al., 1975; Hill et al., 1976; Ugurbil et al., 1977).

Linear Electric-Field Effect. The LEFE for type I copper in decuprolaccase is shown in Figure 5. The magnitude of the shift, much larger than for simple copper complexes, falls within a range for known tetrahedral complexes of Cu^{2+}. At low magnetic field, as observed for stellacyanin and azurin, the shift at the $E_{\perp}H$ setting is larger than at the $E_{\parallel}H$ setting, suggesting that an odd component of crystal field is oriented perpendicular to $g_{\parallel}$ (see Peisach and Mims, submitted, for an analysis of this phenomenon). One can also observe a direct correlation of the size of the shift with the molar extinction coefficient ascribed to the cysteine–copper charge transfer (Table I). This is not altogether surprising, since the optical transition probability and the LEFE will both depend on the magnitude of the electric dipole matrix elements between the ground state and the charge-transfer excited state. A number of other factors are also involved in the LEFE, however (Mims, 1976), and such comparisons are only useful when made between complexes with the same general structure.

TABLE I: Extinction Coefficients and LEFE Shift Parameters at the Low-Field End of the $E_{\perp}H_0$ Curve for Blue Copper Proteins.

Protein	λ_{max} (nm)	ϵ (M^{-1} cm^{-1})	$\sigma \times 10^9$ (V cm^{-1})
Azurin	625	3500[a]	0.70[d]
Stellacyanin	604	3820[b]	0.85[d]
Decuprolaccase	614	5700[c]	1.25

[a] Brill et al. (1968). [b] Peisach et al. (1967). [c] Malmström et al. (1970). [d] Mims and Peisach (1976).

In summary, we have demonstrated that both types I and II copper in *Rhus vernicifera* laccase are bound to imidazole ligands. From comparison with a model compound, it is suggested that the types I and II sites each contain a single imidazole. Imidazole ligation to type II copper of ceruloplasmin is also demonstrated. The symmetry of the type I site in laccase resembles that of the sites in stellacyanin and azurin and the magnitude of the LEFE scales with the optical extinction coefficients in these three cases. All these results lend support to the belief that the type I sites in copper oxidases are similar to those found in dark-blue proteins containing a single copper.

[3] We have studied the nuclear modulation effect of a Cu^{2+} complex, kindly supplied by Dr. R. Bereman, in which a pentadentate chelator with only nitrogen ligands is bound to the metal ion. Thus, planar and axial ligand positions are taken up. Modulation patterns of the type we observe for imidazole ligation are not seen.

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