ON THE NATURE OF COPPER IN TWO PROTEINS OBTAINED FROM RHUS VERNICIFERA LATEX

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INTRODUCTION. Two blue copper-containing proteins have been obtained from lac acetone powders of Rhus vernicifera (Omura, 1961). One of these, laccase, is an aryl diamine and diphenol oxidase and contains more than one copper atom per molecule. The other, a yet unnamed blue protein, contains a single copper atom per molecule. It is the purpose of this communication to describe the nature of copper in both of these proteins, using electron paramagnetic resonance (EPR).

EXPERIMENTAL. Laccase was prepared by modifying a procedure described by Omura (1961) which will be discussed more fully in a future communication. The blue protein was purified as follows: A partially purified extract of 150 g of Rhus venicifera lac acetone powder was applied to a 4 x 50 cm Amerlite CG-50 column. Laccase was eluted with approximately 1 liter of 0.05 M sodium phosphate buffer, pH 5.5, containing 0.2 M NaCl. The column was then eluted with six liters of 0.2 M Na_2HPO_4. Twenty ml fractions were collected, and those that were visibly blue were combined. The volume of combined

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fractions at this point was 1 liter. This was dialyzed against 0.05 M sodium phosphate buffer, pH 5.5 with two changes of buffer. This solution was then applied to a 4 x 40 Amberlite CG-50 column equilibrated with 0.05 M sodium phosphate buffer, pH 5.5. The column was washed with two liters of buffer and the protein eluted from the column using a continuous gradient of sodium phosphate buffer to a limiting concentration of 0.4 M. Those fractions whose optical absorption (A) at 604 mμ was greater than 0.01 were combined and the solution was ultrafiltered to a residual volume of 40 ml. This was then dialyzed against 0.05 M sodium phosphate buffer, pH 5.5. The intense blue solution was then passed through a 1 x 12 cm DEAE cellulose column equilibrated with the same buffer, to remove a yellow impurity. The final solution had a ratio of A_{604}/A_{280} of 0.173 and an $A_{604}/\mu g$ Cu ratio of 0.0615 measured with a Beckman DU spectrophotometer.

EPR experiments were run at 77° K and at liquid He temperatures with a superheterodyne microwave spectrometer (Feher, 1957), operating near 9,200 mc/sec. One Kc/sec modulation field was used to detect the derivative of the resonance signal. A 12 inch Varian magnet was used to produce the magnetic field. The derivative plots obtained by this technique were graphically integrated and the areas under the curves were obtained by weighing the cutouts. In this way, the intensity of the microwave absorption was related to the concentration of divalent copper in a standard $CuSO_4$ solution in 60 per cent glycerine.

RESULTS. The EPR spectrum of 0.5 ml of blue protein is shown in Fig. 1. Qualitatively, the same spectrum is obtained at 77°K and 1.95° K. Unlike ionic copper, the blue protein exhibits an unresolved hyperfine spectrum in the parallel direction even when examined at

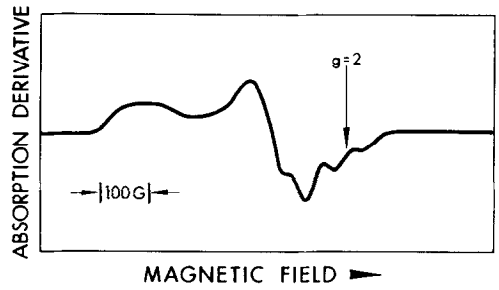

Fig. 1. EPR spectrum of Rhus vernicifera blue protein taken at 77°K.

1.95° K, but shows a well resolved hyperfine pattern in the perpendicular direction. Comparing the area of the EPR spectrum of a standard $CuSO_4$ solution in glycerine-water with that for blue protein indicates that the sample of protein examined with the EPR system contains 78 ± 8 μg of divalent copper. Chemical analysis using the method of Felsenfeld (1960) indicates the presence of 72 μg of copper in this solution. Fig. 2 is the EPR signal for Rhus vernicifera laccase which also has an unresolved parallel hyperfine pattern. The spectrum shows that the copper is bound at a non-axial site. The integration of the EPR curve resulting from a laccase sample containing 78 μg of copper shows the presence of 55 ± 5 μg of divalent copper. The magnetic constants for both copper proteins are shown in Table I.

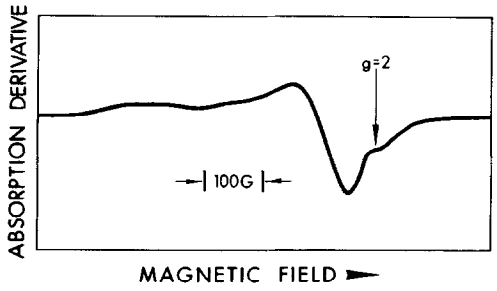

Fig. 2. EPR spectrum of Rhus vernicifera laccase taken at 77°K.

TABLE I. MAGNETIC CONSTANTS FOR RHUS VERNICIFERA COPPER PROTEINS

Laccase		Blue Protein	
g_x=2.00	A_x=<0.0007 cm^{-1}	$g_{\|\|}$=2.30	$A_{\|\|}$=0.0040 cm^{-1}
g_y=2.09	A_y=<0.0007 cm^{-1}	$g_\perp$=2.04	$A_\perp$=0.0058 cm^{-1}
g_z=2.27	A_z= 0.0040 cm^{-1}		

DISCUSSION. From early EPR studies of ceruloplasmin and other copper proteins, it was suggested that the very intense blue color of the molecule was due to a pair of coppers of mixed valency in close proximity (Beinert, et al., 1962). Since that time, Mason (1963), studying blue proteins from Pseudomonas aeruginosa and Pseudomonas denitrificans, showed that the same intense blue color could be found in a molecule where a single divalent copper was present. This was confirmed by Broman et al. (1963) with azurin, and is reported here for a blue protein derived from Rhus vernicifera. In all these cases, the parallel hyperfine splitting constants for the protein copper were much smaller than for ionic copper. In fact, the values presented here for Rhus vernicifera blue protein and laccase are the lowest yet observed for any copper protein, while the perpendicular hyperfine splitting for the blue protein is the largest observed for any copper protein.

The relation between intensity of blue color and copper content for various copper proteins is shown in Table II. Those proteins possessing a single, presumably divalent, copper per molecule have an A/μg Cu ratio ranging from 0.016 to 0.062. If one assumes that the chromophoric centers of ascorbic acid oxidase and the various laccases are

TABLE II RELATION BETWEEN BLUE COLOR AND COPPER CONTENT

Protein	Source	A. max.	A/μg Cu	Cu/protein	References
Cerebrocuproin	Human	665	0.00483	2	Porter and Folch (1957)
Erythrocuproin	Human	655	0.00313	2	Kimmel, et al., (1959)
Ascorbate oxidase	Squash	605	0.0193-0.0206	8	Poillon and Dawson (1963)
Ceruloplasmin	Human	610	0.020-024	8	Blumberg, et al., (1963)
Laccase	Polyporus versicolor	610	0.0157	4	Mosbach (1963)
Laccase	Rhus vernicifera	614	0.0218	6	This work
Laccase	Rhus succedanea	609	0.0180	6	Omura (1961)
Plastocyanin	Spinach	597	0.0772	2	Katoh, et al., (1962)
Blue Protein	Rhus vernicifera	604	0.0615	1	This work
Azurin	Bordetella	625	0.0551	1	Sutherland and Wilkinson (1963)
Blue Protein	Pseudomonas denitrificans	620	0.0165	1	Suzuki and Iwasaki (1962)
Blue Protein	Mung bean	598	0.0168	1	Shichi and Hackett (1963)

due to divalent copper, as has been shown for ceruloplasmin (Blumberg, et al., 1963), then the A/μg divalent copper ratios for these proteins are also well within these limits. From these considerations, one can make predictions as to the nature of the two copper atoms in plastocyanin. If the coppers are bonded in a fashion similar to that of copper in Rhus vernicifera blue protein, then they both must be divalent.

A final point of this study involves the valency of copper in Rhus vernicifera laccase. From previous studies, (Omura, 1961), and confirmed by us, this protein contains six copper atoms. EPR measurements indicate that four of these six are divalent, based on Omura's molecular weight determination of 141,000 and a copper content of 0.25 per cent. This is in direct contrast to the findings of Nakamura (1958), who reports that all the copper in this protein is divalent. Polyporus laccase has a molecular weight of 62,000 and possesses four copper atoms per molecule (Mosbach, 1963). Two of these copper atoms are divalent (Broman et al., 1962; Ehrenberg et al., 1962). EPR data for Polyporus versicolor laccase [axial site g =2.197, g_m=2.048, A =0.009 cm^{-1}, (Malmström and Vänngård, 1960)] are quite different from our data for Rhus vernicifera laccase. From these findings, one must conclude that the laccases from Rhus and Polyporus are not the same. The only similarities that exist may be due to their oxidase substrate specificities, which for the Rhus vernicifera enzyme have been studied in our laboratories. These results will appear elsewhere.

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