and potassium bromides show interaction with IV in ethanolic solution; potassium, which is relatively easily dehydrated, appears more strongly complexed. Furthermore, the stabilities of the KBr complexes decrease in the order II, IV, I, and III (see Table IV) which is in agreement with the stability order of KCl complexes with these polyethers in methanol as found potentiometrically[8] (where cyclohexyl-15-crown-5 has been used in place of I). However, the fact, that $(I)_2$–KBr complex but not (IV)–KBr can be isolated shows that the isolation possibility of a complex is not necessarily related to its solution stability. (See the stability order above.) Isolation of only the (II)–KNCS complex from a 1:1:1 reaction mixture of II, KNCS, and NaNCS (see Table II) from ethanol also suggests this because the differences in R–MX interaction forces for (II)–KNCS and (II)–NaNCS systems, as found paper chromatographically, is only marginal.

Acknowledgments. The author is grateful to the late Professor Sir Ronald Nyholm under whose guidance and inspiration the earlier part of this work was started, to Professor M. R. Truter who provided necessary help and facilities during that work, and to Professor M. M. Bokadia who provided facilities to work in this department for the execution of the later part of this work.

Stereoelectronic Properties of Metalloenzymes. I. A Comparison of the Coordination of Copper(II) in Galactose Oxidase and a Model System, *N*,*N*′-Ethylenebis(trifluoroacetylacetoniminato)copper(II)

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Abstract: The complex, *N*,*N*′-ethylenebis(trifluoroacetylacetoniminato)copper(II), is proposed as a model of the equatorial coordination of the copper(II) in galactose oxidase. The spin Hamiltonian parameters of galactose oxidase (g_{xx} = 2.058, g_{yy} = 2.048, g_{zz} = 2.273, A_{xx} = 28.8 G, A_{yy} = 30.1 G, and A_{zz} = 176.5 G) are quite similar to the proposed model (g_{xx} = 2.0451, g_{yy} = 2.0349, g_{zz} = 2.2043, A_{xx} = 30.7 G, A_{yy} = 31.6 G, and A_{zz} = 194.5 G) differing only in the magnitude of g_{zz} and A_{zz}. This difference is ascribed to the presence of a strong π-bonding axial ligand in galactose oxidase. A calculation of the copper d–d electronic transitions in both model and enzyme based upon the observed esr parameters is in good qualitative agreement with the optical spectra. This agreement suggests both that the model is an appropriate one for galactose oxidase and that the assignment of the spin parameters for each system is correct. Based upon comparisons to the known copper ligation in the model system, possible features of the copper–protein complex are suggested.

Galactose oxidase (galactose, O_2 oxidoreductase EC 1.1.3.9, hereafter referred to as GOase) is a copper-containing enzyme which catalyzes the conversion of primary alcohols to the corresponding aldehydes concomitant with the reduction of O_2 to H_2O_2.[2-9] The molecular weight of this enzyme has been established at 68,000 ± 3000 daltons with one copper atom per molecule.[8,9] The nature of the copper(II) site and the mechanism of the enzymatic reaction[6,7,10] have received some attention, but until recently no definitive information has been obtained. Ettinger and Kosman[11] have reported a detailed CD study of the enzyme in the presence of its substrates and products. Also, the optical spectrum of the enzyme from 350 to 950 nm has been correlated with the CD spectrum.[12] The electron spin resonance spectrum of GOase was reported by Blumberg,[13] but, as previously noted,[14] the conditions of his experiment, when duplicated in our laboratory, inactivate the enzyme. The electron spin resonance spectrum of GOase has recently been employed in this laboratory to establish the nonexistence of a cuprous (Cu^+) intermediate in the enzymatic reaction.[14]

Copper-containing proteins, since initial studies were

(1) Author to whom correspondence should be addressed in the Department of Chemistry, State University of New York at Buffalo.

(2) J. A. D. Cooper, W. Smith, M. Bacila, and H. Medina, *J. Biol. Chem.*, **234**, 445 (1959).

(3) G. Arigad, D. Amaral, C. Asensio, and B. L. Horecker, *J. Biol. Chem.*, **237**, 2736 (1962).

(4) R. A. Schlegel, C. M. Gerbeck, and R. Montgomery, *Carbohyd. Res.*, 7, 193 (1963).

(5) G. I. Zancan and D. Amaral, *Biochem. Biophys. Acta*, **198**, 146 (1970).

(6) G. A. Hamilton, J. DeJersey, and P. K. Adolf in "Oxidases and Related Redox Reactions," M. Morrison, *et al.*, Ed., University Park, Baltimore, Md., 1973, p 103.

(7) D. J. Kosman, L. Kwiatkowski, M. J. Ettinger, and J. D. Brodie, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **32**, 550 (1973).

(8) D. J. Kosman, M. J. Ettinger, E. J. Massaro, and R. Weiner, submitted for publication.

(9) D. Amaral, L. Bernstein, D. Morse, and B. L. Horecker, *J. Biol. Chem.*, **238**, 2281 (1963).

(10) L. D. Kwiatkowski and D. J. Kosman, *Biochem. Biophys. Res. Commun.*, **53**, 715 (1973).

(11) M. J. Ettinger and D. J. Kosman, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, **32**, 55 (1973).

(12) M. J. Ettinger, submitted for publication.

(13) W. E. Blumberg, B. L. Horecker, F. Kelly-Falcoz, and J. Peisach, *Biochem. Biophys. Acta*, **96**, 336 (1965).

(14) D. J. Kosman, R. D. Bereman, M. J. Ettinger, and R. S. Giordano, *Biochem. Biophys. Res. Commun.*, **54**, 856 (1973).

Table I. Spin Hamiltonian Parameters for Galactose Oxidase (GOase) and *N,N'*-Ethylenebis(trifluoroacetylacetoniminato)copper(II) ((TFAA)$_2$Cu)[a]

	A_{zz}	A_{xx}	A_{yy}	g_{zz}	g_{xx}	g_{yy}	$\langle a\rangle_{calcd}$[a]	$\langle g\rangle_{calcd}$[a]
GOase	176.5	28.8	30.1	2.273	2.058	2.048	78.4	2.125
(TFAA)$_2$Cu	194.5	30.67	31.60	2.2043	2.0451	2.0349	85.59 (85.6)	2.0947 (2.0977)
	$\langle a\rangle$(^{14}N)		13.9					

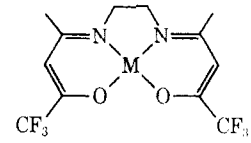
[a] Hyperfine splittings in gauss. [b] Calculated values (observed).

made,[15] have been grouped into two categories: "blue" copper proteins—a classification based on their unusually high extinction coefficients in the visible spectrum—and "nonblue" proteins.[16] Since the esr and optical properties of a metal complex are closely related, a similar classification based on the magnitude of the Z component of the metal hyperfine coupling constant has also been made. One class (designated type 1 Cu^{2+}) has A_{zz} smaller than 100×10^{-4} cm^{-1} and corresponds to the "blue" proteins and the other (type 2 Cu^{2+}) has A_{zz} larger than 140×10^{-4} cm^{-1} and corresponds to the "nonblue" proteins.[16] Type 1 Cu^{2+} has attracted a large amount of interest because of its unique spectral properties. Type 2 Cu^{2+} has been less well characterized because several proteins which contain this type copper also contain type 1 Cu^{2+}.[16]

Galactose oxidase is the only known type 2 Cu^{2+} protein which contains only one Cu^{2+} except for uricase.[17,18] Thus, this metalloenzyme seems to be an ideal choice for a detailed investigation of type 2 Cu^{2+} proteins.

Various model systems for type 1 Cu^{2+} proteins have been investigated.[16] Most of the Cu^{2+} complexes involve coordination to nitrogen ligands where the coordination geometry is distorted tetrahedral.[16] Blumberg has related the optical transitions, their intensities and activities, and the g factors by an appropriate choice of symmetry and crystal field parameters in a quantitative model of type 1 Cu^{2+} proteins.[19]

Since an understanding of the role of copper in any protein must be based on the knowledge of the local environment of the metal,[20] we report here a detailed study of the esr spectrum of the type 2 Cu^{2+} protein,[16] galactose oxidase, and the esr and optical spectra of the most appropriate model of a number of potential ones which we have been studying[21]—*N,N'*-ethylenebis(trifluoroacetylacetoniminato)copper(II) (hereafter referred to as (TFAA)$_2$Cu). The choice of (TFAA)$_2$Cu as a model for the electronic properties of the Cu(II) in GOase was based on several considerations: (1) the x and y portions of the spin Hamiltonian parameters should be as near to those of GOase as possible (this naturally assumes the model complex must exhibit rhombic symmetry); (2) the A_{zz} value for the model system should be higher than that of GOase—a value near 20 G difference was sought (see discussion and ref 31); (3) the optical spectrum of the model system must show some features which might be resolved.



Answers to several key questions concerning GOase were sought: (1) what is the nature of the copper coordination, (2) do valid models exist for type 2 Cu^{2+} proteins? Answers to these questions would allow us to gain insight into the structure–function properties of type 2 Cu^{2+} in metalloenzymes.

Experimental Section

The galactose oxidase samples used in this study were isolated and purified to homogeneity in these laboratories using procedures developed from literature methods.[8,22,23] The GOase solutions ranged in concentration between 15 and 45 mg/ml depending on the time of concentration ($2–7 \times 10^{-5}$ M) and were dialyzed against a sodium phosphate buffer (0.1 M, pH 7.0) containing 1 M $(NH_4)_2SO_4$. This procedure stabilizes the GOase in the freezing process required to obtain electron spin resonance spectra. The enzyme samples were assayed before and after esr measurements to establish their enzymic activity. Under no circumstances did loss of activity occur. All buffers were prepared copper-free by elution from columns of Chelex-100 (BioRad).

GOase was assayed *via* O_2-uptake measurements in an air atmosphere at 20 ± 2° using a Gilson differential respirometer equipped with Model No. 5 all-glass volumometers. Enzyme was added to give an O_2 uptake of 8 μl of O_2/min at a shaking rate of 140 oscillations/min. The concentration of D-galactose (Sigma) was 0.2 M in pH 7.0, 0.1 M sodium phosphate buffer.

(TFAA)$_2$Cu^{II} was obtained as a kind gift from Professor Paul McCarthy, Department of Chemistry, Canisius College.

X-Band esr spectra were obtained on frozen glasses at 120°K by employing a Varian variable-temperature control apparatus with liquid nitrogen as coolant. A Varian V-4502-19 spectrometer was used in conjunction with a Magnion proton oscillator Gauss meter and a Hewlett-Packard frequency counter to obtain accurate measurements of the magnetic field and microwave frequency. Spectra were scanned slowly. No attempts were made to apply second-order corrections.

Optical spectra of (TFAA)$_2$Cu were determined in an ethanol solution at room temperature and a KBr matrix at room temperature and 77°K employing a specially constructed cell.

Results and Discussion

The spin Hamiltonian parameters for GOase and (TFAA)$_2$Cu are given in Table I. The electron spin resonance spectra of GOase and (TFAA)$_2$Cu are shown in Figures 1 and 2, respectively. The spectrum of GO-

(15) B. Malmström, R. Mosbach, and T. Vänngard, *Nature (London)*, **183**, 321 (1959).

(16) T. Vänngard in "Biological Applications of Electron Spin Resonance," H. M. Swartz, J. R. Bolton, and D. C. Borg, Ed., Wiley-Interscience, New York, N. Y., 1972, p 411, and references therein.

(17) H. M. Mahler in "The Enzymes," P. D. Boyer, *et al.*, Ed., 2nd ed, Vol. 8, Academic Press, New York, N. Y., 1970, p 285.

(18) D. G. Priest, *et al.*, submitted for publication.

(19) W. Blumberg in "Biochemistry of Copper," J. Peisach, P. Aisen, and W. Blumberg, Ed., Academic, New York, N. Y., 1966, p 49.

(20) R. Malkin and B. G. Malmström, *Advan. Enzymol. Relat. Subj. Biochem.*, **33**, 177 (1970), and references therein.

(21) More than 20 different copper(II) complexes and compounds were investigated. The criteria for the model were that the esr spectra exhibit rhombic symmetry, have spin Hamiltonian parameters A_{xx} and A_{yy} near that of GOase, and yield an observable optical spectrum. Many copper(II) complexes, even though the gross symmetry is rhombic, do not display esr spectra characteristic of rhomboic distortions: R. D. Bereman and R. S. Giordano, in preparation.

(22) D. Amaral, F. Kelly-Falcoz, and B. L. Horecker, *Methods Enzymol.*, **9**, 87 (1966).

(23) Sh. Bauer, G. Blauer, and G. Avigad, *Proc. Isr. J. Chem.*, **5**, 126 (1967).

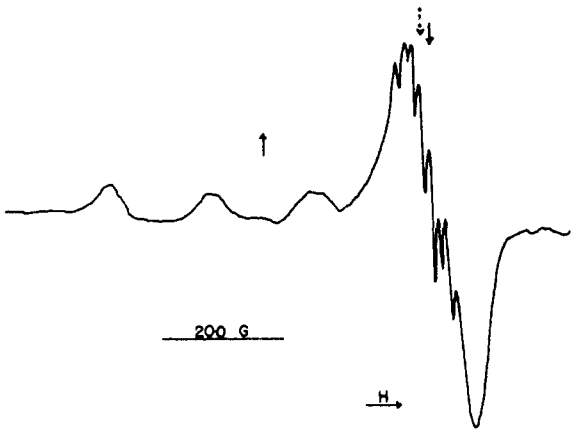


Figure 1. Electron spin resonance spectrum of galactose oxidase (frozen glass = 120°K). Solid arrow up represents position of g_{zz}. Solid arrow down represents position of g_{yy}. Dashed arrow down represents position of g_{xx}.

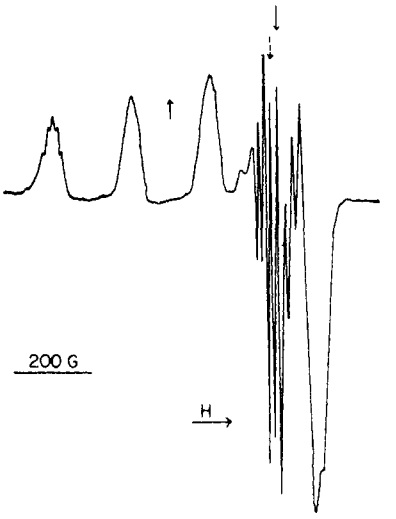


Figure 2. Electron spin resonance spectrum of bis(trifluoroacetylacetoniminato)ethylenediaminecopper(II). Solid arrow up represents position of g_{zz}. Solid arrow down represents position of g_{yy}. Dashed arrow down represents position of g_{xx}.

ase is typical of Cu^{2+} systems in local environments of C_{2v} and D_{4h} symmetry as normally found for pseudo-square-planar environments[24] except that in this case the anisotropy in the x and y directions is detected. Anisotropy in the x and y directions has been noted before for other copper-containing enzymes and models.[24]

Although the esr spectra of square-planar and pseudo-square-planar copper(II) systems have received a large amount of attention, only the assignment of the lines in the parallel region are straightforward. Some discrepancies exist in the interpretation of the lines found in the perpendicular region.[16,24,25] Since several alternatives exist in the assignment of the lines in this region and since a great deal depends on the validity of the interpretation, these assignments in the perpendicular region of the spectrum for both $(TFAA)_2Cu$ and GOase are discussed separately.

If we concern ourselves initially with the spectrum of $(TFAA)_2Cu$ (Figure 2), it is obvious that these lines in the perpendicular region are not due to ^{14}N hyperfine splittings since more than the maximum number of five expected for two equivalent nitrogens are present. The anisotropy in the copper hyperfine splitting constants and g values would be expected to be small for this compound. In fact, quite similar compounds have been studied where no anisotropy existed, that is $g_{xx} = g_{yy} = g_{\perp}$ and $A_{xx} = A_{yy} = A_{\perp}$.[25] Two possibilities for the assignments exist. (A) Two sets of four lines side by side with A values approximately equal to 15 G which will yield g values which are significantly different. (B) Two sets of four overlapping lines with A values approximately equal to 30 G which will yield g values which are very nearly equal. The second of these alternatives is much more reasonable since it yields g values which are quite similar and A values which are also quite similar. Since only frozen glass spectra are available, the assignments of g_{xx} and g_{yy} must be arbitrary; we have in all cases assigned $g_{xx} > g_{yy}$.

The use of the esr parameters to calculate positions of the d–d transitions in metal complexes has proven quite valid in a number of cases (see below).[26] The first alternative assignment above yields optical values which are not realistic. Perhaps most informative in our assignment here is the isotropic solution spectrum. Since the isotropic hyperfine splitting constant, $\langle a \rangle$, is equal to the average of the anisotropic values, it can be shown that again sets of overlapping lines are more reasonable than sets of lines side by side (see Table I).

Essentially the same reasoning as discussed above is applicable to GOase except two additional points need to be discussed. That the lines in the perpendicular region are due to ^{14}N splittings (or other nuclei) is not obvious. However, these lines are more intense and more well-defined than the well-resolved lines associated with the parallel region of the spectrum. This is certainly not *typical* of ligand hyperfine splitting.

Some insight into the correct assignment of the lines in this region can also be gained in the calculation of the hyperfine values from the spectrum. The difference between the magnetic field positions of the outside pair of lines divided by three must equal exactly the difference between the inside pair of lines. If a good fit is obtained as in this case, a proper assignment seems likely. Alternative assignments did not give consistent sets of A values in these regions. An isotropic spectrum was not obtainable at any temperature. Presumably, relaxation phenomena were such that the esr signal was broadened and lost. Again, however, it is possible to calculate the positions of the d–d optical spectrum of GOase, and alternative assignments did not give values near the observed values (see below).

The types of ligands bound to Cu^{2+} can be determined to some extent in any system by carrying out a simple molecular orbital calculation employing the spin Hamiltonian parameters. The procedure is similar to that employed by Maki, *et al.*,[27] and reviewed briefly here for completeness. Real d orbitals were chosen as a basis set and the spin-orbit interaction Hamiltonian, $\sum_K L_K S_K$, was applied as a first perturbation on the spin

(24) J. P. Fackler, Jr., J. D. Levy, and J. A. Smith, *J. Amer. Chem. Soc.*, **94**, 2436 (1972).
(25) H. R. Gersmann and J. D. Swalen, *J. Chem. Phys.*, **36**, 3221 (1962).
(26) F. L. Urbach, R. D. Bereman, J. A. Topich, M. Hariharan, and B. J. Kalbacher, submitted.
(27) A. H. Maki, N. Edelstein, A. Davison, and R. H. Holm, *J. Amer. Chem. Soc.*, **86**, 4580 (1964).

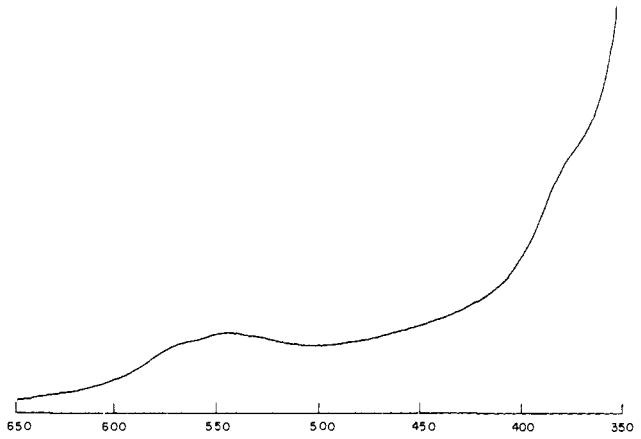
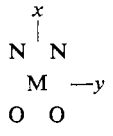


Figure 3. Optical spectrum of bis(trifluoroacetylacetoniminato)-ethylenediaminecopper(II) in a KBr matrix at 77°K. Units are in nm (ordinate is arbitrary).

Hamiltonian ($=\beta \mathbf{S}g\mathbf{H} + \mathbf{I}A\mathbf{S}$), where $\mathbf{H}$ is the magnetic field, and $\mathbf{S}$ and $\mathbf{I}$ are the electron and nuclear spin operators, respectively. Depending on the ground-state configuration chosen (one unpaired electron in the d_{xy} orbital for Cu^{2+} using the coordinate system below), various sets of equations relate g and A values to P and K. ($P = g_e g_n \beta_e \beta_n \langle r^{-3} \rangle$, where g_e and g_n are the electron and nuclear g factors, respectively, β_e and β_n are Bohr and nuclear magnetons, respectively, and $\langle r^{-3} \rangle$ is the inverse cube electron–nuclear distance. K is the Fermi



contact term.) For the systems discussed here, the equations are[27]

$$g_{xx} = 2 - 2\alpha_3 \quad (1)$$

$$g_{yy} = 2 - 2\alpha_2 \quad (2)$$

$$g_{zz} = 2 - 8\alpha_1 \quad (3)$$

$$A_{xx} = P[-2\alpha_3 - K + {}^2/_7 + {}^3/_7\alpha_2] \quad (4)$$

$$A_{yy} = P[-2\alpha_2 - K + {}^2/_7 + {}^3/_7\alpha_3] \quad (5)$$

$$A_{zz} = P[-8\alpha_1 - K - {}^4/_7 - {}^3/_7(\alpha_2 + \alpha_3)] \quad (6)$$

Solving these equations for P and K for GOase yielded values of 0.0263 cm^{-1} and 0.423, respectively. For $(TFAA)_2Cu$, values of 0.0257 cm^{-1} and 0.428 for P and K were obtained. Maki, *et al.*,[27] have noted as we have[28] that values of P are characteristic of the types of ligands or atoms bound in the equatorial sites. For example, with four sulfur atoms, P falls in the range of 0.016–0.020 cm^{-1}.[27-29] It would certainly appear that the Cu^{2+} in GOase has similar endogenous ligands to that of the model, $(TFAA)_2Cu$, namely two nitrogen and two oxygen atoms. The high values of P for the two systems investigated here can also be associated with rather ionic σ-bonds formed between the metal and the in-plane ligands.[27] For the $(TFAA)_2Cu$, weak hyperfine splittings due to two equivalent ^{14}N atoms were detected on the A_{zz} lines. This splitting was 13.9 G. If such a hyperfine structure exists for GOase, the lower signal to noise ratio would make its detection extremely difficult.

The terms α_1, α_2, and α_3 above in eq 1–3 are defined as

$$\alpha_1 = \frac{\xi_{eff}}{\Delta E_{(xy \rightarrow x^2-y^2)}}$$

$$\alpha_2 = \frac{\xi_{eff}}{\Delta E_{(xy \rightarrow yz)}}$$

and

$$\alpha_3 = \frac{\xi_{eff}}{\Delta E_{(xy \rightarrow xz)}}$$

If one chooses an appropriate value of the effective spin–orbit coupling constant, an estimate for the values for the "d–d" transitions of GOase and the $(TFAA)_2Cu$ system can be made. If, as an initial estimation, we assume the spin–orbit coupling constant (ξ_{eff}) is lowered from the free ion value (880 cm^{-1}) to the same extent as P is lowered from the free ion value (0.035 cm^{-1}),[30] then calculated values of $\Delta E_{(xy \rightarrow x^2-y^2)}$, $\Delta E_{(xy \rightarrow yz)}$, and $\Delta E_{(xy \rightarrow xz)}$ become respectively 20.4, 23.9, and 28.9 kK for GOase. Similarly, values of 25.3, 28.7, and 37.1 kK are obtained for $(TFAA)_2Cu$. The optical spectrum of $(TFAA)_2Cu$ at 77°K in a KBr matrix, as shown in Figure 3, has one asymetric peak at 18.4–17.7 kK which appears to have two components and a weak shoulder on a charge transfer band at 26.3 kK.

While the correspondence between calculated and observed values is not good, the calculated values are quite similar with two low energy bands close together and one at higher energy. The ratios of the observed to the calculated energies are approximately 0.70. Application of this factor to the calculated bands of GOase allows us to predict transitions of 14.3, 16.7, and 20.1 kK for the three transitions mentioned above. Hence, the observed absorption transitions[12,23] which occur at 12.9, 15.9, and 22.5 kK should be assigned to the "d–d" transitions $\Delta E_{(xy \rightarrow x^2-y^2)}$, $\Delta E_{(xy \rightarrow yz)}$, and $\Delta E_{(xy \rightarrow xz)}$, respectively.

The difference in A_{zz} and g_{zz} between the model and GOase has been accounted for by the presence of a strong π-bonding ligand in one of the axial coordination sites in GOase.[31]

Since there is such a sharp contrast between "blue" and "nonblue" proteins, that is, $A_{zz} > 140$ G for "nonblue" proteins and $A_{zz} < 100$ G for "blue" proteins, the tendency has been to infer that these Cu^{2+} sites have different coordination geometries. However, a persuasive argument can be made that the differences in A_{zz} values for "blue" and "nonblue" proteins are in part due to differences in ligand type rather than solely to differences in degree of distortion from square planar geometry and must also be considered. The "blue" copper proteins are distinguished by having large extinction coefficients in the visible spectrum and rather small values of the copper hyperfine coupling constant, A_{zz}. Although both of these characteristics have been attributed to unusual coordination geometry, they might also be due

(28) B. J. Kalbacher and R. D. Bereman, *Inorg. Chem.*, **12**, 2997 (1973).
(29) P. C. Savino and R. D. Bereman, *Inorg. Chem.*, **12**, 173 (1973).
(30) B. R. McGarvey, *J. Phys. Chem.*, **71**, 51 (1967).
(31) R. S. Giordano, R. D. Bereman, D. J. Kosman, and M. J. Ettinger, *J. Amer. Chem. Soc.*, **96**, 1023 (1974).

to a large amount of covalent bonding in the complex. The mixing of s and p orbitals (or hybrid) into copper d orbitals would increase the transition probability of the "d–d" transitions. The increase in covalency attributed to the mixing of metal and ligand orbitals also decreases the unpaired electron density at the metal center and results in lower values for the copper hyperfine coupling constants.

The differences between the optical properties of "blue" and "nonblue" cupric copper cannot be equated to merely differences in degree of distortion of the copper ligand field. In support of this contention is the observation that the optical activity associated with the transition near 610 nm for GOase is as large or larger than that reported for "blue" Cu^{2+}.[12] The interpretations given here suggest that some contribution related to the chemical identity of the ligand must also be taken into account while rationalizing the spectral difference between copper types.

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Stereoelectronic Properties of Metalloenzymes. II. Effects of Ligand Coordination on the Electron Spin Resonance Spectrum of Galactose Oxidase as a Probe of Structure and Function

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Abstract: The addition of various potential copper ligands to solutions of galactose oxidase produces distinctive changes in its esr spectrum. The most strongly bound ligands appear to be those capable of forming π-bonds to Cu^{2+} such as cyanide. A substrate, galactose, competes with this ligand for the Cu^{2+} site. Hydrogen peroxide, a product of the enzymatic reaction, appears to bind at the copper site although its effect on the esr spectrum is small. The experiments indicate that only a single coordination site in the copper–protein complex is readily accessible to exogenous ligands and that this site is normally occupied by a water or hydroxide molecule. The manner in which the esr spectrum changes in the presence of strong π-bonding ligands suggests that a strong π-bonding ligand may occupy the nonlabile axial coordination site in the protein–metal complex.

The esr spectrum of galactose oxidase (galactose, O_2 oxidoreductase EC 1.1.3.9, hereafter referred to as GOase) has been analyzed in detail.[2] Since the cupric ion in this enzyme is essential to its activity,[3,4] its ability to bind exogenous ligands may indicate the manner in which the metal participates in the redox reaction catalyzed. Substrates and products could form either inner or outer sphere complexes with the metal or, perhaps, bind not to the metal at all but interact with the copper indirectly through protein moieties. The well-resolved copper optical activity of GOase[5] has been used to detect the alteration in the metal's environment caused by galactose, one of the substrates, and the two products of the reaction, H_2O_2 and galactohexodioldose.[6] Oxygen, the other substrate, does not bind to the free enzyme.[6] Experiments have also been reported on the effects of galactose, oxygen, and the dialdehyde on the esr spectrum of GOase.[7] Since coordination of galactose has no effect on the esr spectrum, it was inferred that it displaces a similar ligand from the copper atom, such as a H_2O molecule or OH^- ion.

In this paper, we report a series of experiments specifically designed to determine the accessibility of the Cu^{2+} in GOase to added ligands. The affinity of the metal ion for ligands of different binding types was tested with the aim of possibly discerning the nature of the endogeneous ligand. Furthermore, the ability of galactose to compete with other exogeneous ligands to

(1) Author to whom correspondence should be addressed in the Department of Chemistry, State University of New York at Buffalo.

(2) R. S. Giordano and R. D. Bereman, *J. Amer. Chem. Soc.*, **96**, 1019 (1974).

(3) D. Amaral, L. Bernstein, D. Morse, and B. L. Horecker, *J. Biol. Chem.*, **238**, 2281 (1963).

(4) F. Kelly-Falcoz, H. Greenberg, and B. L. Horecker, *J. Biol. Chem.*, **240**, 2966 (1965).

(5) M. J. Ettinger, *Biochemistry*, submitted for publication.

(6) M. J. Ettinger and D. J. Kosman, *Biochemistry*, submitted for publication.

(7) D. J. Kosman, R. D. Bereman, M. J. Ettinger, and R. S. Giordano, *Biochem. Biophys. Res. Commun.*, **54**, 856 (1973).