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

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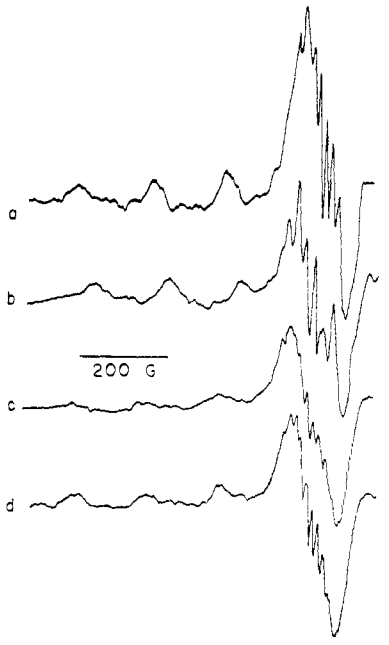

Figure 1. (a) Electron spin resonance (esr) spectrum of galactose oxidase (GOase), (b) esr spectrum of 1:1 molar ratio CN^-:GOase, (c) esr spectrum of 10:1 molar ratio N_3^-:GOase, (d) esr spectrum of 100:1 molar ratio N_3^-:GOase. (Concentrations of GOase varied between 2–3 $\times$ 10^{-5} *M* in these determinations.)

the copper was tested to determine if galactose coordination occurs directly to the copper. Finally, we reasoned that these ligand experiments may yield information which would account for the differences reported between the esr spectrum of GOase and a low molecular weight model which has two nitrogen atoms and two oxygen atoms in the planar coordinating positions.[2]

Experimental Section

The galactose oxidase was prepared, and the X-band esr spectra were determined in the same manner as previously reported.[2] Enzyme concentrations ranged between 15 and 45 mg/ml (2–7 $\times$ 10^{-5} *M*) depending on the preparation.

Standard solutions of all ligands investigated were made to a concentration such that 10 μl of solution when added to 100 μl of GOase yielded either 1:1, 10:1, or 100:1 ligand to GOase molar ratios. Solutions were mixed 5–10 min before esr spectra were determined.

In all cases, the enzyme *regained* full activity when the exogeneous ligands were removed by dialysis. All ligands except urea and thiourea appear to be inhibitors.

Results

The spin Hamiltonian parameters for GOase in the presence of the various molar ratios of ligands are presented in Table I. These ligands can be placed into three classes of apparent affinity. The first includes those ligands which form 1:1 complexes with the copper in GOase. The members of the second group saturate the copper at 10:1 or 100:1 molar ratios as ascertained by no further changes in esr spectra. Lastly, certain ligands, like the halides, do not reach saturation levels at a molar ratio of even 100:1.

The spectra in Figure 1 illustrate how these classes are distinguished experimentally. Figure 1a is the esr spectrum of native GOase. In the presence of a 1:1 molar ratio of CN^-, a spectrum represented by Figure 1b is observed; further increases in the CN^- concentration cause no further alteration of the spectrum. Figure 1c illustrates the GOase spectrum at a 10:1 molar ratio of N_3^-; Figure 1d is the same ligand at a 100:1 molar ratio. The broadened spectrum in Figure 1c is characteristic of the presence of more than one type of Cu^{2+} in a solution, *i.e.*, copper atoms both ligated and unligated to N_3^-. At a 100:1 molar ratio of N_3^-, however, the copper sites are saturated and the sharpened, yet altered spectrum, Figure 1d, results. The most weakly bound ligands, the halides, cause some broadening, as in Figure 1c, at even 100:1 molar ratios. Thus, for these ligands, even these concentrations are below the saturation concentration. On the other hand, the two neutral ligands, urea and thiourea, cause a complete change in the spin Hamiltonian parameters at a 1:1 molar ratio as did CN^-. Addition of more ligand affects no further spectral change, even up to 8 *M* urea.

Table I. Spin Hamiltonian Parameters for Galactose Oxidase and Galactose Oxidase in the Presence of Various Ligands[a]

Ligand present (ratio)[b]	A_{zz}	A_{xx}	A_{yy}	g_{zz}	g_{xx}	g_{yy}
None	176.5	28.8	30.1	2.273	2.058	2.048
CN^- (1:1)	155.8	41.6	45.2	2.226	2.048	2.035
CN^- (10:1)	158.0	42.7	44.5	2.229	2.048	2.035
N_3^- (1:1)	166.8	27.2	27.9	2.262	2.049	2.040
N_3^- (10:1)	170.6	27.6	27.5	2.254	2.048	2.040
N_3^- (100:1)	176.3	28.2	28.3	2.253	2.049	2.039
SCN^- (1:1)	171.9	31.6	29.8	2.262	2.053	2.042
SCN^- (10:1)	172.0	30.4	30.1	2.273	2.052	2.042
Urea (10:1)	171.8	26.8	29.7	2.274	2.058	2.049
Thiourea (10:1)	169.8	28.4	28.8	2.271	2.058	2.049
F^- (100:1)	184.4	21.7	16.2	2.272	2.109	2.047
Br^-	180.8	27.8	28.7	2.276	2.060	2.050
H_2O_2	177.8	28.4	29.8	2.275	2.060	2.050

[a] Hyperfine splittings in gauss. [b] Molar ratio (ligand:GOase).

Hydrogen peroxide is one of the less strongly bound ligands (Table I). Saturation is approached at 100:1 molar ratio as indicated by the esr spectrum. The concentration of peroxide under these conditions is 20 m*M*, while the kinetically determined product inhibition constant for peroxide is 13 m*M*.[8]

The presence or absence of oxygen has no effect on the GOase esr spectrum[7] nor does the concentration of oxygen alter the binding of other ligands. In contrast, while anerobic addition of galactose to ten times it Michaelis constant concentration does not effect the GOase esr spectrum,[7] this concentration is sufficient to effect competition with CN^- at the copper site. Figure 2a represents the esr spectrum of GOase when 0.2 *M* galactose is present. Added CN^- (1:1) produces the broadened spectrum in Figure 2b in contrast to the completely changed spectrum (Figure 1b) for CN^- without galactose present. This, as noted above, would be expected if the CN^- and galactose were competing for the same coordination site.[9]

Discussion

The ligands whose effects on the GOase esr spectrum have been studied herein fall into four chemical classes. These include neutral ligands (urea and thiourea), strong π-bonding ligands (CN^-), strong field ligands

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or hard bases (F^-), and weak field ligands or soft bases (Br^-, N_3^-, and SCN^-). Of these, CN^- and N_3^- are reported to be inhibitors of GOase enzymatic activity.[3,8]

The binding of urea and thiourea are striking with respect to the similarities in their effects on the esr spectrum of GOase and their binding stoichiometry. Because they result in an identical spectrum (Table I), their ligation with the copper must not be *via* the oxygen or sulfur atoms. These two atoms, if coordinated to the copper, would have different effects on the copper esr spectrum. This suggests that ligation is *via* an amide nitrogen. The effect of both ligands is specific because no further alteration in the esr spectrum is observed up to 100:1 molar ratios. Even in 8 *M* urea GOase exhibits a spectrum identical with that seen in 0.2 m*M* urea (100:1 molar ratio). Related to this is the fact that the enzyme in 8 *M* urea is active when diluted into an assay mix containing no urea.[4] Thus, urea and thiourea appear to bind at the copper atom, and GOase is apparently resistant to the commonly observed denaturation effects of these reagents.

The only strong π-bonding ligand investigated, CN^-, was the most informative probe for two reasons. It produces a unique change in the esr spectrum of GOase which argues strongly that CN^- is bound directly to the Cu^{2+}. The value of A_{zz} decreases dramatically while values of A_{xx} and A_{yy} increases dramatically from those of free GOase (Table I). More notable, however, is that the spectral changes occur upon the addition of 1 mol of ligand per mole of GOase. The esr spectra of 10:1 and 100:1 samples are identical with that of the 1:1 sample. This indicates that the association constant of CN^- to Cu^{2+} in this enzyme is large. Of greater significance mechanistically is the observation that only one site is available for exogeneous ligand binding. That is, it is difficult to imagine that, if CN^- in a 1:1 molar ratio system produces a unique spectrum, while in a 100:1 system no further change occurs, more than one coordination site in GOase is available to exogeneous ligands. There must be only one weakly bound and easily displaced ligand in free GOase. Thus, although the esr spectrum of GOase is characteristic of a pseudo-square-planar environment, only one of the remaining two axial sites is available for coordination. The other axial position is presumably blocked by strong coordination to a protein ligand and/or by the stereoelectronic properties of the enzyme–copper complex.

The F^- anion is the only ligand which produces an increase in the A_{zz} value and a decrease in the A_{xx} and A_{yy} values. As a very electronegative ligand, F^- should exhibit different effects from those caused by CN^-, a strong π-bonding type ligand. Significantly, the affinity of GOase copper for F^- is considerably less than for CN^-. Even at 100:1 molar ratios, F^- has not yet saturated the copper site. Consequently, the π bonding of which CN^- is capable but F^- is not is apparently quite important in stabilizing coordination in the labile axial position.[8]

The remaining ligands, N_3^-, Br^-, and SCN^-, each produce a unique spectrum when added to GOase, but the spin Hamiltonian parameters are not vastly different from GOase. However, one of these ligands, SCN^-, which has some π-bonding capabilities also forms a stable 1:1 complex with GOase. This further

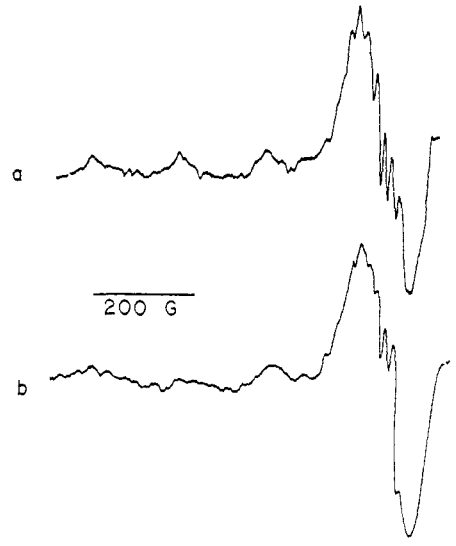

Figure 2. (a) Esr spectrum of GOase in 0.20 *M* galactose, (b) esr spectrum of 1:1 molar ratio CN^-:GOase in 0.20 *M* galactose (GOase was 3.0×10^{-5} *M*).

substantiates the hypothesis that only one labile site at Cu^{2+} in GOase exists and that the most stable complexes are formed with ligands that have π-bonding capabilities. In general, though, the N_3^-, Br^-, and SCN^- ligands are like H_2O in their coordinating ability and are near H_2O in the spectrochemical series. If the coordination site(s) which is labile contains a ligand like H_2O which is displaced in the enzymatic reaction, then the small changes in the esr spectra induced by this class of ligands are in order. That is, changing Br^- for H_2O would not be expected to dramatically change the electronic effect at Cu^{2+}.

A similar argument has been made for the insensitivity of the GOase spectrum to the addition of galactose.[7] However, we have established here by competition experiments with CN^- that galactose probably binds directly to the copper site, exchanging with CN^-. This result correlates with the observation that galactose dramatically decreases the copper optical activity exhibited by GOase.[6]

The binding of H_2O_2 to the Cu^{2+} indicated by the esr spectral changes is also corroborated by an analogous circular dichroic experiment[6] and is further evidence that *this* product of the enzymatic reaction does compete for the galactose site as deduced from kinetic inhibition studies.[9] The H_2O_2 effect on the esr spectrum was too small to be useful as an indicator of galactose binding in a competition experiment as carried out with CN^-. The fact that its effect is small places H_2O_2 in the weak field ligand class, at least at a pH of 7.0.

Although the displaced ligand in each case is presumed to be a water molecule, the pH dependence of the esr spectrum of added ligands needs to be determined. The displaced ligand might as easily be a OH^- ion.

N,*N*′-Ethylenebis(trifluoroacetylacetoniminato)copper(II) has been proposed as a model for the copper coordination in GOase.[2] Of the six anisotropic spin Hamiltonian parameters, only A_{zz} and g_{zz} differ significantly between the two systems; A_{zz} was about 20 G lower for GOase. Significantly, it is the A_{zz} parameter which is most changed when exogeneous ligands coordinate to the copper site in GOase. In particular,

axial coordination by a strong π-bonding ligand (CN^-) lowers A_{zz} by approximately 20 G (Table I). Thus, the one *nonlabile* axial coordination site in GOase could be occupied and stabilized by a protein ligand capable of π bonding. This coordination would, therefore, account for the 20 G difference from GOase in A_{zz} in the model system and for the single coordination site available to exogeneous ligands in GOase.[2] Since the 20 G difference in the A_{zz} values for enzyme and model can be rationalized wholly on the basis of an extra axial π-bonding ligand in GOase, the model does appear to be an excellent one for the in-plane ligands to the copper in GOase.

The inference that the nonlabile endogeneous axial ligand to the copper in GOase has π-bonding character suggests that a sulfhydryl group may be the ligand involved. While one does not normally think of possible protein ligands, –S, –O, and –N, as capable of forming strong π bonds, a π-bond effect has been demonstrated for sulfur ligands.[10,11] Moreover, the presence of a cysteine sulfur in the axial position in GOase is a definite possibility. There is one free sulfhydryl group in GOase which is titrable only in the apoenzyme after it is denaturated.[3,4,8] Therefore, not only is a free sulfhydryl group present in GOase, it and its coordination position are also quite inaccessible in the native protein. The competition by galactose for CN^- binding, the availability of only one axial site, and the lack of effects of oxygen on the esr spectrum substantiate some inferences made from CD spectra about the binding of substrates to GOase. Namely, galactose must bind prior to oxygen in the enzymatic reaction scheme. Furthermore, the esr results are consistant with outer sphere coordination of the oxygen to the reducing equivalent on the sugar aldiol rather than direct coordination to the copper.

These considerations are in agreement with the observation that no copper oxidation change occurs during the enzymatic reaction. The binding of the sugar aldiol by copper produces a strongly polarized bond such that a weak base like oxygen may carry out the oxidation without an oxidation state change for copper. A mechanism for such a reaction in organic chemistry has been discussed by Corey.[12] A similar polarization–activation–oxidation by outer sphere oxygen seems reasonable here.

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Biphenylene. Internuclear Distances and Their Root Mean Square Amplitudes of Vibration

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Abstract: The molecular structure of biphenylene in the gas phase was investigated by electron diffraction; the sample was maintained at 145°. Resolution of the radial distribution curve indicated that these molecules have a planar conformation. On the basis of the assumed D_{2h} symmetry and the relative bond lengths derived from the X-ray crystal structure, a least-squares analysis of the molecular scattering function gave the following r_g values for the interatomic distances: $(C–C)_{av}$ = 1.418 ± 0.003 Å, $C_1–C_{8b}$ = 1.372 ± 0.012 Å, $C_1–C_2$ = 1.428 ± 0.012 Å, $C_2–C_3$ = 1.370 ± 0.015 Å, $C_{4a}–C_{4b}$ = 1.524 ± 0.006 Å, $C_{4a}–C_{8b}$ = 1.432 ± 0.018 Å, C–H = 1.096 ± 0.009 Å, $\angle C_{8b}–C_1–C_2$ = 115.0 ± 1.2°, $\angle C_1–C_2–C_3$ = 122.5 ± 1.2°, $\angle C_{4a}–C_{8b}–C_1$ = 122.5 ± 0.6°. In the above list the angles were defined in the r_α representation and shrinkage corrections were inserted in the least-squares program. The listed uncertainties represent estimated limits of error. The structural parameters are in good agreement with corresponding values derived from a crystal structure analysis. The benzene rings are distorted, and "bond fixation" is clearly present. The $C_{4a}–C_{4b}$ distances are almost as long as in a normal single bond while the $C_{8b}–C_1$ separations are definitely shorter than the C–C separation in benzene. Another characteristic feature of the structure is the relatively small angle, $\angle C_{8b}–C_1–C_2$. Comparison between the observed and calculated mean square amplitudes suggests that this molecule may be "quasiplanar" due to the large amplitudes of the out-of-plane vibrational modes.

Biphenylene provided the first unambiguous example (Lothrop, 1941) of a molecule containing a formal cyclobutadiene ring and has been the subject of intensive chemical, physical, and theoretical study ever since.[1] According to almost all theoretical models the parent hydrocarbon, cyclobutadiene, should exhibit

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