An Investigation of the Role of the Copper in Galactose Oxidase†

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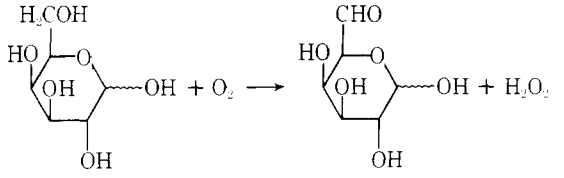
ABSTRACT: Galactose oxidase is a metalloenzyme containing a single copper atom per molecule. The mechanism of action of galactose oxidase is studied in this paper by investigating substrate specificity and activation by peroxidase, and probing the copper site by electron spin resonance (ESR) spectroscopy. Line-shape simulation of ESR spectra are also reported and a comparison is made between observed and simulated spectra for galactose oxidase. A comparison is also reported for the enzyme from various commercial sources and enzyme isolated from a fungus in this laboratory. The results of this investigation suggest that the copper is in an environment of four in-plane nitrogens with axial symmetry.

Galactose oxidase was first isolated from the fungus *Polyporus circinatus* (Cooper et al., 1959). Since then, it has been found to be a metalloprotein having 42,000 g/mol (Kelly-Falcoz et al., 1965; Yip and Dain, 1968) and one copper atom per molecule (Amaral et al., 1963), which catalyzes the reaction of galactose and galactosides with oxygen (Amaral et al., 1966):



The oxidation of the primary alcohol function of C-6 of galactose proceeds stereospecifically with removal of the (*pro-S*)-hydrogen (Maradufu et al., 1971).

The enzyme is relatively nonspecific. Although modification of galactose at position 4 to produce glucose or 4-*O*-methylgalactose abolishes activity, modification of positions 1, 2, or 3 does not (Schlegel et al., 1968). In addition, dihydroxyacetone has been shown to be a substrate (Zancan and Amaral, 1970).

Michaelis–Menten kinetics have been observed for the oxidation of galactose by galactose oxidase, but K_m values ranging from 0.24 to 0.00278 *M* have been reported (Avigad et al., 1962; Guilbault et al., 1968). The Michaelis constant for oxygen has been reported to be 0.6 m*M* (Hamilton et al., 1974), and both sequential and ping-pong kinetic schemes have been proposed (Hamilton et al., 1974; Ettinger and Kosman, 1974). Increased rates have been found in the presence of peroxidase (Kwiatkowski and Kosman, 1973).

The copper of galactose oxidase is largely in the 2+ oxidation state, as shown by electron spin resonance (ESR) spectra (Blumberg et al., 1965), but its ligands are unknown, although titration data indicate that one ligand is a sulfhydryl group (Kelly-Falcoz et al., 1965).

On the basis of inhibition by superoxide dismutase and activation by ferricyanide ion, the reaction has been proposed to proceed by reversible oxidation and reduction of the copper and generation of superoxide anion (O_2^-) (Hamilton et al., 1973); however, there is no indication in ESR experiments that the copper changes valence during the catalytic cycle (Kosman et al., 1973). Superoxide anion could not be detected in the galactose–galactose oxidase–oxygen system, but this does not prove it is not produced, as galactose oxidase was also shown to function as a superoxide dismutase (Cleveland and Davis, 1974), however, at only 0.1% the rate of bovine erythrocyte superoxide dismutase.

The mechanism of action of galactose oxidase was investigated further in three general ways: (1) the specificity of the enzyme was investigated by testing various potential substrates for activity; (2) the activation of the enzyme by peroxidase was investigated; and (3) the environment of the copper site was probed by ESR experiments. From such data, a picture of the copper site can be developed, and evidence pertaining to possible mechanisms can be obtained.

Materials and Methods

Enzymes. Galactose oxidase was prepared from a fungus provided by Dr. D. J. Kosman of the State University of New York Buffalo, which he obtained from the ARS Culture Collection of Peoria, Ill., where it is listed as *Polyporus circinatus* (NRRL-2903). No enzyme is produced by American Test Culture Collection (ATCC 9383) *Polyporus circirnatus*.

Galactose oxidase was also purchased from Miles Laboratories and from Worthington Biochemical Corporation. The Miles enzyme was obtained by them from a laboratory in Sweden and the fungal source could not be established. The Worthington enzyme is isolated from a fungus which they obtained some years ago and it appears to be nearly identical with ours. The white solid obtained from Miles, which produced one band in both disc gel and sodium dodecyl sulfate gel electrophoresis, was used without further purification; the brown powder obtained from Worthington was purified by chromatography on DEAE-Sephadex, as described by Amaral et al. (1966). The fungal enzyme was

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prepared by the method of D. J. Kosman (private communication).

Catalase, type II peroxidase, and type VI peroxidase were obtained from Sigma Chemical Corporation and used without further purification.

Other Chemicals. α-D-Galactose was obtained from Fisher Scientific Corp. α-Methyl D-galactopyranoside, β-methyl D-galactopyranoside, and α-melibiose monohydrate were purchased from Pfanstiehl Laboratories. Dihydroxyacetone and lithium hydroxypyruvate were obtained from Sigma, glycolaldehyde and 1-hydroxyacetone from Aldrich, 1,2-dihydroxypropane and 1,3-dihydroxypropane from Matheson, and 2-hydroxyacetophenone from Eastman Organic Chemicals.

2-Methylene-1,3-propanediol was prepared by the procedure of Mooradian and Cloke (1945), by Mr. Thomas Kalhorn. β-Galactose was prepared from α-galactose by the procedure of Hudson and Yanovsky (1917).

Assay Methods. Galactose oxidase was assayed by measuring oxygen uptake at 31.5° in a 3.0-ml mixture containing 0.033 *M* α-methyl galactopyranoside and 0.067 *M* phosphate buffer (pH 7.10), after addition of 10–100 μl of galactose oxidase. One unit of galactose oxidase is defined as the amount required to catalyze uptake of 1 μmol of oxygen/hr in this assay. Protein concentrations were determined by the Folin–Ciocalteu method (Lowry et al., 1951). Disc gel electrophoresis was performed as described by Davis (1964).

Instrumental Methods. Visible and ultraviolet absorption measurements were made on a Durrum PGS recording spectrophotometer. Oxygen consumption was measured on a Model 53 Biological Oxygen Monitor from Yellow Springs Instrument Co. Electron spin resonance experiments were performed on a Varian 9-GHz instrument, Model V4560, with a 100-kHz field modulation unit.

Line-shape simulations of ESR spectra were performed using three programs specifically written for the analysis of spin ½ powder or glass systems. An approximation to the absorption derivative was calculated by numerical evaluation and plotting of the quantity:

$$S'(H) = \sum_i \int P_i(\Omega) G'[H - H_i(\nu, \Omega)] d\Omega \qquad (1)$$

The terms appearing in this equation are defined as follows: i = an index for the number of allowed transitions with relative intensity $P_i(\Omega)$ and resonance field value $H_i(\nu,\Omega)$ for microwave frequency ν (gigahertz) and magnetic field direction is given by $\Omega \equiv (\theta,\phi)$. The element of solid angle $d\Omega$ was taken to be $\sin\theta\, d\theta d\phi$, and $G[H - H_i(\nu,\Omega)]$ was chosen to be a Gaussian-type line-shape function, assumed to have the same form and line width for each transition and value of Ω. The quantity actually used in the evaluation of eq 1 was the Gaussian derivative line-shape function:

$$G'[H - H_i] = -\frac{(H - H_i)}{\sqrt{2\pi}\Delta^3} \exp\left\{-\frac{(H - H_i)^2}{2\Delta^2}\right\} \qquad (2)$$

Here, Δ = the peak to peak derivative half-width, defined as one-half of the line width between derivative extrema (Petrakis, 1967).

The resonance field values $H_i(\nu,\Omega)$ were calculated explicitly in a rotated quantization basis, and were usually used without higher (second) order corrections beyond those terms leading to angular dependence of the effective g values and hyperfine splittings. The general spin hamiltonian from which the $H_i(\nu,\Omega)$ were calculated has the form:

$$\mathcal{H} = \beta \vec{H} \cdot \mathbf{g} \cdot \vec{S} + \vec{S} \cdot \mathbf{A} \cdot \vec{I} + \sum_{j=i}^{n_{lig}} A_N \vec{S} \cdot I_N{}^j \qquad (3)$$

Here, **g** = the g tensor, **A** = the copper hyperfine (HFS) tensor (assumed to be collinear with **g**), and A_N = the isotropic ligand hyperfine splitting (SHFS) coefficient, assumed to have the same value for n_{lig} equivalent nitrogen atoms. The appropriate statistical weights for the superposition of resonance lines at identical field values due to n_{lig} equivalent nitrogens were included in the program for SHFS, so that the number of equivalent, isotropic nitrogen atoms was an optional variable, which could assume integral values between 0 and 6. The second-order perturbation theory expressions for the resonance field values were essentially those of Abragam and Bleaney (1970). Correction terms for the nitrogen SHFS were the same as found in the papers by Abkowitz et al. (1968), Chen et al. (1969), and Hsu (1971). Empirical allowance for line-width anisotropy was included as an option, assuming that the principal source of line-width variation vs. angle was due to unresolved HFS (Pilbrow, 1973; Venable, 1967). Intensity corrections of the allowed transitions, due to g-tensor anisotropy, were programmed using the correction terms for $P_i(\Omega)$ as given by Pilbrow (1969). No allowance was made, in these simulations, for the existence of two isotopes of copper, ^{63}Cu and ^{65}Cu, having natural abundances of 69 and 31%, respectively. The experimental data were digitized, where necessary, and a best fit to the theoretical line-shape function sought by a least-squares technique.

Computer analysis of kinetic data was performed by a least-squares fit of experimental data to the hyperbolic Michaelis–Menten equation.

Results

Galactose oxidase from different sources differed in activity. Maximum specific activity for the Worthington preparation was about 2000 units/mg, similar to the 1600 units/mg reported by Kosman using a different assay system (Kosman et al., 1975), but much larger than the 300 units/mg obtained for galactose oxidase from Miles Laboratories which was homogeneous by disc gel electrophoresis.

Solutions of Miles galactose oxidase up to 15 mg/ml have no visible color, and therefore a molar absorptivity of less than 300/(*M* cm)[1] near 600 nm, but the molar absorptivity of the Worthington enzyme is probably higher, perhaps even as high as the 1000/(*M* cm) reported by Ettinger (1974).

Substrate Specificity. Since galactose in solution exists as an equilibrium mixture of both α and β anomers, β-galactose was prepared from the commercially available α form (Wolfrom et al., 1954), and both forms were tested as substrates by addition of the solid to start the reaction. An initial rate was obtained in each case which was consistent with both forms being substrates.

If both forms are substrates, they should be competitive inhibitors of each other. This proposition was tested using α- and β-methyl D-galactopyranosides, for which mutarotation is not possible, as substrates. The results presented in Table I suggest that the two forms of methyl galactopyranoside do indeed act as competitive inhibitors of one another. This presumably applies also to galactose itself, and be-

[1] This value was calculated assuming an absorbance of 0.1 at a concentration of 15 mg/ml.

Table I: Oxidation of α- and β-Methyl Galactosides.

Substrate	K_m (M)	V_m (μmol of O_2/min)
α-Methyl D-galacto-pyranoside	0.0263 ± 0.0045	0.463 ± 0.028
β-Methyl D-galacto-pyranoside	0.0467 ± 0.0042	0.901 ± 0.032
50:50 mixture of α,β-galactopyranosides		
Explt	0.0299 ± 0.0017	0.8130 ± 0.017
Calcd		0.628

cause rates obtained using galactose as substrate are therefore complicated by the presence of two active substrates in solution, α-methyl galactopyranoside was chosen as the standard substrate for determination of specific activity.

Since dihydroxyacetone is a substrate (Zancan and Amaral, 1970), other similar compounds were tested for activity; lithium hydroxypyruvate, 1-hydroxyacetone, dihydroxyacetone monomer, glycolaldehyde, 2-methylene-1,3-propanediol, and hydroxyacetophenone as well as 1,2-dihydroxypropane and dihydroxyacetone dimer were found to be active. Also, reported activities for glycerol, 1,3-dihydroxypropane (Hamilton et al., 1974), melibiose (Schlegel et al., 1968), and 3,4-dimethoxybenzyl alcohol (Kwiatkowski and Kosman, 1973) were confirmed. DL-Dihydroxyphenylalanine, tris(hydroxymethyl)aminomethane, and 1-propanol were inactive. Michaelis constants for some of these substrates are presented in Table II.

Peroxidase Effect. The rate increases observed in the presence of peroxidase during manometric measurement of oxygen uptake (Kwiatkowski and Kosman, 1973) were confirmed during oxygen monitor assay. Using Miles galactose oxidase, a twofold increase in the rate of oxygen uptake was obtained when galactose was the substrate, as shown in Figure 1. This increase was observed only when the two enzymes were mixed before starting the reaction when dihydroxyacetone was the substrate. In addition, the rate increase was independent of the concentration of peroxidase over a range of peroxidase concentration from 0.013 to 1.3 times that of galactose oxidase, and was not observed when peroxidase was replaced by other iron-heme proteins like catalase or ferricytochrome *c*.

The effects of peroxidase and catalase on the Michaelis constant of melibiose were tested; the results, presented in

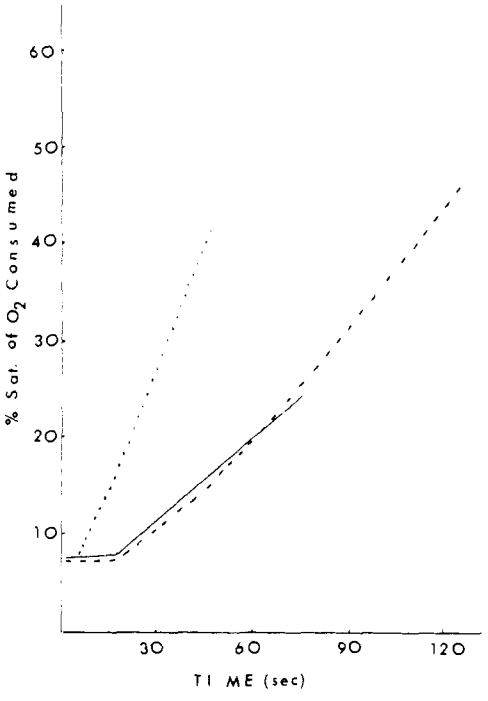

FIGURE 1: Oxidation of galactose in the presence of peroxidase. Reaction mixtures contain 0.0033 M galactose–0.067 M phosphate buffer (pH 7.10), and 6×10^{-8} M Miles galactose oxidase, in a total volume of 3.0 ml: (—) reaction mixture contains no additional components; (- - -) reaction mixture contains 9×10^{-9} M peroxidase added before galactose oxidase; (• • •) reaction mixture contains 9×10^{-9} M peroxidase premixed with galactose oxidase.

Table III, indicate that while catalase does not affect the Michaelis constant of melibiose, peroxidase, with or without the chromagen, decreases the K_m by a factor of almost three.

A further indication of an interaction of some type between galactose oxidase and peroxidase is seen in the shift of the visible absorption spectrum of peroxidase from an absorbance maximum at 405 nm to a maximum at 422 nm in the presence of excess galactose oxidase; simultaneously, maxima are produced at 525 and 560 nm. Since the shift of absorption spectrum does not occur in a nitrogen atmosphere, the product of the interaction is probably the peroxidase–peroxide compound II, which has absorbance maxima at 418, 527, and 557 nm (Paul, 1963). On addition of substrate, either galactose or dihydroxyacetone, the spec-

Table II: Michaelis Constants of Various Substrates.[a]

	Oxygen Monitor Assay			Coupled Assay		
Substrate	K_m (M) (% Error)	V_m/Unit [μmol of O_2/(min Unit)]	Rel Act.[b]	K_m (M) (% Error)	V_m (ΔA/min)	Rel Act.[c]
Galactose	0.008 ± 0.003 (37)		(85)[d]	0.0295 ± 0.0059 (20)	1.99 ± 0.32	100
α-Methyl galactoside	0.0263 ± 0.0045 (17)	0.0298	100			118[d]
β-Methyl galactoside	0.0467 ± 0.0042 (8.9)	0.058	193			
Dihydroxyacetone monomer	0.0121 ± 0.0007 (5.8)			0.110 ± 0.034 (34)	10.8 ± 2.0	540
α-Melibiose	0.0385 ± 0.006 (15)					
	0.056 ± 0.014 (25)	0.0413	138			
2-Methylene-1,3-propanediol	0.169 ± 0.015 (9)	0.033	110			
Lithium hydroxypyruvate	0.0793 ± 0.0027 (3)	0.020	67	0.0218 ± 0.0022 (10)	0.0816 ± 0.004	41

[a] All values determined in the presence of phosphate buffer. [b] Calculated as V_m(substrate)/V_m(α-methyl galactoside) × 100. [c] Calculated as V_m(substrate)/V_m(galactose) × 100. [d] Relative activity of 118 for α-methyl galactoside was used to calculate a relative activity for galactose.

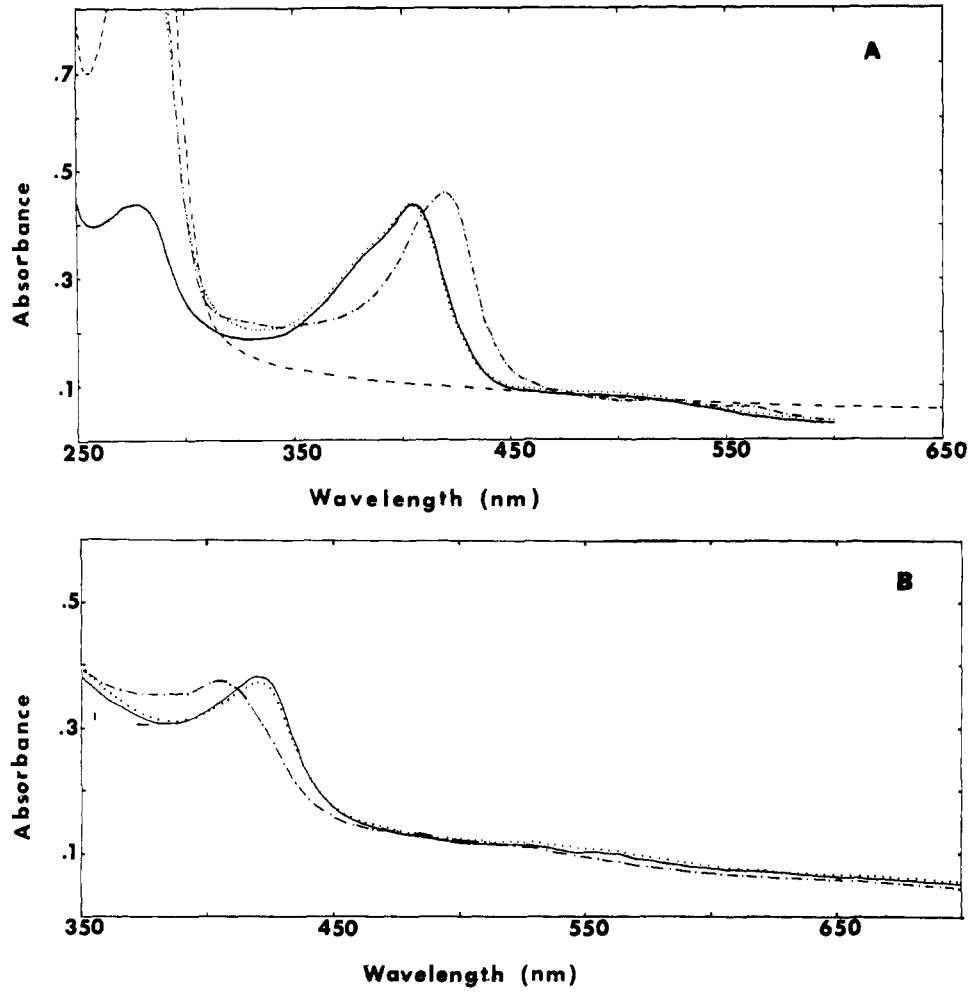

FIGURE 2: (A) Visible spectra of peroxidase–galactose oxidase mixtures: (- - -) 2×10^{-5} *M* Worthington galactose oxidase; (—) 4×10^{-6} *M* peroxidase in a nitrogen atmosphere; (• • •) mixture containing 4×10^{-6} *M* peroxidase and 1×10^{-5} *M* Miles galactose oxidase in a 3.0-ml volume, under a nitrogen atmosphere; (- • -) mixture contains 5×10^{-6} *M* peroxidase and 6×10^{-6} *M* galactose oxidase (Miles) in the presence of air. (B) Spectral changes observed upon the addition of galactose to mixtures of galactose oxidase and peroxidase: (—) 5.0×10^{-6} *M* peroxidase and 1.1×10^{-5} *M* galactose oxidase in 3.0 ml of pH 7.1 phosphate: (• • •) immediately upon the addition of 3.3×10^{-3} *M* of galactose; (- • -) 30 min later.

Table III: Effect of Peroxidase on Oxidation of Melibiose.

Added Compound	Michaelis Constant for Melibiose (*M*)
None	0.0385 ± 0.006
Peroxidase	0.0113 ± 0.0017
Peroxidase and *o*-toluidine	0.0109 ± 0.0006
Catalase	0.0322 ± 0.0008

trum gradually returns to a maximum at 405 nm. These results are shown in Figures 2A and B.

Again, galactose oxidases from Worthington and Miles are not identical. The results presented above apply to the Miles enzyme; the Worthington enzyme gave its maximum rate increase of 1.4-fold only when peroxidase was mixed with galactose oxidase before starting the reaction, and did not show a shift of absorbance maximum to 422 nm even after 18-hr incubation of excess galactose oxidase with peroxidase.

ESR Experiments. The experimental ESR spectrum for Miles galactose oxidase in solution is presented in Figure 3; also presented is a computer-fitted spectrum calculated assuming axial symmetry, four nitrogen ligands around the copper, and the following parameter values: $g_{\parallel} = 2.299$, $g_{\perp} = 2.0731$, $A_{\parallel} = 0.0175$ cm^{-1}, $A_{\perp} = 0.0005$ cm^{-1}, $\Delta H_{\perp} =$ 7.7 G, $\Delta H_{\parallel} = 7.3$ G, and $A_N = 0.00141$ cm^{-1} at 9.262 GHz. The splitting pattern of this spectrum is slightly different from that reported by Kosman et al. (1973) and Hamilton et al. (1973) for Worthington galactose oxidase. In agreement with the results of Kosman et al. (1973) though, the experimental spectrum did not change on removal of oxygen.

At a 30:1 excess of galactose oxidase, the presence of peroxidase eliminates the superhyperfine structure in the parallel region of the ESR spectrum, as shown in Figure 4. Replacement of the nitrogen ligands is unlikely at such low concentrations of peroxidase; therefore, peroxidase must interact to increase the line width or change the nitrogen superhyperfine splitting constant.

On the other hand, excess KCN appears to be capable of removing a nitrogen ligand, as shown by the changes in the superhyperfine splitting pattern given in Figure 5.

Discussion

As shown in Table IV, differences in specific activities, behavior during disc gel electrophoresis, visible absorption molar absorptivities, peroxidase activation, and ESR spectra suggest that the enzymes from Miles Laboratories and Worthington Biochemicals are not identical. In view of the confusion over the identity of the fungus commonly used to

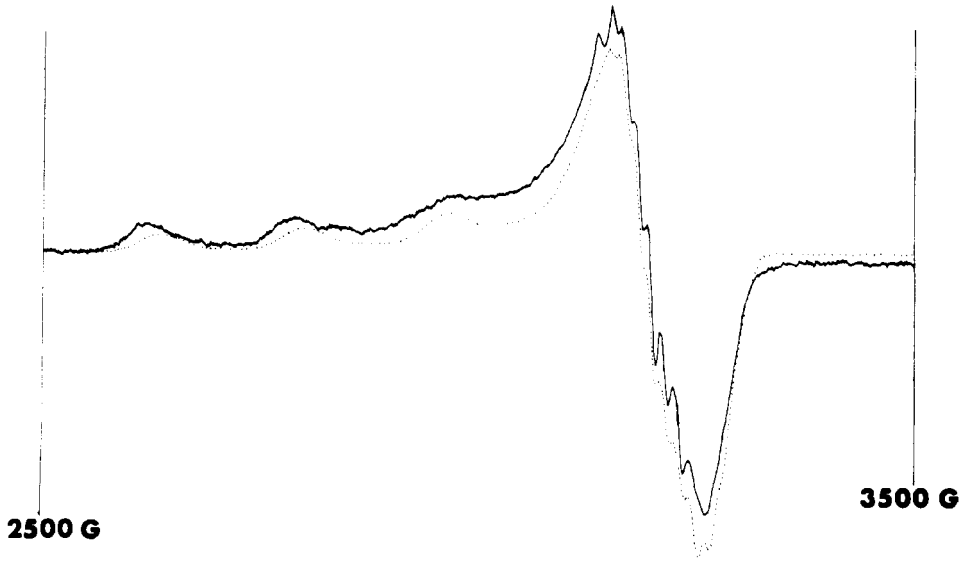

FIGURE 3: Computer fitting of ESR spectrum of galactose oxidase: (—) experimental galactose oxidase spectrum (Miles, 16.7 mg/ml) at 9.262 GHz, 51 mW, and 2.5 G modulation amplitude; (· · ·) calculated galactose oxidase spectrum, using these parameters: $g_{\parallel} = 2.299$, $g_{\perp} = 2.0731$, $A_{\parallel} = 0.0175\ cm^{-1}$, $A_{\perp} = 0.0005\ cm^{-1}$, $\Delta H_{\parallel} = 7.3$ G, $\Delta H_{\perp} = 7.7$ G, and $A_N = 0.001408\ cm^{-1}$, at 9.262 GHz. Splitting by one copper and four nitrogens was included.

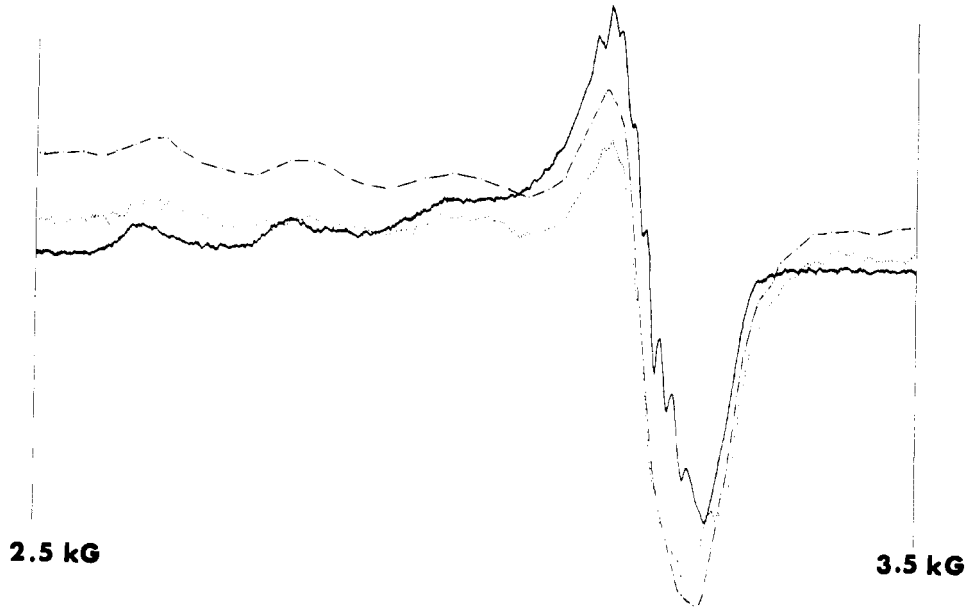

FIGURE 4: ESR spectra of peroxidase-galactose oxidase mixtures: (—) galactose oxidase solution (Miles, 16.7 mg/ml) at 9.262 GHz, 51 mW, and 2.5 G modulation amplitude; (· · ·) mixture contaning 4×10^{-4} *M* galactose oxidase and 1×10^{-5} *M* peroxidase, at 9.262 GHz, 55 mW, and 3.15 G modulation amplitude, after 24 hr incubation; (· · ·) mixture containing 4×10^{-4} *M* galactose oxidase and 4×10^{-5} *M* peroxidase, at 9.265 GHz, 57 mV, and 3.15 G modulation amplitude, immediately after mixing.

extract the enzyme[2] and the report that galactose oxidase is produced in the culture media of several fungi (Nobles and Madhosingh, 1963; Gancedo et al., 1967), it is quite possible that the two commercial preparations come from different fungi, in which case the differences would not be unexpected. It is interesting to note that, based on specific activities, the Worthington preparation is more effective than the Miles enzyme as a galactose oxidase, while Miles enzyme is ten times more effective as a superoxide dismutase; still, each enzyme does have both activities.

The only absolute requirement for activity of a potential substrate appears to be the presence of a primary alcohol group, but the adjacent positions must have certain structural features. The 4 position of galactose and the corresponding 3 position of dihydroxyacetone appear to be critical. Glucose, 4-*O*-methylgalactose, 4-deoxygalactose, and dihydroxyacetone phosphate are not substrates, but 4-fluorogalactose and 4-aminogalactose are substrates (Maradufu and Perlin, 1974), suggesting that the group at the 4 position might be required to accept a hydrogen bond from the enzyme.

Substrates for galactose oxidase therefore appear to have a primary alcohol function adjacent to an "activating" group (C—O, C=O, or C=C) with the next position (the 4 position of galactose or the 3 position of dihydroxyacetone)

[2] The original source of the enzyme was stated to be *Polyporus circinatus*, but later reports indicated it was actually *Dactylium dendroides*. However, Amaral et al. (1966) were unable to detect any enzyme from authenic *Dactylium*.

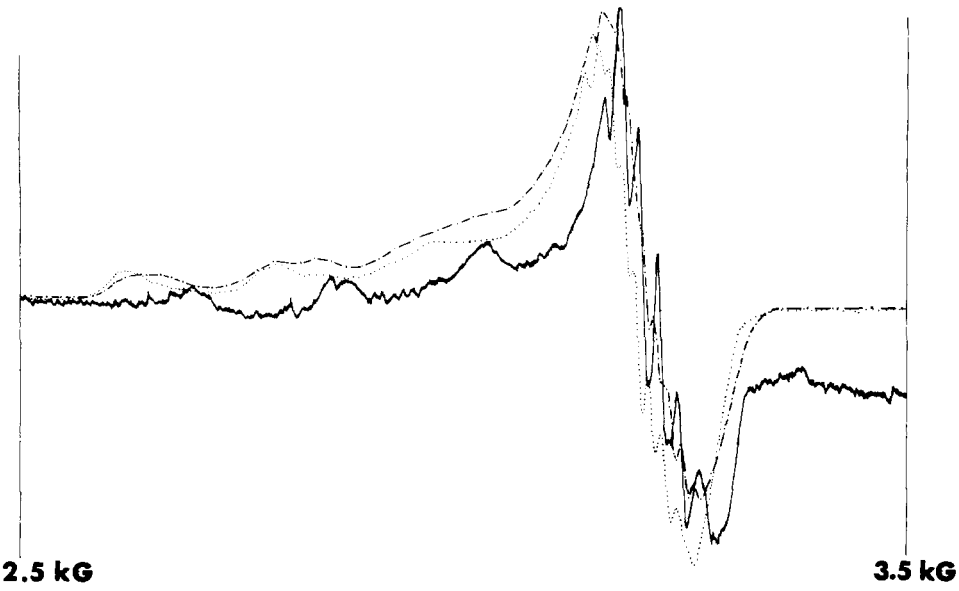

FIGURE 5: ESR spectrum of galactose oxidase in the presence of inhibitors: (···) galactose oxidase solution (Miles, 16.7 mg/ml) at 9.262 GHz, 51 mW, and 2.5 G modulation amplitude;(—) mixture containing 4×10^{-4} *M* galactose oxidase and 7×10^{-3} *M* KCN, at 9.266 GHz, 84 mW, and 3.15 G modulation amplitude; (- · -) mixture containing 6×10^{-4} *M* galactose oxidase and 4×10^{-3} *M* NaN_3, at 9.263 GHz, 50 mW, and 7.6 G modulation amplitude.

Table IV: Comparison of Galactose Oxidases from Different Sources.

Enzyme Source	λ_{max} (ϵ)	Sp Act.	Disc Gel Behavior	Peroxidase Effect		Dismutase Act.[a] (*M*)	ESR Spectrum
				Enhancement	A_{420}		
Miles	(<300)[b]	300 (O_2)	1 band	2.1	Yes	4×10^{-7}	Axial, 4 N
Worthington	n.d.	2000 (C) 2000 (O_2)	1 or 2 bands	1.4	No	1×10^{-5}	
Fungus	630 (1085)[c]	1900 (C)	2 bands				Probably axial, different from Miles

[a] Concentration of galactose oxidase required to produce 50% inhibition of cytochrome *c* assay; Cleveland and Davis (1974). [b] Calculated assuming maximum absorbance around 600 nm to be 0.1 at a concentration of 15 mg/ml, at which concentration no visible color is seen. [c] Value from Kelly-Falcoz et al. (1965).

preferentially occupied by a group capable of being cis to the primary alcohol function and able to accept a hydrogen bond. The low activities of substrates like glycolaldehyde, 1-hydroxyacetone, and 4-deoxygalactose may be a consequence of the inability to bind properly at this position.

The ESR spectrum of the fungal galactose oxidase has been interpreted by Kosman in terms of rhombic symmetry, with hyperfine splitting by copper giving rise to the fine structure; as shown in Figure 6A, a spectrum calculated using Kosman's parameters does not fit his experimental spectrum. A much better fit of his spectrum can be obtained by assuming axial symmetry and splitting by four nitrogen ligands, as shown in Figure 6B. Kosman's spectrum of fungal galactose oxidase thus appears to differ from that of the Miles enzyme largely in the value for A_N, the hyperfine splitting constant for nitrogen.

The ESR spectrum of Miles galactose oxidase, having an $A_{\parallel}$ of 0.0175 cm^{-1}, is consistent with its molar absorptivity of less than 300/(*M* cm) and allows classification of galactose oxidase as a "non-blue" protein (Malkin and Malmstrom, 1970); generally, this implies an ESR spectrum and molar absorptivity similar to those of copper–amino acid complexes having a relatively high symmetry. The ESR spectrum reported by Kosman for the fungal galactose oxidase is also consistent with "non-blue" copper site, but its reported molar absorptivity of about 1000 is inconsistently large. The spectrum of Miles galactose oxidase was simulated using one, two, three, and four nitrogen ligands to obtain the best fit in the perpendicular, parallel, and superhyperfine patterns.

The best fit of calculated to experimental ESR spectrum of Miles galactose oxidase, Figure 3, suggested that the copper site has four nitrogen ligands. The titration data of Kelly-Falcoz et al. (1965) would suggest that the fifth ligand is probably sulfur, and the sixth could then be oxygen, either from O_2 or H_2O. Neither S nor O_2 has a nuclear spin so neither should show ESR splitting. The sources of the nitrogen ligands would probably be histidine or lysine, both of which have been detected in galactose oxidase (Kelly-Falcoz et al., 1965); these are probably indistinguishable in the ESR. The site resembles that of bovine erythrocyte SOD, which has recently been shown by X-ray crystallography to have four nitrogen ligands to the copper (Fridovich, personal communication); the ESR splitting pattern for SOD at pH 11 is practically identical with that of galactose oxidase (Simonyan and Nalbandyan, 1972).

The loss of superhyperfine lines in the perpendicular region of galactose oxidase upon the addition of cyanide from 9 to 7 would suggest that cyanide ion displaces a nitrogen ligand; at the threefold excess used, it could well displace

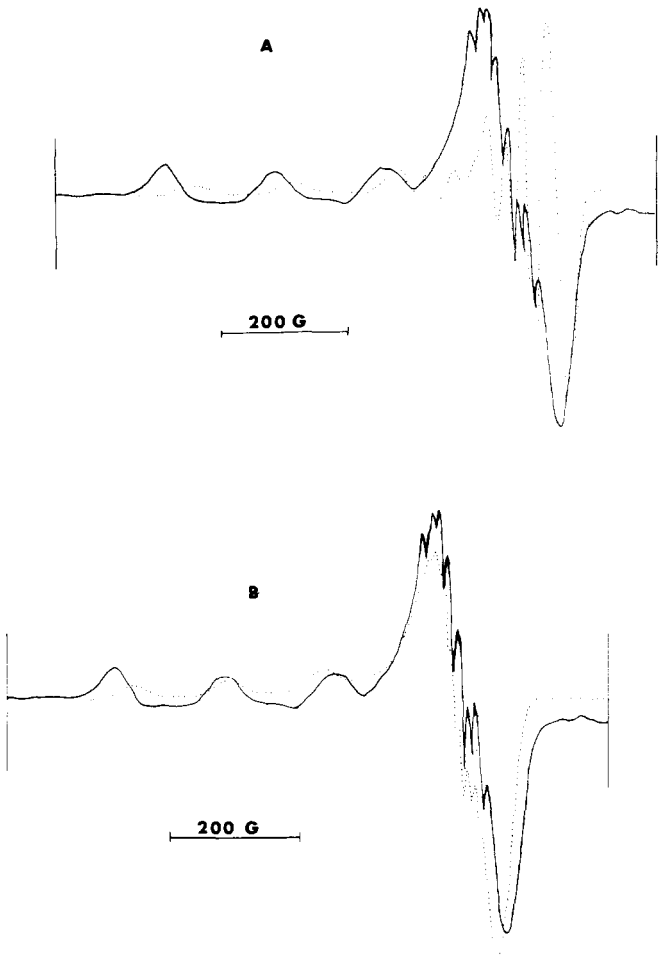

FIGURE 6: (A) Rhombic simulation of Kosman's ESR spectrum of galactose oxidase: (—) Kosman's experimental spectrum of galactose oxidase, as previously published (Kosman et al., 1973); (···) computer-simulated rhombic spectrum, using Kosman's parameters: $g_z = 2.273$, $g_x = 2.058$, $g_y = 2.04J$, $A_z = 0.01872$ cm^{-1}, $A_x = 0.00278$ cm^{-1}, $A_y = 0.00288$ cm^{-1}, $\Delta H_z = 25$ G, $\Delta H_x = \Delta H_y = 7$ G, at 9.084 GHz. (B) Axial simulation of Kosman's ESR spectrum of galactose oxidase: (—) Kosman's experimental spectrum of galactose oxidase, as previously published (Kowman et al., 1973); (···) computer-simulated axial spectrum, using these parameters: $g_\parallel = 2.302$, $g_\perp = 2.070$, $A_\parallel = 0.0175$ cm^{-1}, $A_\perp = 0.0012$ cm^{-1}, $\Delta H_\parallel = 10$ G, $\Delta H_\perp = 7$ G, and $A_N = 0.00125$ cm^{-1}, at 9.084 GHz. Splitting by four nitrogens was assumed.

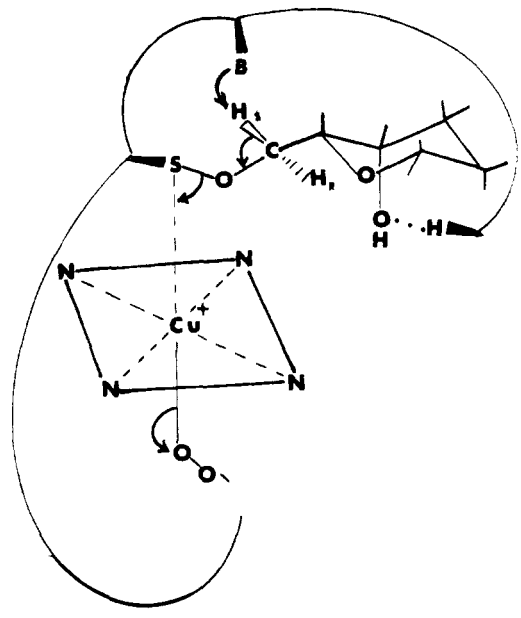

FIGURE 7: Binding of galactose to active site of galactose oxidase.

the fifth and sixth ligands first. However, Giordano et al. (1974) find that the ESR change is complete at a 1:1 mole ratio of KCN to enzyme; they interpret this as indicative of only one site for binding cyanide ion. In either case, the disruption of the copper site is the probable source of cyanide inhibition.

Based on increased rates of galactose oxidase reactions in the presence of ferricyanide and inhibition by superoxide dismutase, Hamilton et al. (1974) have proposed a mechanism in which a Cu(III) form of galactose oxidase is the actively catalyzing species. The resting state is assumed to be a mixture of active Cu(III) and inactive Cu(II) forms, since catalysis could not occur without an oxidizing agent if all the enzyme is in the Cu(II) form.

The observed diminution of the ESR signal on addition of ferricyanide to galactose oxidase, which has been interpreted as supporting the Cu(III) mechanism, could also be a consequence of formation of a spin-paired complex of galactose oxidase with ferricyanide, especially since ferricyanide is in large excess. However, this would not explain the observed increase in rate in the presence of ferricyanide.

Assuming the active form to be Cu(II) which does not change during the catalysis, the enzyme can be resolved of its copper and reconstituted to approximately full activity by Cu(II). A mechanism which would also fit the observed data is one in which a thioperoxide is formed as shown in Figure 7. Thioperoxide formation in the oxidation of alcohols to aldehydes is supported by the model system of Corey and Kim (1972) for the sulfonium ion oxidation of alcohols to aldehydes and Bennett's (private communication) studies of Co(II) phthalocyanine–cysteine complexes which bind oxygen and oxidize cysteine to cystine.

The observed inhibition of galactose oxidase by mercaptoethanol would be consistent with reaction of either the sulfhydryl group or the alcohol group of mercaptoethanol with an active sulfenyl ion to produce a dead-end complex.

ESR evidence indicates that peroxidase in much less than a 1:1 ratio abolished the superhyperfine lines, presumably by broadening the line width or changing the splitting constant. Coupled with the observation that the rate increases are independent of the concentration of peroxidase over a wide range, this suggests that peroxidase acts catalytically. The fact that increased rates are obtained for all substrates only if the two enzymes are premixed suggests that some interaction of peroxidase, galactose oxidase, and perhaps oxygen is responsible, and the visible spectra changes show that under some conditions such interaction occurs.

The observation that the enzyme removes the (*pro-S*)-hydrogen exclusively (Maradufu et al., 1971) suggests that the alcohol substrate is bound at two places and donates the H^+ to a specific third group. Such a pattern would be obtained if the enzyme binds the primary alcohol group at the sulfur and contributes a hydrogen bond to the group at the 4 position. The poor substrates lacking a group to receive this hydrogen bond would be substrates only because the geometry would allow their equivalent of the 4 position to sit in the enzyme site without binding. It is quite possible though, that for these substrates, the enzyme is not specific for the (*pro-S*)-hydrogen. The H^+ would then be removed by a group spatially arranged to pick off only the (*pro-S*)-hydrogen, as shown in Figure 7. This binding picture is con-

sistent with the nonspecific requirements for groups bonded to C-5.

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