# ON THE ROLE OF A CUPROUS ION INTERMEDIATE IN THE GALACTOSE OXIDASE REACTION[1]

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ABSTRACT The $Cu^{+2}$ electron spin resonance spectrum of galactose oxidase (galactose:$O_2$ oxidoreductase, E.C. 1.1.3.9) indicates that the metal is in a pseudo-square planar environment. The electron g values are: $g_{zz}$ = 2.273, $g_{xx}$ = 2.058 and $g_{yy}$ = 2.048. The copper nuclear hyperfine constants are (in Gauss): $A_{zz}$ = 176.5, $A_{xx}$ = 28.8 and $A_{yy}$ = 30.1. This spectrum is unaltered in either intensity or g or A values under conditions which cause the inhibition of galactose oxidase by superoxide dismutase. No combination of substrates (galactose and $O_2$) and oxidant traps (superoxide dismutase and catalase) results in the reduction of the cupric ion resonance. Thus, a $Cu^{+1}$-enzyme does not appear to be a stable intermediate along this enzyme's reaction path.

Galactose oxidase (GOase)[2] is a copper containing enzyme which catalyzes the conversion of primary alcohols to their corresponding aldehydes concomitant with the reduction of $O_2$ to $H_2O_2$ [1-6]. Hamilton *et al.*[5] have postulated a mechanism for this enzyme in which an enzyme - $Cu^{+1}$ intermediate is formed via the dismutation of a $Cu^{+2} \cdot HO_2^-$ complex to a $Cu^{+1} \cdot HO_2^{\cdot}$ one. Their most compelling evidence for this hypothesis is the inhibition of GOase by superoxide dismutase (SDase )[2], generally regarded as a good probe of the involvement of $O_2^{\cdot-}$ in diverse chemical and enzymatic reactions[7]. We report in this paper, however, that under those conditions which allow inhibition of GOase by SDase, the characteristic electron spin resonance (ESR)[2] spectrum of GOase is not altered. We conclude that a cuprous ion enzyme is not a viable enzyme intermediate and probably is not formed at all.

## Materials and Methods:

The galactose oxidase [8,9] and equine superoxide dismutase[10] used were isolated and purified following literature procedures. Catalase (Grade A,

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[2]Abbreviations used: galactose oxidase, GOase; superoxide dismutase, SDase; electron spin resonance, ESR.

Cal Bio Chem) was dissolved in 0.1 M phosphate buffer (pH 7.0) to 10,000 units/ml and dialyzed against the buffer prior to use. SDase solutions (18 mg, or ca 50,000 units/ml[10]) were prepared similarly. The GOase solutions (15-22 mg/ml) were dialyzed against the phosphate buffer containing 1 M $(NH_4)_2SO_4$. This concentration of neutral salt stabilizes the GOase in the freezing and thawing procedures. The enzyme was assayed (see below) before and after all ESR experiments to establish its viability.

All solutions were prepared copper-free by elution from columns of Chelex-100 (BioRad).

X-band ESR spectra were determined at 110°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. GOase was assayed via $O_2$-uptake measurements in an air atmosphere at 20 ± 0.2° using a Gilson Differential Respirometer equipped with Model No. 5 all-glass volumometers. The concentration of D-galactose (Sigma) was 0.2 M in pH 7.0, 0.1 M phosphate buffer.

Results:

The ESR spectrum of GOase is presented in Figure 1. This spectrum is similar to but significantly better resolved than that reported by Blumberg et al.[11]. A comparison of the copper nuclear hyperfine constants and electron g-values from the two laboratories is given in Table I.

Briefly stated, under no conditions was this spectrum altered in either intensity or characteristic coupling and g-values. ESR spectra were recorded of the following frozen solutions:

A. Aerobic addition of galactose - An enzyme solution was made up to 0.4 M in galactose by addition of 2 M substrate in the buffer. The reported Michaelis constant for galactose is 38 mM[5].

B. Anaerobic addition of galactose - An enzyme solution was deoxygenated

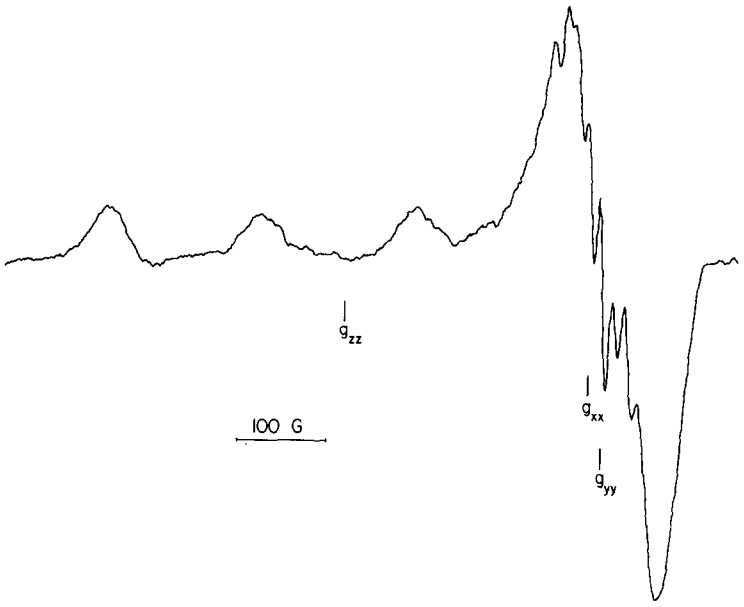


Figure 1: ESR Spectrum of Galactose Oxidase. Conditions: 22 mg/ml enzyme in 0.1 M phosphate (pH 7.0), 1 M $(NH_4)_2SO_4$ at 110°K. Instrument settings: microwave power, 70 mW; modulation amplitude, 5G; signal level, x1000; scanning rate, 100 G/min.

TABLE I

| This work | Blumberg et al[11] |
|---|---|
| $g_{zz}$ = 2.273; $g_{xx}$ = 2.058; $g_{yy}$ = 2.048 | $g_{\parallel}$ = 2.28; $g_{\perp}$ = 2.04 |
| $A_{zz}$ = 176.5; $A_{xx}$ = 28.8; $A_{yy}$ = 30.1 | $A_{\parallel}$ = 178.5 (A values in Gauss) |

Table I: Experimental g- and A- values for galactose oxidase

by stirring in a nitrogen atmosphere for 1 hr. Again, 2 M galactose (deoxygenated) was added to 0.4 M; based upon the amount of $H_2O_2$ produced (assayed by the anaerobic addition of peroxidase and o-dianisidine[9]) less than $10^{-6}$ M $O_2$ remained in the solution.

C. Presence of galactohexodialdose - The addition of catalase (1,000 units) to the preceding solutions and allowing the oxidation of galactose to

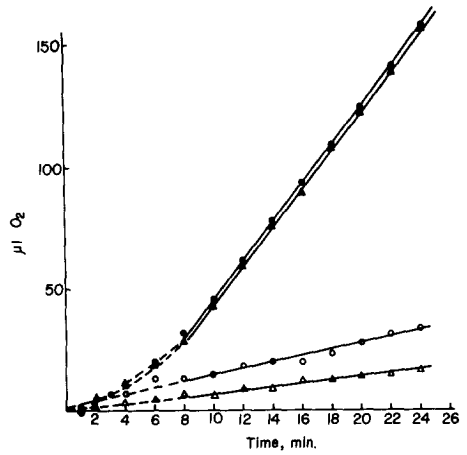


Figure 2: $O_2$-Uptake by Galactose Oxidase. These data were obtained using a Gilson Differential Respirometer. Conditions: 1 x $10^{-7}$ M galactose oxidase, 0.2 M galactose in 2.0 ml air saturated 0.1 M phosphate buffer, pH 7.0 at 20 ± 0.2°C. Shaking speed: 140 oscillations/min. All enzymes were added to the closed, equilibrated system through rubber septums via syringe. ● ——— ●, galactose oxidase alone; ▲ ——— ▲, galactose oxidase frozen and thawed from ESR experiments performed in the absence of other enzymes; o ——— o, galactose oxidase in the presence of 540 units[10] SDase; Δ ——— Δ, galactose oxidase in the presence of 540 units SDase and 200 units catalase. In these latter two experiments, galactose oxidase was added last. Full lines indicate portions of curves from which rate data were obtained.

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go to completion did not result in an alteration of the ESR spectrum. While the amount of product aldehyde actually present was not determined, based upon previous analyses of such reaction mixtures[3], the reaction was at least 80% complete. Air was bubbled through these solutions to insure an adequate concentration of $O_2$. Aldehyde at ca 0.3 M, then, does not alter the spectrum.

D. Aerobic addition of catalase and/or SDase - These proteins when added separately or in sequence did not alter the spectrum. In all experiments, these enzymes were added in 10 λ to 0.2 ml of GOase (at 21 mg/ml) to give 1,000 units of catalase or 270 units SDase in 0.21 ml of sample. These samples were incubated for up to 30 min prior to freezing.

E. Aerobic addition of galactose to samples containing catalase and SDase - In these experiments galactose was present at 0.2 M, while catalase and SDase were at the concentrations stated above. Incubation prior to freezing was again for up to 30 min and samples were also thawed, incubated

further, and refrozen. Spectra of such samples obtained over a period of 1-2 hrs did not change. Under these conditions of galactose, oxygen, catalase and SDase concentrations, $O_2$-uptake is inhibited to greater than 90% as measured in a separate respirometry experiment. This is illustrated by the $O_2$-uptake curves in Figure 2.

## Discussion

Our attribution of the hyperfine structure in the $g_\perp$ region of the ESR spectrum of GOase to anisotropy in the $g_\perp$ and $A_\perp$ values is based upon the intensity of these hyperfine lines and their absence in the $g_\parallel$ resonances. Although the spectrum presented in Figure 1 has been run at 70 mW, the shapes of the $g_\perp$ and $g_\parallel$ lines are not altered as the microwave power is reduced (to as low as 10 mW). Consequently, the absence of superhyperfine (due to ligand nuclei) in the $g_\parallel$ region is not due to selective saturation. Our assignments require that the enzyme-copper complex have pseudo-square planar geometry.

The maintenance of copper electron resonance in the resting enzyme upon addition of galactose or galactohexodialdose, a reaction product[2], suggests, by itself, that neither compound alters the protein-cupric ion complex in any way that significantly perturbs the electronic structure of that complex. Thus, these derivatives, if they bind to the copper, must do so axially, replacing a quite similar ligand, probably water. An in-plane (and inner-sphere) interaction is definitely ruled out. The fact that both compounds dramatically alter the copper optical activity exhibited by GOase strongly suggests that they do bind at the metal site[12].

The principal finding is that under conditions which lead to inhibition of GOase activity by SDase, the cupric ion resonance is again not diminished or altered. It must be emphasized that this demonstration that the copper remains as $Cu^{+2}$ cannot be taken as evidence against the involvement of the superoxide radical in the galactose oxidase reaction. These experiments only show that GOase when inhibited by SDase still contains cupric ion. The

inhibition is neither caused by nor causes reduction of the metal to the cuprous state. Since we have included catalase to scavenge the $H_2O_2$ produced by either GOase or by the dismutation of $HO_2^{\cdot}$, the reoxidation of a cuprous-ion enzyme by $H_2O_2$[5] is not responsible for the stability of the enzyme-bound cupric ion concentration.

The lack of an ESR-detectable reduction of the copper under several reaction conditions apparently rules out a cuprous enzyme intermediate. This is consistent with a sequential mechanism characterized by a ternary complex of galactose, $O_2$, and enzyme-bound copper in which the copper serves as a template (and perhaps a Lewis acid) but is not directly involved in the electron transfer reaction. However, kinetic results in this laboratory on product inhibition and the dependence of reaction velocity on oxygen concentration[13] together with the effects of peroxidase and catalase on GOase activity[6,14] indicate that the overall kinetic scheme must be somewhat more complex than simple sequential. The results presented in this communication are currently being integrated with kinetic and circular dichroism data to arrive at a scheme from which all of these observations can be rationalized.

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