THE VALENCE OF COPPER AND THE ROLE OF SUPEROXIDE IN THE D-GALACTOSE OXIDASE CATALYZED REACTION

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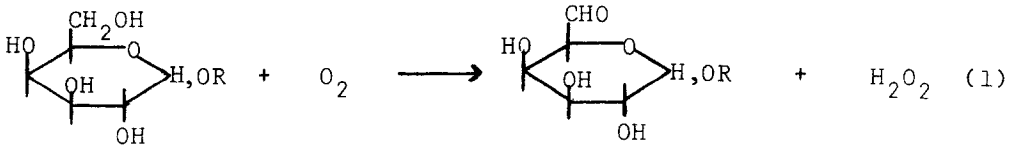
Received September 27, 1973

SUMMARY: Evidence for the involvement of Cu(III) in an enzymic reaction (that catalyzed by D-galactose oxidase) is reported. Superoxide dismutase inhibits the rate of the D-galactose oxidase catalyzed reaction and causes a small increase in the EPR signal due to Cu(II). Both ferricyanide and superoxide activate the enzyme (frequently 4 fold or greater) and cause a decrease (to essentially zero in some cases) in the intensity of the EPR signal. These and other results suggest that in its catalytic cycle the enzyme oscillates between Cu(I) and Cu(III) with superoxide bound to a Cu(II) state being only a fleeting intermediate. The Cu(III) enzyme is apparently the oxidant which converts the primary alcohol function of galactose to an aldehyde.

D-galactose oxidase (galactose: O_2 oxidoreductase; 1.1.3.9), which catalyzes the reaction of eq. 1, was discovered by



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Cooper et al. (1) in 1959 but most of its properties were subsequently described by Horecker and his coworkers (2). The enzyme has a molecular weight of ca. 55,000 daltons and contains 1 atom of copper per molecule as its sole cofactor. Early EPR data (3) indicated that the majority of the copper in the resting enzyme exists in the Cu(II) state, and that the

TABLE I. Effects of Some Additives on the Rate of the Galactose Oxidase Catalyzed Reaction[a]

Additives				Rate of O_2 uptake[c]
EDTA	SOD	$Fe(CN)_6^{-3}$	$O_2^{\cdot -}$[b]	(nmoles/min/ml)
-	-	-	-	~4
+	-	-	-	12
-	+	-	-	~1
+	+	-	-	~1
-	-	+	-	23
+	-	+	-	23
+	+	+	-	24
+[b]	-	-	+	20

[a]Reaction Conditions: 25°C; 0.01 to 0.02M phosphate, pH 7.0 to 7.1; $[O_2] = 2.5 \times 10^{-4}$M; [galactose] = 0.09M; [catalase] = 2µg/ml; [galactose oxidase] = 2×10^{-9}M; and when present, [EDTA] = 5×10^{-4}M; $[Fe(CN)_6^{-3}] = 10^{-3}$M; [SOD] = 10^{-9}M. [b]$O_2^{\cdot -}$ generated photochemically in situ by a flavin-EDTA system (7) consisting of 10mM EDTA and 3.3µM flavin (3-carbethoxymethyl-10-methylisoalloxazine). [c]Rate measured using a Gilson Oxygraph equipped with a Clark electrode; the reported rate is twice the observed rate because catalase is present.

addition of galactose does not appreciably change the intensity of the EPR signal. In an earlier report (4) we briefly noted that superoxide dismutase (SOD) (5) inhibits, and ferricyanide increases the rate of the D-galactose oxidase catalyzed reaction. These and other effects have now been investigated further and are reported here.

RESULTS AND DISCUSSION

Kinetic Data. The effects of various additives on the rate of the D-galactose oxidase catalyzed reaction are summarized in Table I. In the absence of all additives the rate

varies from day to day but in the presence of EDTA a reproducible rate is obtained. Trace metal ion impurities, whose concentrations could vary, thus apparently cause some inhibition. The absence of any effect of EDTA on the inhibition by SOD indicates that the trace metal ions are probably inhibiting the reaction by the same mechanism that SOD does, i.e., by removing $O_2^{\cdot-}$ from solution. It is known that trace metal ions catalyze the disproportionation of $O_2^{\cdot-}$ while EDTA complexes of these ions do not (6). In those experiments (Table I) showing inhibition by SOD, the rate starts off more rapidly but slows to the reported rate after a few minutes reaction time.

The activation by ferricyanide shows saturation-type kinetics, and at 10^{-3}M ferricyanide the rate is near the maximum achievable. Since both ferricyanide and $O_2^{\cdot-}$ activate the enzyme by comparable amounts, ferricyanide apparently performs the same function that $O_2^{\cdot-}$ usually does. The lack of any effect of EDTA and SOD on the ferricyanide activation is consistent with this conclusion. The following two observations indicate respectively that neither the $O_2^{\cdot-}$ nor the ferricyanide is reacting stoichiometrically when they increase the rate of O_2 uptake: (1) the control for the final experiment listed in Table I contained no galactose oxidase and less than 5% as much O_2 reacts compared to when the enzyme is present (the same amount of $O_2^{\cdot-}$ should be formed from the flavin system in both experiments), (2) in the experiment where all of EDTA, SOD, and ferricyanide are present, more than 300 molecules of O_2 react per molecule of ferricyanide converted to ferrocyanide (limit of detection).

All of the above kinetic experiments taken together thus indicate that removal of $O_2^{\cdot-}$ from the actively catalyzing system gives an inactive form of the enzyme, while the addition of $O_2^{\cdot-}$

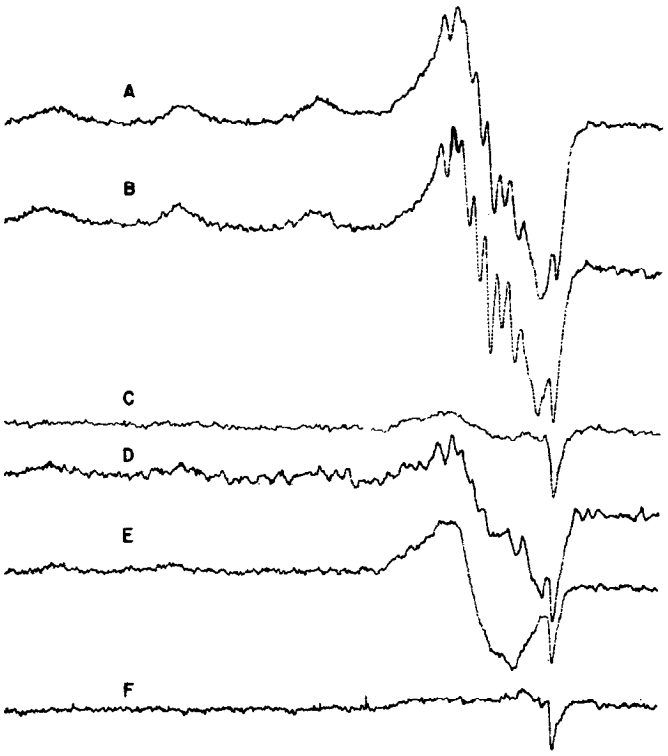
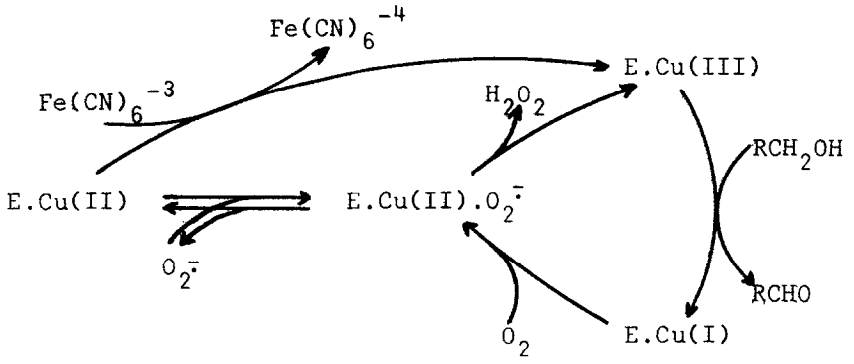


Figure 1. EPR spectra of galactose oxidase under various conditions. Spectra were taken on a Varian E-9 EPR Spectrometer under the following conditions: Frequency, 9.15 GHz; microwave power, 30mW; modulation amplitude, 10 gauss; time constant, 0.3 sec.; scanning rate 125 gauss/min; temperature, 87°K. Samples contain: 65μM galactose oxidase (prepared as previously described[4]), 0.02M phosphate buffer (pH 7.1), 2μg per ml catalase, and the following additives: A, none; B, 0.1M galactose; C, 1.0mM ferricyanide; D, 0.1M galactose and 1.0mM ferricyanide after 2 min at room temperature; E, 33μM flavin (3-carbethoxymethyl-10-methylisoalloxazine), 10mM EDTA, exposed to room light for 2.5 min.; F, background cavity signal.

or ferricyanide increases the amount of an active form. The resting enzyme, and the actively catalyzing system in the absence of additives, apparently have a mixture of active and inactive forms. The observed stoichiometry of the activation, and the lag in the inhibition by SOD, indicate that at least 300 turnovers occur with the active form (or forms) of the enzyme before a molecule of $O_2^{\cdot-}$ leaks out to give the inactive form. In order to clarify the nature of the inactive and active forms some EPR experiments were performed.



Scheme 1

EPR Data. Spectra A and B of Fig. 1 confirm the earlier report (3); a large proportion of the resting enzyme must exist as Cu(II) and galactose does not appreciably change the intensity of the signal. However, sharpening of the superhyperfine splitting on adding galactose is seen. The system which gives spectrum B is essentially anaerobic because the dissolved O_2 would be exhausted after 4 turnovers. Thousands of turnovers are required in order to obtain the SOD inhibition. Thus, for EPR experiments with SOD present a tenfold lower concentration of galactose oxidase was used, and the EPR spectra (not illustrated) were correspondingly poorer. However, under other conditions similar to those of B in Fig. 1, but with SOD (10^{-7}M) present, the intensity of the Cu(II) signal increases 20 to 30% after 1000 to 10,000 turnovers have occurred. This result, taken in conjunction with the kinetic results, implies that a Cu(II) form of the enzyme is inactive catalytically.

Spectrum C (Fig. 1) shows that the Cu(II) signal almost completely disappears when ferricyanide at a concentration which gives essentially complete activation is added to the resting enzyme. Thus a Cu(III) species, or possibly a Cu(II) in close

proximity to a radical, must be a catalytically active form. The species is relatively stable because the ferricyanide can be dialyzed out over a period of hours with only a slight return of the Cu(II) signal (experiment performed by G. C. DuBois). When amounts of ferricyanide comparable to the amount of resting enzyme are mixed, the decrease in the Cu(II) signal indicates that the Cu(III) form has only a slightly higher reduction potential than ferricyanide.

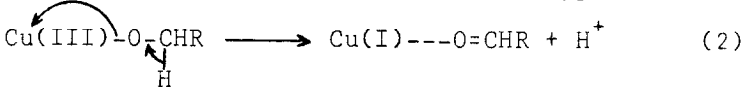
The addition of ferricyanide to galactose oxidase with galactose present causes a decrease in the intensity of the Cu(II) signal (spectrum D, Figure 1) but some of it remains after 2 min at room temperature. Ferricyanide can replace O_2 as an electron acceptor (at a rate less than 10^{-3} that of O_2), so under the conditions of D the enzyme is turning over and a Cu(II) form is an expected intermediate. The addition of $O_2^{\cdot-}$ to the resting enzyme causes a decrease in the Cu(II) signal (spectrum E) but also the superhyperfine structure is lost. This signal is not altered by adding ferricyanide and is probably due to an EDTA complex of Cu(II). Thus, under these conditions some of the copper is lost from the enzyme, probably from a Cu(I) state which is believed to be labile. This could accumulate under the O_2 starvation conditions of the experiment.

Mechanism. All the kinetic and EPR results are consistent with the minimal mechanism shown in Scheme 1. It is proposed that the Cu(III) and Cu(I) forms of the enzyme are in the usual catalytic cycle with $O_2^{\cdot-}$ bound to a Cu(II) enzyme being also a fleeting intermediate. The $O_2^{\cdot-}$ apparently dissociates from this intermediate once in several hundred or thousand turnovers to give a Cu(II) enzyme which is out of the catalytic cycle and inactive catalytically. Only when it is reacted with a one-

electron reagent (ferricyanide, $O_2^{\cdot-}$, etc.) can it return to the cycle as indicated. The resting enzyme is presumably a mixture of the Cu(II) and Cu(III) forms with the Cu(II) form usually predominating (about 75% of the total). In the actively catalyzing system an equilibrium between the inactive Cu(II) form and the cycle intermediates is apparently established; the position of the equilibrium (and thus the catalytic rate) would be expected to depend on conditions, including the involvement of unknown trace one-electron reagents either generated by, or present in the complex reaction system.

Some non-enzymic Cu(III) compounds are known, and Cu(III) is believed to be the oxidant in a chemical system (8) very analogous to the galactose oxidase reaction. Thus, treatment of various Cu(II) amine complexes with superoxide leads to the oxidation of the ligand to aldehyde and ammonia. The superoxide is apparently involved in the conversion of the Cu(II) complex to a Cu(III) complex as proposed for the present enzymic reaction.

The conversion of alcohol to aldehyde by the Cu(III) enzyme probably proceeds as shown in eq. 2. This type of mechanism has



(2)

been previously discussed (9,10) and is closely analogous to that for the chromate oxidation of alcohols (9,11).

In view of the relatively mild conditions ($O_2^{\cdot-}$ or ferricyanide) required to convert the Cu(II) form of galactose oxidase to a Cu(III) form, one suspects that Cu(III) may be an intermediate in many other cuproenzyme catalyzed reactions as well. The conclusion, that the lack of an EPR signal for copper enzymes indicates the copper is present as Cu(I), or that two Cu(II)'s are in close proximity, must now be considered inoperative.

Acknowledgement: This research was supported by a research grant from the National Institute of Arthritis, Metabolic and Digestive Diseases. C.R.H. is an established investigator of the American Heart Association.

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