ON THE ROLE OF SUPEROXIDE RADICAL IN THE MECHANISM OF ACTION OF GALACTOSE OXIDASE[1]

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Abstract The inhibition of galactose oxidase by superoxide dismutase is a function of the method of assay, nature of substrate, and composition of incubation and assay mixtures, as well as the concentration of dismutase. A reasonable level of inhibition is attained only when superoxide dismutase is present prior to the onset of catalysis although this effect is not observed under all conditions tried. Peroxidase activates galactose oxidase and blocks its interaction with either superoxide dismutase or catalase. These results further obscure the possible role of superoxide radical in the galactose oxidase reaction.

Hamilton et al. (1) have reported that O_2-uptake by galactose oxidase (GOase)[2] is inhibited by superoxide dismutase (SDase)[2]. In our own work on GOase, we have used for routine assays a procedure which couples the H_2O_2 produced by the oxidase to the oxidation of o-dianisidine as catalyzed by peroxidase (2). Under these assay conditions we found that the dismutase had no effect on the oxidase reaction. To better understand this inconsistency as well as the mechanism of action of GOase, we attempt here to delineate the conditions under which this inhibition can occur.

Materials and Methods

The galactose oxidase (2,3) and equine superoxide dismutase (4) used were isolated and purified in these laboratories following literature procedures. Catalase (Grade A) was from CalBioChem, peroxidase (Type II) and crystalline bovine serum albumin (BSA)[2] were from Sigma. All protein solutions were prepared in 0.1 M,

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[2]Abbreviations used: galactose oxidase, GOase; superoxide dismutase, SDase; bovine serum albumin, BSA.

pH 7.0 phosphate. Galactose was a Sigma product. 3,4-Dimethoxybenzyl alcohol and 3,3'-dimethoxybenzidine (o-dianisisine) were from Aldrich.

A Gilson Differential Respirometer equipped with Model No. 5 all-glass volumometers operating at a shaking rate of 140 oscillations/min was used to measure O_2-uptake in air-saturated solutions at 20 ± 0.2°. Spectrophotometric assays were performed on a Perkin-Elmer 124 equipped with a scale expander. All assays were performed in 0.1 M, pH = 7.0 phosphate buffer containing 0.028 M galactose or 0.05 M 3,4-dimethoxybenzyl alcohol (in 10% DMF) and o-dianisidine (2), peroxidase (2), or catalase when so indicated.

RESULTS

Respirometry - If SDase is incubated with GOase or is present in sufficient quantity in the substrate mixture prior to reaction initiation by addition of GOase, inhibition of oxygen uptake is observed, confirming the observation of Hamilton et al. (1). 50% inhibition occurs when 0.07 mg/ml (200 units) (4) SDase is present in the reaction flask. However, addition of equivalent or greater amounts of SDase after the reaction has begun has no effect upon GOase; no inhibition results. Typical respirometry data is presented in Figure 1. Furthermore, inclusion of peroxidase at the same concentration used in the coupled assay, 50 µg/ml, eliminates the lag seen in Figure 1A and the inhibition by SDase seen in Figure 1B; these data are presented in Figures 1C and 1D. This "explains" why inhibition by SDase is not observed when monitoring the GOase reaction in the coupled assay medium which contains peroxidase. The 3-fold rate enhancement of O_2-uptake induced by peroxidase should also be noted in Figures 1C and 1D.

Direct Assay - The oxidation of 3,4-dimethoxybenzyl alcohol by GOase was followed by the appearance of 3,4-dimethoxybenzaldehyde

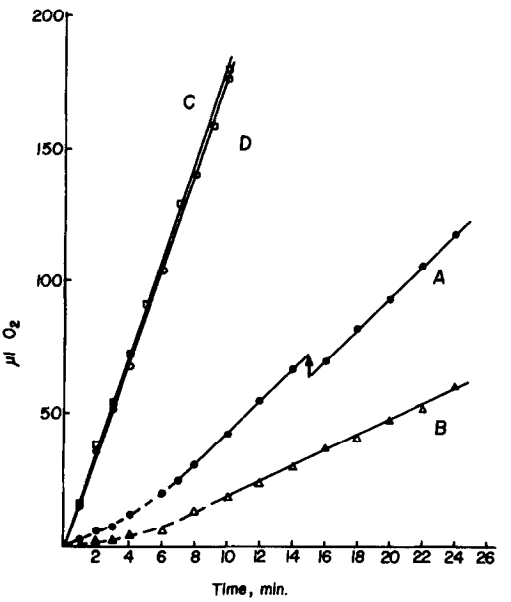
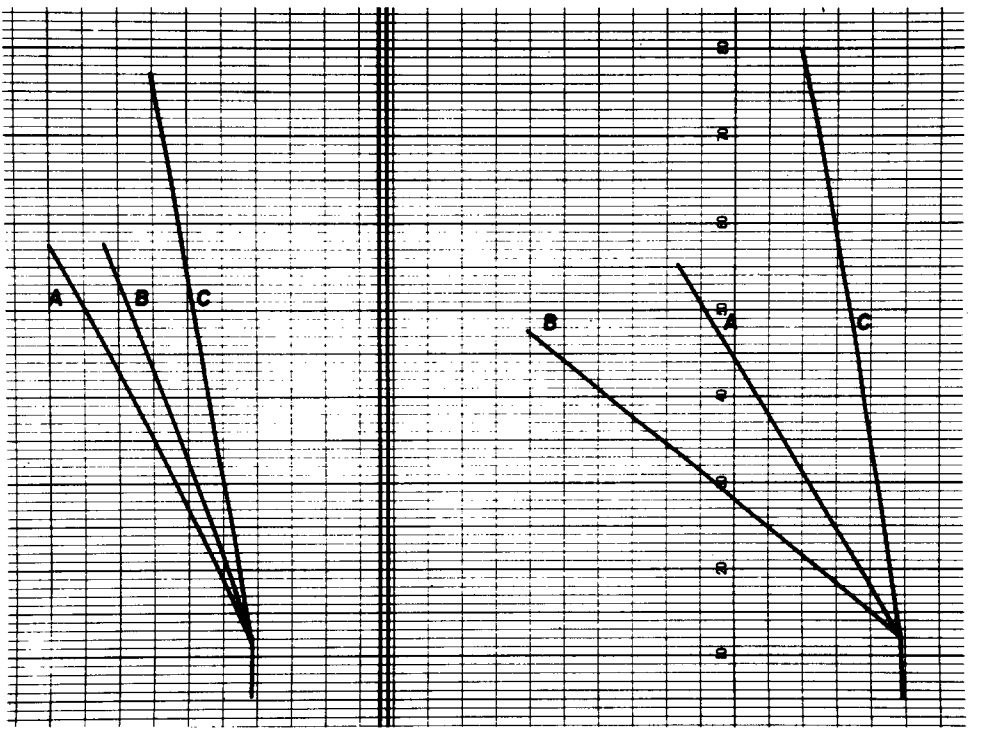


Figure 1: O_2-uptake curves for galactose oxidation by galactose oxidase. Conditions: 1 x 10^{-7} M galactose oxidase, 0.028 M galactose in air-saturated, 0.1 M, pH 7.0 phosphate buffer at 20 ± 0.2°. (A) ●——●, no initial additions, 270 units SDase added at 15 mins (arrow); (B) Δ——Δ, 200 units SDase added prior to addition of GOase; (C) □——□, 50 μg peroxidase added prior to addition of GOase; (D) o——o, 200 units SDase, 50 μg peroxidase added prior to addition of GOase. All additions were made through rubber septum via syringe. The data points are the average of at least two experiments.

monitored at 340 nm (Fig. 2A). SDase, when present in the substrate mixture or added after initiation of reaction, was without effect at levels below 500 units/ml in the final assay solution. Fifty percent inhibition was observed at a concentration of 4300 units/ml, or 1.6 mg SDase per ml of assay medium (Fig. 2B). However, this was 50% of the rate observed in the presence of 1.6 mg/ml of BSA, a rate which was 3-fold greater than that in the absence of these large quantities of added protein (cf. Fig. 2A and 2C). GOase, itself, is present in these solutions at 1 μg/ml. The "inhibition" by SDase, therefore, may represent a weaker "activation" when compared to the effect of BSA.

On the other hand, when 1.6 mg/ml SDase was incubated with GOase in the absence of alcohol substrate (and then present in the assay



Figures 2 and 3: Effect of SDase, BSA and peroxidase on the oxidation of 3,4-dimethoxybenzyl alcohol by GOase. Conditions: 2 x 10^{-7} M GOase in air-saturated, 0.1 M phosphate, pH 7.0 containing 0.05 M 3,4-dimethoxybenzyl alcohol in 10% DMF. The reactions were initiated by addition of GOase solutions and were monitored at 340 nm; full scale deflection is 0 - 0.1, chart speed 6 divisions/min.

Left, Figure 2: A) addition of GOase alone; B) addition of GOase alone to reaction mixture containing 1.6 mg/ml SDase; C) addition of GOase alone to reaction mixture containing 1.6 mg/ml BSA. The labeling of the traces is correct - see Results, Direct Assay.

Right, Figure 3: A) addition of GOase alone; B) addition of GOase preincubated with 1.6 mg/ml SDase; C) rate obtained in the presence of 1 µg/ml peroxidase; both trace A) and B) convert to this upon addition of peroxidase to either reaction or incubation mixtures.

medium at 1.6 µg/ml), 50% inhibition of 3,4-dimethoxybenzaldehyde oxidation was observed. However, if as little as 1 µg of peroxidase is present in either the incubation mixture or substrate solution, the inhibition is abolished and the rate enhancement induced by peroxidase is observed as before (Fig. 3).

Coupled Assay - As mentioned previously, when assayed by a coupled

assay employing peroxidase, GOase is not demonstrably inhibited by SDase. The above results indicate that under no circumstances will SDase effect GOase in the presence of peroxidase. A futher indication of the unusual relationship between GOase and peroxidase is the observation that catalase, when added to the coupled assay mixture after initiation of the GOase reaction, does not retard the oxidation of o-dianisidine by the GOase-produced H_2O_2 as catalyzed by peroxidase. In a separate experiment, the addition of an equivalent amount of catalase to an assay mixture for peroxidase does result in a decreased color yield due to the action of catalase on the hydrogen peroxide.

DISCUSSION

Under the most favorable conditions, 50% inhibition of the GOase reaction is observed at 1.6 μg/ml SDase in the reaction medium, or 4-5 units of SDase activity. This level of inhibition occurs only when the two enzymes are incubated together in the absence of substrate at an SDase concentration of 1.6 mg/ml prior to the onset of alcohol oxidation. Otherwise, the method of assay determines the 50% level of GOase inhibition. 0.07 mg/ml SDase is sufficient to cause this inhibition in the O_2-uptake experiments using galactose while 1.6 mg/ml is required in the direct assay using an aromatic alcohol.

The effect of SDase on GOase must be viewed in light of the demonstrated stimulation of GOase activity by high concentrations of BSA and μg amounts of peroxidase. Also, the seemingly symbiotic relationship between peroxidase and GOase which proscribes the catalysis by catalase of the dismutation of GOase-produced hydrogen peroxide is yet another puzzling feature of galactose oxidase and its mechanism of action.

The stimulatory effect of high protein concentrations may be

due to one or more of several factors. These include de-adsorption of GOase from the surfaces of the reaction vessel and concentration dependent conformational changes that are inhibited by maintaining adequate levels of protein in the solution.

The interesting relationship of GOase and peroxidase does not involve a protein concentration dependent property of GOase since the peroxidase effect occurs at 50 μg/ml levels or less. The evidence suggests that peroxidase scavenges H_2O_2 directly from GOase and thereby accelerates the turnover by the latter enzyme as observed. This is consistent with the hypothesis that the rate-limiting step in the GOase reaction is the dissociation of H_2O_2 from the enzyme (1). Hamilton pointed out that this ligand might well dissociate slowly and postulated that the formation of $O_2^{\cdot-}$ facilitated the eventual expultion of hydrogen peroxide from the enzyme (1).

As a probe of the intermediancy of $O_2^{\cdot-}$, SDase might be expected to provide evidence for such a mechanism (5). While at exceedingly high levels of SDase the on-going GOase reaction is inhibited, only if the two enzymes are incubated together prior to the onset of GOase-mediated catalysis does SDase exhibit significant activity. This suggests that some initiating event of the GOase reaction is inhibited by SDase, perhaps requiring $O_2^{\cdot-}$. A reductive activation of the pseudomonad and hepatic tryptophan dioxygenases by $O_2^{\cdot-}$ has been postulated (6). The inhibition of GOase, however, does not involve the metal, since SDase does not alter the characteristic GOase ESR spectrum (7). In conclusion, the experiments described herein raise more questions than they answer. The role of $O_2^{\cdot-}$ in the GOase reaction remains obscure and now not only the effect of SDase, but also that of peroxidase on GOase needs to be explained as well.

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