Superoxide Radical Inhibits Catalase*

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Catalase was inhibited by a flux of $O_2^-$ generated in situ by the aerobic xanthine oxidase reaction. Two distinct types of inhibition could be distinguished. One of these was rapidly established and could be as rapidly reversed by the addition of superoxide dismutase. The second developed slowly and was reversed by ethanol, but not by superoxide dismutase. The rapid inhibition was probably due to conversion of catalase to the ferro-oxy state (compound III), while the slow inhibition was due to conversion to the ferryl state (compound II). Since neither compound III nor compound II occurs in the catalatic reaction pathway, they are inactive. This inhibition of catalase by $O_2^-$ provides the basis for a synergism between superoxide dismutase and catalase. Such synergisms have been observed in vitro and may be significant in vivo.

The inhibition of catalase by $O_2^-$ would provide an explanation for several otherwise puzzling reports of synergism between catalase and superoxide dismutase. Thus, the auto-inactivation of xanthine oxidase was partially prevented by 10 μg/ml of catalase. Superoxide dismutase, which offered very little protection when acting alone, markedly enhanced the protective effect of catalase (1). Similarly, superoxide dismutase did not prevent lysis of erythrocytes from vitamin E-deficient rats by autoxidizing dialuric acid, but substantially increased the protection offered by catalase (2). In both of these cases the effect of catalase could have been blunted by a suppression of catalatic activity by $O_2^-$, and this effect could have been relieved by superoxide dismutase.

The hemes of catalase lie deeply buried and are accessible to solvent by way of a narrow channel lined with hydrophobic residues (3). This structure helps explain the marked preference of catalase for small substrates (4) and the acquisition of activity toward larger substrates afforded by mild denaturation (5). How then explain the reported inhibition of native catalase by NADH plus phenazine methosulfate (6)? An answer may lie in the known production of $O_2^-$ by the aerobic interaction of these compounds (7). $O_2^-$ is small enough to gain access to the hemes of catalase and would convert the resting enzyme to the ferro-oxy state (compound III), which is known to be inactive (8). There is a precedent for such a reaction. Thus $O_2^-$ is known to react rapidly with horseradish peroxidase, yielding the relatively inactive compound III (9, 10). This inhibition of peroxidase was relieved by superoxide dismutase (10), and this effect was used as the basis for an assay of superoxide dismutase (11). We have investigated the possibility that $O_2^-$ might inhibit catalase and here present evidence that it does so in two distinct ways.

Materials and Methods

Xanthine oxidase was isolated from unpasteurized cream (12), while the manganese-containing superoxide dismutase was isolated from Escherichia coli B (13). This superoxide dismutase was used throughout because, unlike the corresponding iron and copper/zinc enzymes, it is not susceptible to inactivation by $H_2O_2$ (14–17). Crystalline bovine liver catalase was obtained from Sigma. This material was free of superoxide dismutase activity and was used directly. Acetaldehyde from Eastman was distilled daily into a chilled receiver. Catalase was assayed by the method of Beers and Sizer (18) as described by Aebi (19), using Gilford 200 or Amino DW-2 spectrophotometers.

Results

Rapid Inhibition of Catalase by $O_2^-$.—The aerobic xanthine oxidase reaction, which is known to generate large amounts of $O_2^-$ (20), was used as the source of this radical. Acetaldehyde was routinely used as the electron donor in place of xanthine, in order to avoid interferences due to the strong ultraviolet absorbances of xanthine and of urate, but xanthine could be, and occasionally was, used without substantially affecting the results. As shown in Fig. 1, initiation of the xanthine oxidase reaction rapidly inhibited the activity of catalase. Superoxide dismutase prevented this inhibition, when present from the outset, and largely reversed it when added 2.5 min after the inception of the xanthine oxidase reaction.

Increasing the concentration of xanthine oxidase increased the inhibition of catalase, to a limit of 40% at pH 7.0 and 75% at pH 10.0. The inhibition seen at any given level of xanthine oxidase was greater at pH 10.0 than at pH 7.0. This is shown in Fig. 2. Xanthine oxidase produces much more $O_2^-$ at pH 10.0 than at pH 7.0 (20), moreover, $O_2^-$ is more stable at the higher pH (21). The greater inhibition of catalase at pH 10.0 than at pH 7.0 is thus consistent with the effector being $O_2^-$, and the protection by superoxide dismutase establishes this point. Since the rate of production of $O_2^-$ by the xanthine oxidase reaction would be a first order dependent of the concentration of this enzyme, whereas elimination of $O_2^-$ by the spontaneous dismutation would depend upon the square of [$O_2^-$], we can expect the steady state level of $O_2^-$ to approach some limit, as the concentration of xanthine oxidase was raised. Furthermore, a shown in Fig. 1, the inhibition of catalase was rapidly reversed by superoxide dismutase, implying a reversible reaction of $O_2^-$ with catalase. The limiting level of $[O_2^-]$ approached at high rates of production of this unstable radical and the reversibility of the $O_2^-$–catalase reaction account for the limiting inhibitions of catalase shown in Fig. 2.

The Effect of Superoxide Dismutase.—The manganese-containing superoxide dismutase, which is not susceptible to inhibition by $H_2O_2$ (14–17), was tested over a range of concentrations for its ability to prevent inhibition of catalase by the xanthine oxidase reaction. As shown in Fig. 3, 10 μM super-

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**Fig. 1. Rapid inhibition of catalase by O$_2^-$**. Reaction mixtures contained 10 mM H$_2$O$_2$, 30 mM acetaldehyde, 0.1 mM EDTA, and 50 mM sodium carbonate at pH 10.0 and at 23 °C. In all cases reactions were started at 0 time by adding catalase to 0.87 nm. Line 1 is the course of the catalatic reaction in the absence of xanthine oxidase, while line 2 was obtained when both 4.3 nM xanthine oxidase and 8.3 nM Mn-superoxide dismutase were present from the outset. Line 4 was recorded after adding xanthine oxidase (4.3 nM) at arrow I, while line 3 was obtained after adding xanthine oxidase (4.3 nM) at arrow I followed by Mn-superoxide dismutase (8.3 nM) at arrow II.

**Fig. 2. Effects of pH and of [xanthine oxidase] on the rapid inactivation of catalase.** Reaction mixtures contained 10 mM H$_2$O$_2$, 30 mM acetaldehyde, 0.1 mM EDTA, 50 mM buffer salts, and the indicated concentrations of xanthine oxidase. Percentage of catalase activity was calculated from the initial rates of consumption of H$_2$O$_2$, followed at 240 nm after the addition of catalase to 0.7 nm. Potassium phosphate provided buffering at pH 7.0 and 7.8, while sodium carbonate was used at pH 10.0. The symbols indicate: ■, pH 7.0; ▲, pH 7.8; △, pH 10.0 with 0.1 mM xanthine in place of acetaldehyde and with the reaction followed at $A_{240\text{~nm}} - A_{283.4\text{~nm}}$; ○, pH 10.0. The isosbestic point for the xanthine → urate conversion at pH 7.0 is 283.4 nm.

Oxidized dismutase prevented 50% of the initial inhibition imposed by 8.6 nM xanthine oxidase acting on acetaldehyde at pH 10.0, while 4.0 nM provided complete protection. Given its molecular weight of 45,000, 1.0 nM Mn-superoxide dismutase contains 0.045 μg/ml. To eliminate the remote possibility of a nonspecific protein effect, bovine serum albumin was tested at 100 μg/ml and had no effect. Mannitol at 10 mM and diethylene triamine pentaacetic acid at 0.1 mM were also unable to influence this inhibition of catalase. Replacement of acetaldehyde by 0.1 mM xanthine did not significantly alter the inhibition of catalase by xanthine oxidase. All of this is in accord with the conclusion that O$_2^-$ reacts rapidly and reversibly with catalase and in so doing suppresses catalatic activity.

**Inhibition of Catalase by Acetaldehyde**—During the course of these experiments it was noted that acetaldehyde was itself able to inhibit catalase. As shown in Fig. 4, this inhibition appeared to be competitive with H$_2$O$_2$. Under the conditions used to routinely assay catalase, 10 mM acetaldehyde caused 20% inhibition, and 30 mM caused 60% inhibition. This inhibition of catalase by acetaldehyde was a constant factor in our measurements and did not interfere with studies of the inhibition caused by O$_2^-$.

Slow Inhibition of Catalase by O$_2^-$—The rapid and reversible inhibition of catalase by O$_2^-$, shown in Fig. 1, was followed by a slow and apparently irreversible inhibition. This was explored by incubating catalase with the xanthine oxidase-acetaldehyde reaction at 25 °C in a shaking water bath and, at intervals, removing aliquots for assay of residual catalase activity. These aliquots were diluted 300-fold into buffered H$_2$O$_2$ for assay. As shown in Fig. 5, there was a progressive inhibition of catalase. This was more pronounced at pH 10.0 than at pH 7.0, as can be seen by comparing lines 4 and 6. Superoxide dismutase, present at 4.2 nM during the incubation, provided almost complete protection at pH 7.0 (line 2).


Fig. 5. Slow inhibition of catalase by $O_2^-$. Incubation mixtures contained 10 mM acetaldehyde, 0.1 mM EDTA, and 50 mM potassium phosphate at pH 7.0 or 50 mM sodium carbonate at pH 10.0, 0.25 mM catalase at 23°C. Xanthine oxidase, when present, was at 5.2 nm, and Mn-superoxide dismutase, when present, was at 4.2 nm. At intervals, 10-μl aliquots of these incubation mixtures were diluted 300-fold into assay mixtures containing 10 mM H$_2$O$_2$, 0.1 mM EDTA, and 50 mM potassium phosphate at pH 7.0 and at 23°C. Residual catalase activity was measured in terms of the initial rate of H$_2$O$_2$ consumption, followed at 240 nm. Incubation conditions were: lines 1 and 4, pH 7.0 without and with xanthine oxidase, respectively; line 2, pH 7.0 with xanthine oxidase plus superoxide dismutase; lines 3 and 6, pH 10.0 without and with xanthine oxidase, respectively; line 5, pH 10.0 with xanthine oxidase plus superoxide dismutase. The open squares on line 6 were obtained when the incubation mixture was assayed in the presence of 4.2 mM superoxide dismutase.

Fig. 6. Ethanol prevents and reverses the slow inhibition of catalase by $O_2^-$. Incubation at pH 10.0 and assay conditions were as described in the legend of Fig. 5, with the following changes in the incubation mixtures. Line 1, no xanthine oxidase; line 2, with xanthine oxidase plus 16.3 mM ethanol; line 3, xanthine oxidase present from time 0 and 16.3 mM ethanol added at the arrow; line 4, xanthine oxidase plus 1.1 mM ethanol; line 5, xanthine oxidase without ethanol, and provided substantial protection at pH 10.0 (line 5). Since the manganese superoxide dismutase is less active at pH 10.0 than at pH 7.0 (23), this is not surprising. Whereas superoxide dismutase present during the incubation protected the catalase, it failed to reverse this inhibition when added to the catalase assay mixture (open squares on line 6). Control experiments, in which xanthine oxidase was omitted, indicated that catalase was completely stable for 3 h at pH 7.0 (line 1), but not at pH 10.0 (line 3). Mannitol, at 100 mM, did not prevent this slow inactivation.

It appeared possible that the slow and apparently irreversible inhibition of catalase by $O_2^-$ was due to this radical acting as a univalent reductant toward catalase compound I. In that case the accumulated product would be catalase compound II, and it might be converted back into active catalase by ethanol. This seemed reasonable because catalase is competent to act as a peroxidase toward ethanol. Fig. 6 demonstrates that 16.3 mM ethanol largely prevented the slow inactivation of catalase (lines 2 and 5), while ethanol added after 90 min of reaction reversed the inactivation which had already occurred (line 3).

**DISCUSSION**

Catalase is inhibited by $O_2^-$ in two distinct ways. One of these is a rapid inhibition, which can be prevented and reversed by superoxide dismutase. The second is a slow inhibition, which can be prevented but not reversed by superoxide dismutase and which can be both prevented and reversed by ethanol. It is likely that the rapid inhibition is due to a reversible reaction of $O_2^-$ with the native catalase, yielding compound III. This reaction involves a univalent reduction of the ferric center to a ferro-oxy compound. Thus

\[
\text{Fe(III)} + O_2^- \leftrightarrow \text{Fe(II)} - O_2 \quad (1)
\]

**Catalase Compound III**

The slow inhibition was probably due to a reaction of compound I of catalase with $O_2^-$. Catalase would have first been converted to compound I by reaction with the H$_2$O$_2$, which is also a product of the xanthine oxidase reaction. This may be written

\[
\text{Fe(III)} + H_2O_2 \rightarrow 2 H_2O + \text{Fe(V)} \quad (2)
\]

**Catalase Compound I**

\[
\text{Fe(V)} + O_2^- \rightarrow O_2 + \text{Fe(V)} \quad (3)
\]

**Compound I Compound II**

Since compound II does not occur on the catalatic reaction pathway, it represents an inactive form of the enzyme, and its accumulation would be associated with inactivation. It is possible that compound II might be reduced to the active trivalent state by reacting with another $O_2^-$. Thus

\[
\text{Fe(IV)} + O_2^- \rightarrow O_2 + \text{Fe(III)} \quad (4)
\]

**Compound II Catalase**

In that case the slow inactivation of catalase by $O_2^-$ should approach a limit defined by the ratio of the rate constants for Reactions 3 and 4. The data shown in Fig. 5 do indeed suggest that there was such a limit, but it should not be taken as being well defined by the data in Fig. 5, since the rate of production of $O_2^-$ by the xanthine oxidase reaction might well have declined markedly over the several hours of these incubations.

Ethanol both prevented and reversed the slow inactivation of catalase, whereas superoxide dismutase prevented, but did not reverse, this inactivation. It follows that the inactive compound II was not spontaneously reactivated by endogenous reductants under our conditions, and that reactivation by ethanol was due to univalent reduction of the compound II. This conclusion seems to contradict the results of Nicholls (24), which indicated that ethanol acted as a divalent reductant of compound I and not as a univalent reductant of compound II. This apparent disagreement may be explained by differences in experimental conditions. Thus Nicholls (24), of necessity, worked at high concentrations of catalase, so that conversions to compounds I and II could be followed spectrophotometrically. In contrast, we worked at concentrations of xanthine oxidase much smaller, which were suitable for measurement of catalatic activity. Ultraacentrifugation of the com-
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Compound II of the catalase of *Micrococcus lysodeikticus* demonstrated a tendency to dissociate into monomeric subunits (25). Let us suppose that the monomeric compound II can be reduced univalently by ethanol, whereas the tetrameric form cannot. Low concentration would favor the monomeric form, whereas high concentration would favor the tetrameric form. In that case we could observe univalent reduction of compound II to the active catalase by ethanol, whereas Nicholls (24) could not.

Ethanol prevented the slow inactivation of catalase in the presence of O₂⁻ plus H₂O₂, and this was probably due to a combination of two factors. In the first case ethanol would compete with O₂⁻ for reaction with compound I and thus would decrease the rate of production of compound II. Thus

\[
\text{Compound I} + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{Catalase} + \text{H}_2\text{O} + \text{CH}_3\text{CHO}
\]

The second factor is the univalent reduction of compound II by ethanol, which has already been invoked to explain the reversal of the slow inhibition of catalase by this alcohol.

These inhibitions (the fast and the slow) provide the basis for synergistic interactions between catalase and superoxide dismutases. In any reaction mixture generating both O₂⁻ and H₂O₂, the effectiveness of catalase would be enhanced by superoxide dismutase, which would prevent the conversion of the active catalase into the relatively inactive compounds II and III. Since the Cu/Zn and the Fe superoxide dismutases can be inactivated by H₂O₂ (14–17), one also has the basis for a reverse synergism in which the catalase prevents inactivation of the superoxide dismutase. Catalase and superoxide dismutases clearly constitute a mutually protective set of enzymes. This cooperative interaction of superoxide dismutase and catalase is distinct from that which derives from prevention of OH⁻ production by the iron-catalyzed interaction of O₂⁻ with H₂O₂ (26–28).

The observations of Marcus and Feeley (6) can now also be explained. Thus, NADH plus phenazine methosulfate, which inhibited catalase and modified its absorption spectrum (6), is a known source of O₂⁻ (7). Marcus and Feeley (6) did report seeing an effect of NADH plus phenazine methosulfate on catalase even under N₂, but that must have been due to insufficiently stringent exclusion of oxygen.

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