Xanthine Oxidase

V. DIFFERENTIAL INHIBITION OF THE REDUCTION OF VARIOUS ELECTRON ACCEPTORS*

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Milk xanthine oxidase can catalyze the reduction of oxygen, cytochrome c, nitrate, ferricyanide, and various quinones and dyes by many aldehydes and purines. When acting upon its substrates, this enzyme can also initiate the aerobic oxidation of sulfite (1, 2) and induce the chemiluminescence of lucigenin and of luminol (3, 4).

The reduction of cytochrome c by milk xanthine oxidase has been the subject of conflicting reports. Thus, Horecker and Heppel (5) found that oxygen was essential to this reaction, whereas Morell (6) reported that cytochrome c reduction was faster in the absence of oxygen. The requirement for oxygen was confirmed by Weber, Lenhoff, and Kaplan (7) who proposed that hydrogen peroxide generated by the enzyme is the immediate reductant of cytochrome c. Maclor, Mahler, and Green (8) reported that inorganic phosphate is specifically required for the reduction of cytochrome c and that removal of molybdenum from the enzyme caused a loss of this activity, but other workers (9, 10) have been unable to repeat these findings. Continued interest in the pathways of electron transport within this complex enzyme prompted a systematic analysis of the influence of a series of inhibitors on the reduction of various electron acceptors by the enzyme in the presence of its substrates. The results of these studies permit an estimate of the minimal number of sites of electron egress from xanthine oxidase.

EXPERIMENTAL PROCEDURE

Materials and Methods—Xanthine oxidase was purified and assayed as described (11). Cytochrome c was the purified, "type III" horse heart preparation of the Sigma Chemical Company. Crystalline catalase was a product of the Worthington Biochemical Corporation. Cytochrome oxidase (12), DPNH-cytochrome c reductase (13), and sulfite-cytochrome c reductase (14) were each prepared as described in the literature. TPNH-cytochrome c reductase (15) was kindly provided by Drs. Charles Williams and Henry Kamin.

Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid disodium salt) was a product of the Eastman Kodak Company. Tris was the primary standard grade, Sigma 121, furnished by the Sigma Chemical Company.

Experiments under controlled gas mixtures were performed in silica cuvettes similar to that described by Lazarow and Cooperstein (16). These cuvettes permitted bubbling the gas mixture through the liquid phase. Known gas mixtures were prepared by mixing tank oxygen and prepurified nitrogen (Matheson Company) under saturated ammonium sulfate. Iron was assayed by the method of Mahler and Elowe (17), and molybdenum, by the method of Dick and Bingley (18). FAD was estimated as the absorbancy at 450 mμ of trichloroacetic acid extracts of the enzyme (19). Unless otherwise stated, all assays were performed in 0.05 M potassium phosphate, pH 7.8, containing 10−5 M EDTA.

Studies of the chemiluminescence of DBA++ induced by xanthine oxidase (3, 4) were kindly performed by Mr. Lorraine Greenlee. The experiments here reported were performed in 0.10 M sodium carbonate buffer at pH 10.0 containing 500 μg per ml of bovine serum albumin. Light emission was measured with a Farrand photofluorometer operated as a photometer. Since inhibition of light emission could be due either to the action of the inhibitor on the enzyme or to quenching of the chemiluminescent process itself, all inhibitors were tested for quenching of the hydrogen peroxide-induced luminescence of DBA++. None of the inhibitors tested exhibited such quenching effects.

Studies of the initiation of sulfite oxidation (1, 2) here reported were kindly performed by Mr. Stuart Levy. In all cases, inhibitors were tested for possible "chain breaking" in an electrode-initiated (2) sulfite oxidation reaction. None of the inhibitors whose effects are to be reported exhibited significant chain breaking under the conditions of these experiments.

Nitrate reduction was followed by measurement of nitrite accumulation under rigorously anaerobic conditions. The possibility of interference in the colorimetric assay of nitrite by the inhibitors tested was eliminated by measurements of the recovery of added nitrite in the presence of the various inhibitors. Nitrate reduction was not observed in the presence of oxygen, and recovery studies eliminated the possibility that the aerobic xanthine oxidase system acts upon nitrite. Although nitrate reduction by milk xanthine oxidase has been reported (20) to proceed optimally at pH 4.6, the experiments herein recorded were performed under the same conditions of buffering used in the other assays.

Since uric acid rapidly reduces ferricyanide (21), assay of ferricyanide reduction by xanthine oxidase plus xanthine under aerobic conditions merely reflects the rate of urate production. To investigate the direct reduction of ferricyanide by xanthine oxidase, it was necessary to use aldehyde substrates or to work under rigorously anaerobic conditions with purine substrates.

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1 The abbreviations used are: DBA++, 10,10-dimethyl-9,9-biacriclidinium; FMN, flavin mononucleotide.
Identical results were obtained by both procedures. Ferricyanide reduction was followed at 420 mμ; methylene blue reduction was followed at 650 mμ under anaerobic conditions. Uric acid formation was routinely measured by the increase in absorbancy at 295 mμ. Oxygen reduction was measured by the formation of uric acid under aerobic conditions.

Inhibition of Xanthine Oxidase by Heavy Metals—Aerobic incubation of xanthine oxidase with xanthine in EDTA-free buffer resulted in loss of 80% of activity within 4 minutes, as judged by the rate of formation of uric acid. Ability to catalyze reduction of cytochrome c disappeared even more rapidly under these circumstances. Complete protection was afforded by the presence of 10⁻⁴ M EDTA. This loss in activity was entirely dependent upon activity of the enzyme; no loss in activity was observed after several hours of storage in EDTA-free buffer, if the EDTA was added immediately before assay. This activity-dependent, irreversible inhibition was also prevented by α,α’-dipyridyl (10⁻⁴ M), o-phenanthroline (10⁻⁴ M), 8-hydroxyquinoline (10⁻⁴ M), pyrophosphate (.01 M), citrate (.01 M), and bovine serum albumin (0.4 mg per ml).

This inhibition is reminiscent of the activity-dependent inhibition by p-chloromercuribenzoate previously reported (22), and the findings suggest contamination by heavy metals in the water and reagents. This was demonstrated by treatment of the stock (1.0 M) phosphate buffer with dithizone (23) in CCl₄ until three successive extracts failed to show the color change indicative of metal complex formation. The buffer was then freed of traces of dithizone by repeated extractions with CCl₄. The CCl₄ was removed by repeated extraction with ether, and residual ether was eliminated by forced aeration. All reagents had been prepared in glass-distilled water. With the use of water and reagents so prepared, the concentration of EDTA required to maintain linear rates in the aerobic and cytochrome c reduction assays for the enzyme was reduced to 5 × 10⁻³ M. However, except in those studies specifically designed to examine the effect of EDTA upon the enzyme, ordinary reagents were employed, and 10⁻⁴ M EDTA was present in all systems.

Oxygen Requirement for Cytochrome c Reduction—Fig. 1 illustrates the effects of varying oxygen tension on the reduction of cytochrome c, and of oxygen itself, by xanthine oxidase. As shown, there is an absolute dependence on the presence of oxygen for the reduction of cytochrome c. Indeed, the value for the Michaelis-Menten constant, Kₘ for the latter process was found to be 8 × 10⁻⁴ M, whereas KₘO₂ for the reduction of oxygen was only 2.7 × 10⁻⁵ M.

The initial rate of cytochrome c reduction in these experiments was not affected by the presence of catalase (50 units per ml) and ethanol (10 mg per ml) although, with less purified preparations of xanthine oxidase, catalase appeared to be essential for linear rates of cytochrome c reduction. This finding appears to be due to the virtual absence of ferrocytochrome c peroxidase activity, presumably that of lactoperoxidase, in the purified xanthine oxidase and to its relative abundance in crude preparations of the enzyme. Anaerobic reduction of cytochrome c was not made possible by the presence of 0.1 M nitrate but, as reported by Mahler, Fairhurst, and Mackler (24), menadione acted as an electron carrier from the enzyme to cytochrome c anaerobically. The apparent Kₘ for menadione in this regard was 8.6 × 10⁻⁴ M. The presence of 2,6-dichlorophenol indophenol or FMN also permitted anaerobic reduction of cytochrome c. The former was effective at quite low concentrations, whereas the apparent Kₘ for the latter was 2.2 × 10⁻³ M. Presumably, each of these carriers is reduced by the enzyme and then, in turn, oxidized by cytochrome c.

Inhibition by Tiron—Tiron, a chelating agent for titanium, Fe⁺⁺⁺, and Mo⁺⁺ (25) was found to be without influence on the reduction of oxygen or dyes by xanthine oxidase in the presence of either aldehyde or purine substrates. However, as shown in Fig. 2, this reagent was found to inhibit cytochrome c reduction competitively with respect to cytochrome c. From these data, Kₘ for cytochrome c was calculated to be 1.7 × 10⁻⁶ M, and Kᵢ for Tiron, to be 5 × 10⁻⁸ M. This inhibition was not competitive with respect to xanthine, salicylaldehyde, or oxygen. When the data of Fig. 2 were plotted according to Johnson, Eyring, and Williams (26), a slope of 1.0 ± 0.4 was obtained, indicating...
Tiron effected a 50% inhibition of oxygen consumption in the affected, as were the relative rates of oxygen and cytochrome c. The iron, molybdenum, and FAD contents of the enzyme were unaltered, and no enzymic activity was observed in any of the usual assays. Moreover, the several changes of water, resulted in no observable diminution in the inhibition by Tiron. Cytochrome c was weakly inhibited by Tiron, but this inhibition was noncompetitive with respect to cytochrome c. For this process, $K_i$ for Tiron was estimated to be approximately $4.4 \times 10^{-3}$ M. The inhibition of rabbit liver aldehyde oxidase, an enzyme whose properties resemble those of milk xanthine oxidase in many respects, will be reported elsewhere.

**Effect of 8-Hydroxyquinoline**—The reduction of cytochrome c by xanthine oxidase has been reported to be accelerated by 8-hydroxyquinoline (27, 28). However, under the conditions employed in these studies, i.e., a highly purified enzyme operating in the presence of buffer containing EDTA, reduction of cytochrome c was weakly inhibited by 8-hydroxyquinoline. The inhibition was competitive with respect to cytochrome c, oxygen, or xanthine; $K_i$ for 8-hydroxyquinoline was calculated to be $2 \times 10^{-4}$ M. Since this effect was not revealing with respect to the mechanism of action of the enzyme, no attempt was made to study the effects of 8-hydroxyquinoline on the operation of the enzyme with other electron acceptors.

**Influence of Tris(hydroxymethyl)aminomethane**—Like Tiron, Tris was observed to inhibit competitively the reduction of cytochrome c by xanthine oxidase with any of its usual substrates, whereas it was without influence on the reduction of oxygen or 2,6-dichlorophenol indophenol. Although ordinarily the reduction of cytochrome c by xanthine oxidase in phosphate-buffered media is linear with time, the presence of Tris introduced a lag. The inhibition of cytochrome c by Tiron was uncompetitive with respect to cytochrome c, oxygen, and xanthine; $K_i$ for 8-hydroxyquinoline was calculated to be $2 \times 10^{-4}$ M. Since this effect was not revealing with respect to the mechanism of action of the enzyme, no attempt was made to study the effects of Tris on the operation of the enzyme with other electron acceptors.

### Table I

<table>
<thead>
<tr>
<th>Contents</th>
<th>$\Delta$nm/min</th>
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</thead>
<tbody>
<tr>
<td>No inhibitor</td>
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</tr>
<tr>
<td>Tiron ($4.5 \times 10^{-4}$ M)</td>
<td>0.022</td>
</tr>
<tr>
<td>Tiron ($4.5 \times 10^{-4}$ M) + 10 MoO$_4^-$</td>
<td>0.024</td>
</tr>
<tr>
<td>Tiron ($4.5 \times 10^{-4}$ M) + 0.42 Fe$^{3+}$ per Tiron</td>
<td>0.038</td>
</tr>
<tr>
<td>Tiron ($4.5 \times 10^{-4}$ M) + 0.84 Fe$^{3+}$ per Tiron</td>
<td>0.072</td>
</tr>
<tr>
<td>Tiron ($4.5 \times 10^{-4}$ M) + 1.26 Fe$^{3+}$ per Tiron</td>
<td>0.082</td>
</tr>
</tbody>
</table>

**Fig. 3.** The effect of the concentration of Tris on the calculated $K_i$ for Tris in the cytochrome c reduction assay of xanthine oxidase. Each cuvette contained 5 $\mu$g of catalase, 0.5 $\mu$g of xanthine, 8 $\mu$g of xanthine oxidase, and the indicated concentrations of Tris, in a final volume of 10.0 ml buffered as in Fig. 1. The concentration of cytochrome c was varied at each concentration of Tris.
of Tris. Since the rate of cytochrome c reduction, in the presence of Tris, remained proportional to the concentration of enzyme even at extremely low concentrations of enzyme, it appeared unlikely that the samples of Tris employed contained traces of a contaminant which served as a stoichiometric inhibitor of the enzyme.

At 0.05 M, Tris also inhibited the luminescence assay with DRA by 95%. Since Tris served as a chain breaker, it was not possible to examine its effects in the sulfite autoxidation system. Nitrate reduction was inhibited approximately 30% in the presence of 0.025 M Tris.

Inhibition of Xanthine Oxidase by Myoglobin—Myoglobin or the globin moiety thereof obtained from pig or horse heart has been shown to inhibit competitively the reduction of cytochrome c by xanthine oxidase, although it is without influence on the reduction of oxygen or dyes (11). At a concentration of 1.0 µg per ml, horse heart myoglobin inhibited by 80% the luminescence of a system containing DBA*, xanthine, and xanthine oxidase. At this concentration, the initiation of sulfite autoxidation by xanthine oxidase in the presence of xanthine was inhibited approximately 33%, the extent of inhibition being proportional to myoglobin concentration.

Influence of EDTA on Xanthine Oxidase—The following studies were conducted in essentially metal-free solutions which were prepared by treatment with dithioerythrite as described above. Variation in the concentration of EDTA from 10⁻⁶ M to 10⁻⁴ M was without effect on the reduction of oxygen or of cytochrome c by xanthine oxidase in the presence of xanthine. Nor did EDTA affect the Tiron-induced inhibition of the reduction of cytochrome c. However, the inhibition of xanthine oxidase by Tris was markedly affected by the presence of EDTA. Thus, in the presence of 10⁻⁴ M EDTA, for Tris was 2.6 × 10⁻⁵ M, and in the presence of 10⁻⁴ M EDTA, Tris; for Tris increased to 1.3 × 10⁻³ M. At 10⁻⁴ M EDTA, the Tris inhibition of cytochrome c reduction by xanthine oxidase was entirely eliminated. In similar fashion, the inhibition by Tris of luminescence in the xanthine oxidase-xanthine-DBA system was also reversed by EDTA, as was the Tris inhibition of nitrate reduction. EDTA did not influence the various inhibitory effects of myoglobin.

Inhibition by 2,4-Dinitrophenol—2,4-Dinitrophenol was found to be an effective inhibitor of xanthine oxidase in each of the assay systems employed. Thus, 1.6 × 10⁻⁴ M dinitrophenol occasioned approximately 50% inhibition of the reduction of oxygen, and of cytochrome c as well as of methylene blue. This inhibition was not competitive with respect to aldehyde or purine substrates, cytochrome c, oxygen, or dyes in the respective assay systems. The influence of dinitrophenol is completely reversible, since activity was restored by brief dialysis against several changes of water.

Inhibition of xanthine oxidase by cyanide, p-chloromercuribenzoate (22), and carbonyl reagents such as semicarbazide and hydroxylamine has been reported previously (27). Cyanide and p-chloromercuribenzoate have been found to inhibit reduction of all of the available electron acceptors for this enzyme (22). The mechanism by which the carbonyl reagents affect the enzyme is unclear. Westerfield, Richert, and Higgins (27) reported that reduction of oxygen by the enzyme was considerably more susceptible to inhibition by semicarbazide than was reduction of cytochrome c. In this laboratory, the inhibition of oxygen reduction effected by this reagent has always paralleled that of cytochrome c reduction. Thus, 3 × 10⁻³ M semicarbazide resulted in approximately 25% inhibition of both cytochrome c and oxygen reduction when the enzyme and inhibitor were preincubated for 30 minutes at room temperature.

Substrate Inhibition of Xanthine Oxidase—It has long been known that xanthine oxidase is susceptible to inhibition by its own substrates at higher concentrations. A previous publication from this laboratory indicated that, in contrast with the reduction of oxygen, reduction of cytochrome c and initiation of sulfate oxidation were found to not be sensitive to inhibition at higher substrate concentrations. These studies have been repeated with the best available preparations of enzyme and of cytochrome c. The initiation of sulfate oxidation was found to be resistant to excessive substrate inhibition, whereas oxygen reduction and cytochrome c reduction exhibited parallel sensitivity.

No adequate explanation of these results is presently available.

DISCUSSION

The dependence upon molecular oxygen of the reduction of cytochrome c by milk xanthine oxidase, originally reported by Horecker and Heppel (5), was confirmed and found to be absolute. The Kₘ for oxygen for reduction of cytochrome c, 8 × 10⁻⁴ M, was approximately 25 times that observed for the reduction of oxygen itself; the latter was found to be 2.7 × 10⁻⁴ M, in reasonable agreement with the value of 5 × 10⁻⁴ M estimated by Otsfufrand and Sturtevant (29). The large difference in Kₘ for oxygen observed for these two processes, the failure of catalase in the presence of ethanol to inhibit reduction of cytochrome c, and the failure of xanthine oxidase to reduce cytochrome c in the presence of a system containing snake venom L-amino acid oxidase (30), L-leucine, and oxygen as a hydrogen-peroxide generator, all appear to preclude the possibility that cytochrome c reduction is simply affected by a reduction product of oxygen such as H₂O₂, as had been suggested by Weber, Lenhoff, and Kaplan (7). No adequate tests have been made of the hypothesis suggested by Horecker and Heppel (5) that the reductant of cytochrome c may be a semiquinoid form of the flavin moiety of the enzyme generated by aerobic oxidation of the reduced flavin. However, the failure of nitrate to support anaerobic reduction of cytochrome c, and the inability of those metal-free flavoenzymes reported to date to reduce cytochrome c, are not in accord with this hypothesis.

The fact that globin is even more effective than myoglobin as a competitive inhibitor of cytochrome c reduction by xanthine oxidase (11) suggests that the binding of cytochrome c to the enzyme is a specific protein-protein interaction which does not directly involve the heme group of cytochrome c. Further, since the structure proposed for cytochrome c (31) places the heme within a crevice in the protein moiety, it would appear likely that the heme of cytochrome c bound to xanthine oxidase may not be in direct conductive contact with its immediate reductant in the enzyme structure. It is suggested, therefore, that the role of oxygen in cytochrome c reduction is to serve as an electron transport bridge across the gap between the heme moiety of cytochrome c and its unidentified reductant in xanthine oxidase. The operation of bridging groups, facilitating otherwise hindered electron transfers, has been demonstrated by Taube et al. (32–35). The electronic structure of oxygen (36) renders it eminently suitable to such a task, and complex ions in which such oxygen bridges occur have been described (37–39). Electron transport bridging groups have been postulated to operate by either (a) "double exchange" (40), in which the brid-
ties, but not of the latter, by Tiron and Tris. The reversal of two sites is also suggested by the inhibition of the former activity, and the induction of DBA++ luminescence occur at the same conditions, has been shown to generate a reactive radical, preceding to be O2-, capable of initiating a free radical chain reaction such as the aerobic oxidation of sulfite (2).

If the reduction of cytochrome c, the initiation of sulfite oxidation, and the induction of DBA++ luminescence occur at the same site on the enzyme, it would be expected that these processes share distinctive properties. The results summarized in Table II show this to be the case. Each of these processes is oxygen dependent, and, presumably, in each instance oxygen mediates electron transport to the external acceptor. If each of the observed processes occurs at the same enzyme site, then one might expect competition between them. Ferricytochrome c, at 2 x 10^-5 M, was observed to cause 55% inhibition of the luminescence generated by the system, xanthine oxidase-xanthine-DBA++. Because of the inhibition by O2 at high concentration, it was not possible to measure Km for oxygen in the xanthine oxidase-DBA++-xanthine luminescence system. However, Km for oxygen for the initiation of sulfite oxidation by xanthine oxidase was found to be 5.5 x 10^-4 M, in reasonable agreement with Km for oxygen for the reduction of cytochrome c and 15 times greater than Km for the reduction of oxygen to peroxide by this enzyme. These findings suggest that the binding site for oxygen for the reduction of cytochrome c, for initiation of sulfite oxidation, and for luminescence in the presence of DBA++ is other than that at which oxygen is reduced with the production of hydrogen peroxide in ordinary aerobic systems. The differentiation of these two sites is also suggested by the inhibition of the former activities, but not of the latter, by Tiron and Tris. The reversal of the latter inhibition by EDTA and other chelating agents and the competitive nature of these inhibitions with respect to cytochrome c suggest the operation of a functional metal at the cytochrome c-reducing site on xanthine oxidase. That this metal may be iron is tentatively suggested from other evidence, viz. stoichiometric amounts of ferric iron prevent the inhibition by Tiron, whereas excess molybdate is without effect; model studies of the reaction of molybdate with Tiron, followed by the change in absorbancy at 385 mu, of the reaction of ferric iron with Tiron, followed by the change in absorbancy at 485 mu, and of the effects in both model systems of EDTA and of Tris have been performed in 0.05 M phosphate buffer at pH 7.8. These experiments indicate that Tiron has much greater affinity for ferric iron than for molybdenum as molybdate, and that the reaction with iron is considerably more rapid than that with molybdate. Tris (0.05 M) did not interfere with the Tiron-molybdate reaction but did interfere with the ferric iron-Tiron reaction, indicating that Tris also has greater affinity for ferric iron than for molybdenum as molybdate. Again, several concentrations of EDTA prevented the ferric iron-Tiron reaction but were without influence on the Tiron-molybdate reaction. To the extent that these metals, within the enzyme structure, are chemically similar to the same metals in solution, these results suggest that iron may be the essential metal at the cytochrome c-reducing site of xanthine oxidase and that the EDTA chelate of this iron is capable of catalyzing reduction of cytochrome c, whereas the chelates formed with Tiron, 8-hydroxyquinoline, and Tris are inactive in this regard.

From the data in Table II it may be inferred that the 2-equivalent reduction of oxygen, quinones, and dyes, as well as the 1-equivalent reduction of ferricyanide, occur at a second site on the enzyme surface. Since reduction of these electron acceptors by the enzyme is not affected by chelating agents such as Tiron, 8-hydroxyquinolone, or Tris, and since metal free flavoproteins are also capable of catalyzing reduction of cytochrome c, whereas the chelates formed with Tiron, 8-hydroxyquinoline, and Tris are inactive in this regard.

The available evidence suggests that nitrate reduction may occur at yet a third site on the enzyme. This process is sensitive to Tiron inhibition, but only at concentrations considerably greater than those required to inhibit reduction of cytochrome c or sulfite oxidation. Moreover, it is completely insensitive to myoglobin and is actually inhibited by oxygen. In view of the reported nature of the nitrate reductases of microorganisms (43-45), it may tentatively be suggested that nitrate reduction by xanthine oxidase occurs at a molybdenum-containing site. It is noteworthy that nitrate reductase prepared from Pseudomonas aeruginosa can utilize hypoxanthine and acetaldehyde as electron donors (45).

The mechanism of excess substrate inhibition of the enzyme remains obscure, although it remains attractive to consider that this relates to the presence in the enzyme of two flavin moieties (1). The observation in the present studies that the Km for cytochrome c is not affected by a 100-fold change in the concentration of xanthine clearly indicates that cytochrome c and xanthine do not share a common binding site on the enzyme surface.

The fact that cyanide and p-chloromercuribenzoate inhibit the reduction of all known electron acceptors by xanthine oxidase, with either purine or aldehyde substrates, suggests that these two inhibitors operate early in the electron transport system of this enzyme and prevent reoxidation of the first flavin moiety, if indeed the latter is the initial acceptor of electrons from the substrate as has been previously suggested (1). It is of interest in this connection to note that the cyanide inhibition of xanthine

### Table II

<table>
<thead>
<tr>
<th>Assay</th>
<th>Inhibitors</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; O&lt;sub&gt;2&lt;/sub&gt;</th>
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<tr>
<td>O&lt;sub&gt;2&lt;/sub&gt; reduction</td>
<td>-</td>
<td>2.7 x 10^{-4} M</td>
</tr>
<tr>
<td>Ferricyanide reduction</td>
<td>-</td>
<td>Not required</td>
</tr>
<tr>
<td>Methylene blue reduction</td>
<td>-</td>
<td>Not required</td>
</tr>
<tr>
<td>Cytochrome c reduction</td>
<td>+</td>
<td>8 x 10^{-4} M</td>
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<td>Chemiluminescence</td>
<td>+</td>
<td>5.5 x 10^{-4} M</td>
</tr>
<tr>
<td>Sulfite oxidation</td>
<td>+</td>
<td>Inhibitory</td>
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</table>
oxidase, which is first order with respect to the concentration of the enzyme, is not prevented by Tiron, 2,4-dinitrophenol, or urea. Hence, the site of cyanide inhibition of the enzyme may be presumed to be distinct from the sites at which these three agents are reversibly bound. The mechanism of the reversible inhibitions of the enzyme by 2,4-dinitrophenol remains entirely obscure.

**SUMMARY**

The various catalytic activities of milk xanthine oxidase and the action thereon of several inhibitors have been studied. The reported observations permit grouping of the electron acceptors and suggest that this enzyme possesses a minimum of three sites on electron egress and that oxygen functions differently at two of these sites, being reduced to hydrogen peroxide at one and acting as a bridge to other electron acceptors, such as cytochrome c, at the other. The nature of these three sites on the enzyme is considered.

**REFERENCES**

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