Xanthine Oxidase Inactivation by Reagents that Modify Thiol Groups

By R. C. GREEN AND P. J. O'BRIEN

Department of Medical Biochemistry, University of Birmingham

(Received 27 February 1967)

1. The presence of xanthine was required for the inhibition of bovine milk xanthine oxidase by *o*-iodosobenzoate, iodoacetamide, hydrogen peroxide or *p*-chloromercuribenzoate. 2. Inactivation by *p*-chloromercuribenzoate was very rapid, was reversed by cysteine and was less in the presence of FAD. Lineweaver-Burk plots showed that the inactivation by *p*-chloromercuribenzoate was competitive with substrate. 3. Inactivation by *o*-iodosobenzoate, iodoacetamide or hydrogen peroxide could not be reversed by cysteine or xanthine. However, the presence of xanthine during the incubation with inhibitor protected the enzyme against *o*-iodosobenzoate but not against iodoacetamide or hydrogen peroxide. 4. *p*-Chloromercuribenzoate protected the enzyme against inactivation by hydrogen peroxide.

Andrews, Griffith, Mead & Stein (1960) reported that giving oxidized soya-bean oil to rats caused a marked fall in the mucosal xanthine oxidase activity. This was attributed to inactivation of the enzyme by a lipid peroxide. Siegel & Weintraub (1952) found that relatively crude enzyme preparations were inactivated by hydrogen peroxide, but according to Bergel & Bray (1959) purified xanthine oxidase is resistant. More recently, Bray & Watts (1966) reported that the presence of xanthine made the enzyme much more sensitive to inactivation by iodoacetamide. This action of xanthine could be simulated by reducing agents, and was apparently due to the exposure of an essential thiol group.

In this paper we have compared the effects of peroxide, *o*-iodosobenzoate, *p*CMB* and iodoacetamide on purified bovine milk xanthine oxidase in the presence of xanthine. A preliminary account of this work has been published (Green & O'Brien, 1967).

METHODS AND MATERIALS

Materials. Bovine milk xanthine oxidase was a highly purified preparation provided by Dr L. J. Hart and Dr R. C. Bray. Linoleic acid hydroperoxide was prepared and purified as described by O'Brien & Frazer (1966). Succinoyl peroxide was prepared by the method of Clover & Houghton (1904). Catalase (type CX10) was obtained from Boehringer Corp. Ltd., London, W. 5. All other reagents were of analytical reagent or laboratory reagent grade.

Enzyme assay. Xanthine oxidase activity was assayed at 23° by following the formation of urate from 0.1 mM-xanthine, equilibrated with air, in 0.05 M-tris-HCl buffer, pH 8.2. The increase in E_{295} was measured with a Unicam SP.700 automatic recording spectrophotometer.

* Abbreviation: *p*CMB, *p*-chloromercuribenzoate.

Inactivation experiments. Preincubation in the presence of xanthine and inhibitors was carried out *in vacuo* to prevent oxidation of the xanthine. The enzyme was tipped from a side arm of the evacuated Warburg vessel into a mixture of buffer, xanthine and inhibitor. After incubation, a sample was taken for assay of enzymic activity. It was confirmed that, by evacuation of the incubation vessel, the enzymic reaction was decreased to such an extent that unchanged xanthine remained even after 1 hr.

Inhibition by *p*CMB was very rapid (Fig. 1). The *p*CMB was simply added to the assay system and the initial reaction rate was immediately measured at various substrate concentrations. With *o*-iodosobenzoate, iodoacetamide or H_2O_2 , the enzyme was preincubated anaerobically for 2-3 min. in the presence of xanthine. Excess of the inhibitor was removed with cysteine (or catalase in the case of H_2O_2), and the enzyme solution was then left until all the xanthine had been oxidized to urate (as shown by E_{295}). Samples of the inhibited enzyme were then assayed immediately in the presence of various substrate concentrations.

RESULTS

Inactivation by hydrogen peroxide. Hydrogen peroxide caused inactivation of xanthine oxidase only in the presence of xanthine (Table 1). Urate (0.05 mM), the reaction product, did not simulate this effect of xanthine, and the presence of oxygen did not increase the inactivation in the presence of xanthine. This indicates that binding of the electron acceptor was not essential for inactivation to occur. Bergel & Bray (1959) showed that inactivation by hydrogen peroxide in the absence of xanthine was obtained only by incubating for 24-70 hr. with high concentrations of peroxide.

Inactivation by hydrogen peroxide was fairly slow (Fig. 1). The rate of inactivation was less at

Table 1. *Inhibition of xanthine oxidase by hydrogen peroxide*

Unless otherwise stated, xanthine oxidase (final concn. $0.1 \mu\text{M}$) was incubated anaerobically for 15 min. with H_2O_2 in the presence of xanthine (0.1 mM) and phosphate (0.05 M) or tris-HCl buffer (0.05 M). Excess of H_2O_2 was removed with catalase before enzyme assay.

Concn. of H_2O_2 (mM)	Buffer	Inactivation by H_2O_2 (%)
0.2*	Tris, pH 8.2	0
0.2	Tris, pH 8.2	57
0.2†	Tris, pH 8.2	40
0.5	Phosphate, pH 8.2	69
0.5	Phosphate, pH 6.1	35

* Control incubation without xanthine.

† With haematin (final concn. $80 \mu\text{M}$).

Fig. 1. Inactivation curves with different inhibitors. Xanthine oxidase ($0.1 \mu\text{M}$) was incubated anaerobically with xanthine (0.1 mM) in 0.05 M -tris-HCl buffer, pH 8.2. Also present in the incubation media were (final concns.): $\circ$, $23 \mu\text{M}$ -pCMB; Δ , 0.2 mM - H_2O_2 ; $\square$, $30 \mu\text{M}$ -*o*-iodosobenzoate; $\bullet$, $36 \mu\text{M}$ -iodoacetamide.

pH 6.1 than at pH 8.2 (Table 1). If the enzyme was treated with hydrogen peroxide so that 50% inactivation occurred within 1 min., no restoration of activity could be obtained by the immediate addition of an excess of cysteine (up to 50 mM). This is in contrast with the inactivation of glyceraldehyde phosphate dehydrogenase by hydrogen peroxide, which is readily reversed by cysteine (Little & O'Brien, 1966b).

A Lineweaver-Burk plot for the hydrogen peroxide-inhibited enzyme (Fig. 2) shows that K_m is unaltered by the inactivation. It was also shown that xanthine does not protect the enzyme from inactivation by hydrogen peroxide (Fig. 6).

Oxidation of thiol groups by hydrogen peroxide is readily catalysed by haem compounds (Little &

Fig. 2. Lineweaver-Burk plots, with xanthine as substrate. $\circ$, pCMB-inhibited; Δ , H_2O_2 -inhibited; $\square$, *o*-iodosobenzoate-inhibited; $\bullet$, no inhibitor. See the Methods and Materials section for details. Initial velocity, v , is expressed in activity units: 1 unit represents $18 \cdot 2 \mu\text{moles}$ of urate formed/min.

O'Brien, 1966b). However, cytochrome *c* or haematin decreased the inactivation of xanthine oxidase by hydrogen peroxide (Table 1).

Action of other peroxides. Organic peroxides oxidize protein thiol groups (Little & O'Brien, 1966a), and inactivate some enzymes that contain essential thiol groups (Little & O'Brien, 1966b). However, purified linoleic acid hydroperoxide (0.1 mM), *tert*-butyl hydroperoxide (0.3 mM), or succinyl peroxide (0.3 mM) had no effect on the enzyme in the presence of xanthine.

Inactivation of *o*-iodosobenzoate. Harris & Hellerman (1956) reported that *o*-iodosobenzoate inhibits xanthine oxidase activity. However, we have found that the presence of xanthine is essential for this inhibition. The initial inactivation was rapid and was followed by a slower phase (Fig. 1). *o*-Iodosobenzoate is a much more effective inhibitor than hydrogen peroxide. *o*-Iodosobenzoate inactivation could not be reversed by adding an excess of cysteine.

A Lineweaver-Burk plot for the *o*-iodosobenzoate-inhibited enzyme (Fig. 2) was similar to that for the hydrogen peroxide-inhibited enzyme. However, if the xanthine concentration was increased above that needed to give maximal inhibition, protection of the enzyme against *o*-iodosobenzoate was observed (Fig. 6).

Inactivation by pCMB. The initial inactivation of xanthine oxidase by pCMB in the presence of xanthine was extremely rapid (Fig. 1). For this reason, the possible involvement of xanthine in the inactivation process cannot be demonstrated unless unbound pCMB is removed before assay. Fridovich & Handler (1958) suggested that xanthine increased the sensitivity of xanthine oxidase to pCMB, but their evidence seems inconclusive since they used cysteine, which brings about immediate reversal of

Fig. 3. Inactivation of xanthine oxidase by *p*CMB. Xanthine oxidase ($0.04 \mu\text{M}$) was incubated for 3 min. with various concentrations of *p*CMB, in 0.05 M -tris-HCl buffer, pH 8.2, with xanthine (0.1 mM).

*p*CMB inactivation, to remove the excess of *p*CMB. However, Brumby, Miller & Massey (1965) have shown that no inactivation of the pig-liver enzyme occurs in the absence of xanthine if the unchanged *p*CMB is removed on a Sephadex column before enzyme assay. No data are available on the number of thiol groups bound by *p*CMB in the presence of xanthine. In the absence of xanthine, one or two thiol groups react within the first few minutes (Bergel & Bray, 1959).

Fig. 3 shows a titration of *p*CMB against enzyme activity in the presence of xanthine. The degree of inactivation was proportional to *p*CMB concentration; the lack of an initial lag indicates that *p*CMB does not react with groups that are not essential for the enzymic activity before it reacts with groups involved in the enzymic activity.

The kinetics of the *p*CMB-inhibited enzyme (Fig. 2) showed that $12 \mu\text{M}$ -*p*CMB caused K_m to increase from 10^{-5} M to 10^{-4} M , whereas V_{max} was unchanged. It seems therefore that *p*CMB was inhibiting competitively.

The inactivation caused by *p*CMB was readily reversed by cysteine. Even after 95% inactivation, cysteine restored the activity to 71% of its original value. In these experiments, the *p*CMB was in contact with the enzyme in the presence of xanthine for only a few minutes before cysteine was added. Bergel & Bray (1959) reported that, after prolonged incubation of the enzyme with *p*CMB, inactivation could not be reversed by GSH, presumably owing to irreversible denaturation of the enzyme.

Protection against hydrogen peroxide inactivation by *p*CMB. This is illustrated in Fig. 4. The enzyme

Fig. 4. Protection of enzyme by *p*CMB. The medium consisted of xanthine (0.1 mM) in 0.05 M -tris-HCl buffer, pH 8.2. Curve I, untreated enzyme; curve II, enzyme inhibited by H_2O_2 ; curve III, enzyme protected against H_2O_2 inhibition by *p*CMB. Arrows denote times of additions as follows (final concns.): E, $35 \mu\text{M}$ -xanthine oxidase; H, 20 mM - H_2O_2 ; C, 42 mM -cysteine; P, 0.2 mM -*p*CMB.

Fig. 5. Lineweaver-Burk plots for iodoacetamide inhibition. The enzyme was preincubated anaerobically for 2–3 min. in the presence of xanthine (see the Methods and Materials section). Final concns.: $\circ$, no inhibitor; Δ , 0.09 mM -iodoacetamide; $\bullet$, 0.36 mM -iodoacetamide; $\square$, 0.90 mM -iodoacetamide. Initial velocity, v , is expressed in activity units (see Fig. 2).

was treated with *p*CMB to give 95% inactivation. Enough hydrogen peroxide was then added so that 95% inactivation of the native enzyme would have occurred in the absence of *p*CMB. Excess of cysteine was then added to remove unused hydrogen peroxide and to remove *p*CMB from the enzyme. The activity was then 45% of that of the native enzyme. As cysteine could not reactivate the enzyme after treatment with hydrogen peroxide in the absence of *p*CMB, it is concluded that *p*CMB partially protects the enzyme from hydrogen peroxide inactivation.

Inactivation by iodoacetamide or iodoacetate. A detailed investigation of the reaction of iodoacetamide with xanthine oxidase has been described by Bray & Watts (1966).

Fig. 6. Effect of xanthine on inactivation of xanthine oxidase. Xanthine oxidase ($0.1 \mu\text{M}$) was incubated *in vacuo* with various concentrations of xanthine in 0.05M -tris-HCl buffer, pH 8.2. Also present during incubation were (final concns.): O, 5mM -H₂O₂; Δ , $13 \mu\text{M}$ -*o*-iodosobenzoate; $\square$, 0.4mM -iodoacetamide. Excess of inhibitor was removed by catalase or cysteine after 5 min. incubation, and enzyme activity was assayed in 0.1mM -xanthine 3 min. later.

Fig. 1 shows the progress of inactivation by iodoacetamide, and the Lineweaver-Burk plots (Fig. 5) show that K_m is not altered by this inhibitor. As with hydrogen peroxide, increasing the concentration of xanthine above that needed to give maximal inactivation did not protect the enzyme against iodoacetamide (Fig. 6).

The rate of inactivation by iodoacetate was about 1.6 times less than that by iodoacetamide, as would be expected for alkylation of a thiol group. Thus the relatively poor reactivity of an essential thiol group with *p*CMB cannot be attributed to a negatively charged environment around the essential thiol group of the enzyme.

Protection of the enzyme with FAD. Addition of 40 moles of FAD/mole of *p*CMB gave 10% protection against *p*CMB inhibition. FAD gave no measurable protection against inactivation by *o*-iodosobenzoate or iodoacetamide.

DISCUSSION

Bray & Watts (1966), using a silver-silver iodide electrode, have shown that in the initial reaction of iodoacetamide with milk xanthine oxidase 1 thiol group/mol. of enzyme probably reacts. Recently, T. Keleti & R. C. Bray (personal communication), with $\text{iodo}[^{14}\text{C}]\text{acetate}$, confirmed that alkylation of a thiol group had occurred. Further, R. C. Bray, A. M. Scott & L. I. Hart (personal communication) have shown that inactivation may be due to alkylation of a thiol group involved in binding FAD to the apoenzyme, since part of the FAD is released during the inactivation. Bray & Watts (1966) have also discussed possible mechanisms for the apparent exposure of this essential thiol group when xanthine oxidase is reduced by xanthine, salicylaldehyde or

dithionite. They reported that the initial rate of reaction with iodoacetamide was followed by a slower phase, during which about 12 thiol groups/mol. of enzyme reacted.

We have found that hydrogen peroxide and *o*-iodosobenzoate are similar to iodoacetamide in that inactivation occurs only in the presence of substrate. Since preincubation of the enzyme with *p*CMB protected the enzyme against inhibition by hydrogen peroxide, it is likely that these two reagents react at the same site, presumably a thiol group.

The rate of inactivation by hydrogen peroxide was faster at an alkaline pH. Little & O'Brien (1967) have shown that such an oxidation of thiol groups is most rapid when the groups are fully ionized. Restoration of the activity of a thiol-dependent enzyme can be obtained with cysteine in some cases if it is added immediately after incubation with hydrogen peroxide (Little & O'Brien, 1966b) or *o*-iodosobenzoate (Rafter, 1957). However, cysteine had no effect on xanthine oxidase inactivated by either of these reagents. It is likely therefore that oxidation of the thiol group to a high oxidation state, such as a sulphonic acid, had occurred, although the possibility cannot be ruled out that inactivation may be due, in part at least, to modification of other amino acid residues such as tyrosine, methionine or tryptophan (O'Brien, 1966).

Certain other enzymes have also been reported to be more sensitive to hydrogen peroxide in the presence of substrate than in its absence. These include glucose oxidase (Kleppe, 1966), trypsin and chymotrypsin (Schachter, Halliday & Dixon, 1963).

Inactivation by *p*CMB differed from inactivation by the other reagents in that it was more rapid, reversed by cysteine, less in the presence of FAD and formally competitive with substrate. The competitive inhibition by benzoate, reported by Bergel & Bray (1956), was effective only at concentrations of about 0.1M (for 75% inhibition), and so the competitive inhibition by *p*CMB cannot be explained by competition due to the benzoate moiety.

Inactivation of D-amino acid oxidase by *p*CMB is also competitive, reversible by glutathione, and less if FAD is present (Frisell & Hellerman, 1957). This inactivation has been attributed to the carboxyl group of the *p*CMB competing with the amino acid substrate for the substrate-binding site, since benzoate is also an effective competitive inhibitor. However, binding of *p*CMB with a thiol group also occurs. The sensitive thiol group is responsible for the binding of FAD to the apoenzyme (Yagi & Ozawa, 1960). It is possible that the inactivation of xanthine oxidase occurs by a mechanism similar to the latter one for D-amino acid oxidase.

The failure of xanthine to protect the enzyme against inactivation by hydrogen peroxide or iodoacetamide seems to rule out the possibility of the sensitive thiol group being situated directly at the substrate-binding site. The approach of iodoacetamide or hydrogen peroxide would not be hindered by the xanthine if the xanthine-binding site and the thiol group were a sufficient distance apart, but, since *p*CMB and *o*-iodosobenzoate are larger molecules, the presence of xanthine might shield the thiol group from these compounds. Since *p*CMB binding is reversible, a formal competitive inhibition is observed. Oxidation of the thiol group by *o*-iodosobenzoate is irreversible, however, and so no formally competitive inhibition is seen (Fig. 2), although xanthine protects against *o*-iodosobenzoate (Fig. 6).

Rabbit-liver aldehyde oxidase is also inhibited by *p*CMB only in the presence of substrate (Rajagopalan & Handler, 1964). However, pig-liver xanthine oxidase is inhibited non-competitively by *p*CMB (Brumby *et al.* 1965). This may indicate that in the pig-liver enzyme the sensitive thiol group is not as close to the substrate-binding site as in the milk enzyme.

The lack of effect of lipid peroxide and other organic peroxides even in the presence of xanthine may be due to steric hindrance. The marked fall in mucosal xanthine oxidase observed on giving oxidized lipid to rats is probably due therefore to a metabolic lesion connected with protein depletion (Litwack, Williams, Feigelson & Elvehjem, 1950), rather than to direct inactivation of the enzyme.

We thank the Nuffield Foundation for financial support, and Dr R. C. Bray for helpful discussion.

REFERENCES

- Andrews, J. S., Griffith, W. H., Mead, J. F. & Stein, R. A. (1960). *J. Nutr.* **70**, 199.
Bergel, F. & Bray, R. C. (1956). *Nature, Lond.*, **178**, 88.
Bergel, F. & Bray, R. C. (1959). *Biochem. J.* **73**, 182.
Bray, R. C. & Watts, D. C. (1966). *Biochem. J.* **98**, 142.
Brumby, P. E., Miller, R. W. & Massey, V. (1965). *J. biol. Chem.* **240**, 2222.
Clover, A. M. & Houghton, A. C. (1904). *J. Amer. chem. Soc.* **32**, 43.
Fridovich, I. & Handler, P. (1958). *J. biol. Chem.* **231**, 899.
Frisell, W. R. & Hellerman, L. (1957). *J. biol. Chem.* **225**, 53.
Green, R. C. & O'Brien, P. J. (1967). *Biochem. J.* **102**, 11f.
Harris, J. & Hellerman, L. (1956). In *Inorganic Nitrogen Metabolism*, p. 565. Ed. by McElroy, W. D. & Glass, H. B. Baltimore: The Johns Hopkins Press.
Kleppe, K. (1966). *Biochemistry*, **5**, 139.
Little, C. & O'Brien, P. J. (1966a). *Biochem. J.* **101**, 11f.
Little, C. & O'Brien, P. J. (1966b). *Biochem. J.* **101**, 13f.
Little, C. & O'Brien, P. J. (1967). *Biochem. J.* **102**, 10f.
Litwack, G., Williams, J. N., Feigelson, P. & Elvehjem, C. A. (1950). *J. biol. Chem.* **187**, 605.
O'Brien, P. J. (1966). *Biochem. J.* **101**, 12f.
O'Brien, P. J. & Frazer, A. C. (1966). *Proc. Nutr. Soc.* **25**, 9.
Rafter, G. W. (1957). *Arch. Biochem. Biophys.* **67**, 267.
Rajagopalan, K. V. & Handler, P. (1964). *J. biol. Chem.* **239**, 2027.
Schachter, H., Halliday, K. A. & Dixon, G. H. (1963). *J. biol. Chem.* **238**, 3134.
Siegel, S. M. & Weintraub, R. L. (1952). *Biochem. J.* **52**, 324.
Yagi, K. & Ozawa, T. (1960). *Biochim. biophys. Acta*, **42**, 381.