The Chemistry of Xanthine Oxidase

REACTION WITH IODOACETAMIDE

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1. The reaction of milk xanthine oxidase with iodoacetamide has been studied with the silver-silver iodide electrode. 2. The reaction proceeds considerably faster in the presence of xanthine than in its absence. Anaerobically, with excess of xanthine, the reaction takes place as a rapid phase in which the enzyme is inactivated and in which approx. 1 thiol group/mol. of enzyme reacts and as a slower phase in which about 12 groups/mol. react. 3. The rapid reaction appears to be first-order with respect to xanthine oxidase and iodoacetamide and independent of the xanthine concentration with more than about 3 mol. of xanthine/mol. of enzyme. 4. The velocity constant of the rapid phase is 0.26 min.^{-1} at 25° and pH 7.0, with 1 mM-iodoacetamide and $17 \mu\text{M}$ -xanthine oxidase. The velocity constant for the slower phase is about one-hundredth of this value. 5. The velocities of both phases increase with increasing pH in the range 5.0–9.6. 6. Xanthine may be replaced by salicylaldehyde without affecting the rate of loss of enzymic activity. With sodium dithionite as reducing agent, the reaction is slightly faster. 7. The possible function of thiol groups in the reaction mechanism of the enzyme is discussed.

The importance of thiol groups in the reaction mechanism of milk xanthine oxidase is implied by the work of Mackworth (1948), Harris & Hellerman (1956), Wills (1956), Peters & Sanadi (1961) and Gilbert (1963). Bergel & Bray (1959) found that, of at least 35 groups in the enzyme that could react with sodium *p*-chloromercuribenzoate, 9 groups reacted relatively rapidly (half-reaction time about 2 hr. at 24°) but without affecting the enzymic activity. In contrast with this, Fridovich & Handler (1958) found that in the presence of the substrate the enzyme was rapidly inactivated by the mercurial. However, they did not study the stoichiometry of the reaction. Their work was confirmed by Brumby, Miller & Massey (1965) with the liver enzyme. These workers also titrated this enzyme amperometrically with phenylmercuric acetate and reported that, when the enzyme was reduced with hypoxanthine, the titre increased by about 4 thiol groups/mol. of enzyme flavine. Gilbert (1962) found that the milk enzyme was stable in the presence of iodoacetamide, but that on adding xanthine rapid inactivation took place.

The present investigation describes quantitative studies, with the silver-silver iodide electrode (Watts, Rabin & Crook, 1961), on the reaction with iodoacetamide of those reactive groupings in the milk enzyme that appear on reduction. A single

thiol group in the molecule is implicated. The possible role of such a group in the catalytic mechanism and the group's relationship to other components of the enzyme are discussed.

MATERIALS

Chemicals. Iodoacetamide (from L. Light and Co. Ltd., Colnbrook, Bucks.) was recrystallized five times from aq. 50% (v/v) ethanol, as described by Watts *et al.* (1961). Phenylxanthine was a gift from Professor F. Bergmann. All other reagents were of A.R. grade where obtainable.

Buffers. Pyrophosphate buffer was prepared from the di- and tetra-sodium salts; acetate buffer was prepared from acetic acid and NaOH (Datta & Grzybowski, 1961); tris-maleate and carbonate buffers were prepared as described by Gomori (1955).

Xanthine oxidase. The enzyme was prepared from milk as described by Gilbert & Bergel (1964) and by Palmer, Bray & Beinert (1964), and high-molecular-weight impurities were removed by gel filtration on a column of Sephadex G-200 (cf. Andrews, Bray, Edwards & Shooter, 1964). The samples used generally had activity/ E_{450} values approx. 100 (cf. Bray, Petersson & Ehrenberg, 1961), but in one case (Fig. 7) the value was only 50. This sample had a molybdenum content, determined by a modification (L. I. Hart, unpublished work) of the dithiol method, of 0.9 atom of Mo/mol. of xanthine oxidase, whereas another sample (Fig. 4), with activity/ E_{450} 100, had 1.3 atoms of Mo/mol. of xanthine oxidase. Xanthine oxidase concen-

trations were calculated from E_{450} values by taking ϵ to be 70000. For anaerobic experiments, the enzyme was concentrated by vacuum-dialysis against a suitable buffer containing EDTA (as shown in the legends to the Figures) and the solution (approx. 0.25 mm) was made anaerobic by evacuating and flushing the tube in which it was contained several times with argon purified with vanadous sulphate (Meites & Meites, 1948). The enzyme was then drawn into a syringe with a Teflon plunger (Hamilton Co., Whittier, Calif., U.S.A.) previously flushed with argon, and was dispensed from this syringe into the reaction vessel (see below).

METHODS

Reaction with iodoacetamide. For the aerobic experiments the reaction was carried out as described by Watts *et al.* (1961), with a silver-silver iodide electrode assembly to follow the production of I^- ions, with a total volume of 4.0–4.7 ml. at 25°. For the anaerobic experiments a special cell was constructed (Fig. 1) and a reaction volume of 2.3 ml. was employed. Reagents, as described in the legends to the Figures, were pipetted into the cell and deoxygenated by bubbling purified argon through the mixture for 15 min. The bubbler (*B* in Fig. 1) was then withdrawn and argon was blown rapidly over the surface of the reaction mixture via G_1 (Fig. 1), to exclude oxygen. (Argon was preferred to nitrogen for this purpose owing to its greater density.) The reaction was initiated by momentarily stopping the stirrer, removing the stopper (*S* in Fig. 1) and adding either

deoxygenated xanthine oxidase solution from a syringe (see the Materials section) or deoxygenated xanthine from a pipette previously flushed with argon. Samples of the reaction mixture for enzyme assay were withdrawn with a pipette in a similar manner and delivered rapidly into an excess of ice-cold cysteine solution (2 mM in 50 mM-pyrophosphate buffer, pH 8.2) to quench the alkylation reaction. In some experiments the iodide production curves showed signs of a short induction period. Since this was probably due to slow mixing of the dense xanthine oxidase solution into the reaction medium, a 0.5 min. correction was applied to the times where appropriate.

The anaerobic technique was similar when iodide production was not followed and only activity measurements were made, but the total volume was decreased to 0.5 ml. Sodium dithionite solution was prepared and standardized as described by Bray *et al.* (1961).

Assay of xanthine oxidase. Activity was determined spectrophotometrically, with xanthine as substrate, as described by Bergel & Bray (1959), on portions of the reaction mixture withdrawn and diluted as described above.

RESULTS

Aerobic experiments

It was found (Fig. 2) that the enzyme liberated I^- ions from iodoacetamide more rapidly when xanthine was added to the reaction mixture than it did in the absence of a substrate. However, after a short period, i.e. at about the time when it would be expected that all the xanthine had been oxidized by the enzyme, the rate of iodide production fell

Fig. 1. Apparatus used for following the anaerobic reaction with iodoacetamide. The constant-temperature water jacket (inlet: W_1 ; outlet: W_2) surrounds a magnetically stirred central reaction chamber, with 1.4 cm. diam. and 2.5 ml. capacity. The iodide electrode, E_1 , is inserted through a standard B14 joint in the top of the vessel and the satd. KNO_3 bridge, E_2 , into which is inserted the calomel reference electrode, is fused through the water jacket into the side of the reaction chamber. Mixing is prevented by a sintered plug. Argon is blown over the surface of the reaction mixture via G_1 or is bubbled through it via a removable polythene bubbling tube, B , which passes through a B10 joint, S . Gas escapes through G_2 . The B10 stopper is also used for making additions to, or removing samples from, the reaction mixture.

Fig. 2. Effect of xanthine on the aerobic reaction of xanthine oxidase ($2.6 \mu M$) with iodoacetamide ($12 mM$). At the times shown by the arrows 0.1 ml. portions of xanthine (equivalent to a final concentration of 0.25 mM) were added. The reaction medium (4 ml. at 25°) was as follows (final concentrations): tris-maleate buffer, pH 7.2, 20 mM; EDTA, 0.4 mM; KI, $1 \mu M$; Tween 80, 0.02% (v/v). At the end of the experiment 14% of the xanthine-oxidase activity remained.

back to its original level. The rate could then be re-elevated by successive further additions of xanthine (Fig. 2). During the period of rapid iodide liberation enzymic activity was progressively lost; negligible activity was lost before xanthine was added. In control experiments, xanthine (final concn. 2.5 mM) added to the reaction mixture in the absence of xanthine oxidase gave a small increase in the background rate of iodide liberation but this was in no way comparable with the effect produced in the presence of the enzyme.

Anaerobic experiments

The aerobic experiments showed that iodoacetamide reacts rapidly with xanthine oxidase only in the presence of the substrate, xanthine. Anaerobic studies were therefore undertaken to obtain further information and to confirm that oxygen was not involved in activating the enzyme towards the reagent.

Effect of xanthine oxidase concentration. Fig. 3 shows the time-course of iodide production at three different xanthine oxidase concentrations, other conditions remaining the same. Xanthine was added in excess at the time indicated and, as in the

Fig. 3. Effect of xanthine oxidase concentration on the anaerobic reaction of xanthine oxidase with iodoacetamide (1.0 mM). The final xanthine oxidase concentrations were: ▲, 8.5 μ M; ○, 17 μ M; ●, 34 μ M. After 18 min. (indicated by an arrow) 0.1 ml. of xanthine (equivalent to a final concentration of 0.44 mM) was added to each sample. The reaction medium (2.3 ml. at 25°) was as follows (final concentrations): tris-maleate buffer, pH 7.4, 40 mM; EDTA, 0.07 mM; KI, 1 μ M; Tween 80, 0.02% (v/v).

aerobic experiments, it caused an immediate increase in the rate of iodide production. After 10–15 min. the rate decreased, but to a value higher than it had been before the addition of substrate. The rates of iodide production at all phases of the reaction increased as the enzyme concentration was raised. Similarly, the extent of the rapid phase of the reaction, which started when the xanthine was added, increased in proportion to the xanthine oxidase concentration.

Effect of iodoacetamide concentration. In general, in the anaerobic work, an iodoacetamide concentration of 1 mM was employed. In one experiment this was increased to 4 mM. The initial rate of the rapid phase of the reaction now too high for accurate measurement but it appeared to be at least 3 times as fast as that at the lower reagent concentration.

Effect of xanthine concentration. A series of experiments was performed with constant xanthine oxidase and iodoacetamide concentrations but with various xanthine concentrations. In Fig. 4(a) the curves of iodide production against time and of activity against time are presented for an experiment with excess of xanthine. Fig. 4(a) shows that the iodide production curve may be expressed as the sum of first-order and zero-order reactions which, when added, give a theoretical curve fitting the experimental data well. In this experiment the loss of activity went to completion and paralleled closely the first-order alkylation reaction. Fig. 4(b) shows a similar experiment at a lower xanthine concentration. Here, the activity loss was only partial but again it appeared to parallel the first-order reaction. In Fig. 5, the initial iodide rate and the extent of inactivation after 10 min. are shown as a function of xanthine concentration for a series of experiments of the above type. Inactivation did not go to completion and initial iodide production rates were submaximal with less than 3–4 mol. prop. of substrate.

Effect of pH. A series of experiments was carried out in the pH range 5.0–9.6, with an excess of xanthine. The stability of the enzyme in the reaction medium in the absence of xanthine at the two extremes of pH was first checked. At pH 9.6 the activity apparently increased by 4% in 11 min., whereas at pH 5.0 it decreased by 8% in 10 min. These changes may be regarded as negligible in comparison with those occurring in the presence of the substrate. Fig. 6 shows an experiment at pH 9.6 in which the data have been analysed in the manner illustrated in Fig. 4(a). The rate of reaction was much higher under these conditions and, further, to obtain a fit to the iodide production curve it was necessary to assume that two first-order reactions, one fast and one slow, were proceeding, rather than a first-order and a slower zero-order reaction. Fig. 7 shows data obtained in a similar manner at a series

Fig. 4. Correlation between iodide production and loss of activity during anaerobic reaction of xanthine oxidase (16 μM) with iodoacetamide (1.0 mM) in the presence of 217 μM-xanthine (a) and of 11 μM-xanthine (b). The enzyme and substrate were mixed anaerobically at zero time. ○, Iodide concentration; ●, xanthine-oxidase activity. In (a) the theoretical line (A) through the experimental points (○) represents the sum of the broken line (B), a zero-order reaction with a velocity of 0.384 μM/min., and the third line (C), a first-order reaction with $k_1 = 0.27 \text{ min.}^{-1}$ and $\Delta I_1 = 15.0 \mu\text{M}$. A similar summation is made in (b), where the broken line (B) is a zero-order reaction with a velocity of 0.150 μM/min. and the third line (C) a first-order reaction with $k_1 = 0.17 \text{ min.}^{-1}$ and $\Delta I_1 = 6.4 \mu\text{M}$. The conditions were as given in Fig. 3 except that the concentration of EDTA was 0.03 mM.

Fig. 5. Effect of xanthine concentration on the anaerobic reaction in a series of experiments similar to those in Figs. 4(a) and 4(b). ○, Xanthine-oxidase activity after 10 min. reaction; ●, initial rate of iodide liberation.

of pH values. Though the slower reaction did not deviate significantly from zero-order kinetics during a period of about 1 hr., except at pH 8.8 and

9.6, nevertheless, first-order velocity constants were calculated from the initial observed zero-order rates by assuming that the extent of the reaction was the same (cf. Fig. 6) at all pH values. Fig. 7 shows, as a function of pH, the velocity constant for the slow phase (k_2 ; curve C), the velocity constant for the fast phase (k_1 ; curve A), the extent of the fast phase (ΔI_1 ; curve B) and the velocity constant of the fast phase as calculated from activity measurements (k_a ; curve A). (The activity measurements were made 3 min. after adding the substrate and it was assumed that complete inactivation occurred). The k_1 and k_a points both fall on curve A, indicating once again parallelism between the rapid phase of iodide production and the inactivation of the enzyme. The velocity constants of both phases of the reaction increased with increasing pH. So also, apparently, did the extent of the rapid phase reaction, which amounted to 0.7 mol. of iodide/mol. of enzyme at pH 5.0 and to 1.3 mol./mol. of enzyme at pH 9.6.

Reaction with uric acid and with phenylxanthine. With 12.8 mol. prop. of uric acid in place of xanthine,

Fig. 6. Anaerobic reaction at pH 9.6 of $17\mu\text{M}$ -xanthine oxidase with 1mM -iodoacetamide and $217\mu\text{M}$ -xanthine. $\circ$, Iodide concentration; $\bullet$, xanthine-oxidase activity. The theoretical line (A) through the experimental points ($\circ$) represents the sum of the interrupted line (B), a first-order reaction with $k_2 = 0.01\text{min}^{-1}$ and $\Delta I_2 = 210\mu\text{M}$, and the third line (C), a first-order reaction with $k_1 = 0.43\text{min}^{-1}$ and $\Delta I_1 = 22.6\mu\text{M}$. The conditions were as given in Fig. 4, except that 70mM -carbonate buffer, pH 9.6, was used in place of tris-maleate buffer.

Fig. 7. Effect of pH on the two phases of the reaction. Experiments were carried out as described in Fig. 6 and the data were analysed as in Fig. 6 or Fig. 4(a). Curve A: $\circ$, k_1 (min^{-1}); $\square$, k_a (min^{-1}) calculated from activity measurements made after 3 min. reaction. Curve B: $\triangle$, ΔI_1 (mol. of thiol group/mol. of xanthine oxidase). Curve C: $\bullet$, k_2 (min^{-1}). The buffers used were as follows: pH 5.03, 70mM -acetate; pH 9.62, 70mM -carbonate; pH 7.38, 40mM -tris-maleate; other pH values, 70mM -tris-maleate.

no increase in the iodide production rate was found at pH 7.5. With 12.8mol. prop. of 8-phenylxanthine at the same pH a brief rapid reaction was observed that corresponded to a ΔI_1 value of $0.06\text{mol. of iodide/mol. of xanthine oxidase}$.

Reaction with salicylaldehyde and with sodium dithionite. Fig. 8 gives the inactivation rates when salicylaldehyde or sodium dithionite was used to reduce the enzyme in place of xanthine. The aldehyde and purine substrates were equally effective, and dithionite gave a slightly higher rate of inactivation.

DISCUSSION

The results show clearly that reaction of approx. 1mol. prop. of iodoacetamide with xanthine oxidase reduced with xanthine can lead to complete

inactivation of the enzyme. Though it has not been proved that the reactive group in the reduced enzyme is a thiol it seems unlikely that any other group would react with iodoacetamide at a rate as high as that observed (cf. Gundlach, Stein & Moore, 1959). The presence of such a group in xanthine oxidase is in full agreement with the literature on the enzyme.

The exact number of thiol groups reacting/mol. of xanthine oxidase is not easy to determine from the stoichiometry of the fast reaction (ΔI_1 ; Figs. 4a and 7), owing to a degree of uncertainty as to the true concentration of the enzyme. The concentration was determined from E_{450} but this measures both 'active' and 'inactive' enzyme (cf. Bray *et al.* 1961). According to Morell (1952) the latter may be slowly reduced by reduced 'active' xanthine oxidase. From Morell's data the velocity

Fig. 8. Anaerobic inactivation of xanthine oxidase ($20\mu\text{M}$) by iodoacetamide (1.0mM) in the presence of xanthine ($200\mu\text{M}$) ($\circ$ and $\square$), salicylaldehyde ($190\mu\text{M}$) ($\bullet$) and sodium dithionite ($180\mu\text{M}$) ($\triangle$). The reaction was carried out in 90mM -tris-maleate buffer, $\text{pH } 5.6$, containing EDTA (0.04mM). Other conditions were as given in Fig. 3. Activities are expressed as a percentage of that found before adding the substrate or dithionite. The continuous line corresponds to the first-order velocity constant, $k_a = 0.108\text{min}^{-1}$, and the broken line to $k_a = 0.135\text{min}^{-1}$.

of this reduction seems to be comparable with the velocity we find for the iodoacetamide-mediated inactivation process. Hence it is uncertain whether 'inactive' xanthine oxidase is participating in the reaction with iodoacetamide or not. However, the fact that two samples of xanthine oxidase with very different specific activities (see the Materials section) gave values for ΔI_1 as close as 0.9 and $1.0\text{mol. of thiol group/mol. of xanthine oxidase at pH } 7.4$ (Figs. 4a and 7 respectively) suggests that both 'active' and 'inactive' enzyme must be taking part and reacting indistinguishably. It is thus reasonable to infer that 'active' xanthine oxidase has 1 reactive thiol group/mol. Comparison with the work of Brumby *et al.* (1965) suggests that substrate reduction activates more thiol groups for reaction with phenylmercuric acetate than it does for reaction with iodoacetamide. Since these authors used the liver enzyme the difference could, of course, simply reflect differences between the liver and milk enzymes. However, mercurial compounds in general, unlike iodoacetamide, are very destructive reagents and reaction is frequently accompanied by gross changes in protein structure, as indeed the data of Brumby *et al.* (1965) show. A similar phenomenon has been observed for yeast alcohol dehydrogenase (cf. Hoch & Vallee, 1959; Rabin, Ruiz Cruz, Watts & Whitehead, 1964). It is therefore suggested that phenylmercuric acetate reacts with the thiol group that is sensitive to iodoacetamide but that in addition it reacts with several more thiol groups, as a consequence of disruption of the structure of the protein by the reagent.

Increasing the pH increases the velocity (k_1) of the rapid phase of the alkylation reaction (Fig. 7) but not to the large extent that would be expected for a freely-ionizing thiol group (Hollaway, Mathias & Rabin, 1964). Further, even at the highest pH values, the velocity of the reaction is still relatively low. Hence it is concluded (cf. Watts & Rabin, 1962; Rabin *et al.* 1964) that the thiol is modified by interaction with another group on the enzyme. Interaction of the thiol group with the substrate (or product) seems to be excluded, since the reaction rate (k_a) is identical with two different substrates and is only slightly different if the enzyme is reduced with sodium dithionite (Fig. 8; see below). The reason for the small but apparently significant increase in the number of groups reacting (ΔI_1) as the pH is raised is not clear.

The experiments of the titration type (Fig. 5), in which the xanthine concentration was varied, may be compared with electron-spin-resonance titrations of the enzyme reported by Ehrenberg & Bray (1965). They found that $4\text{--}5\text{mol.prop.}$ of xanthine was required to obtain complete reduction of the enzyme. The present results suggest that about the same, or possibly a slightly lower, molar ratio of xanthine may be adequate for maximum sensitivity to iodoacetamide. In the presence of excess of xanthine the reaction appeared to be first-order with respect both to iodoacetamide and to xanthine oxidase.

It was not possible to measure iodide production when xanthine oxidase was reduced by salicylaldehyde or sodium dithionite, as these react with the silver-silver iodide electrode. Nevertheless, it is clear that they produce an effect on enzyme inactivation comparable with that given by xanthine (Fig. 8). It is concluded that the grouping reactive towards iodoacetamide is present in reduced xanthine oxidase itself and does not depend on the presence of bound substrate. The fact that sodium dithionite gave slightly more rapid inactivation than did the substrates might be due to side reactions or to a small measure of protection afforded by the substrates. Further indirect evidence that xanthine was simply reducing the enzyme was deduced from the fact that two non-reducing substances capable of interacting with the active centre failed to give any effect comparable with that produced by xanthine. Thus the reaction product, uric acid, had no detectable effect on iodide liberation. Similarly, 8-phenylxanthine, which is a competitive inhibitor of xanthine oxidase with K_i in the range $1\text{--}10\mu\text{M}$ (A. J. Chisholm, unpublished work), gave only an extremely slight enhancement in the rate of iodide production and this might have been due to reducible impurities in the phenylxanthine sample.

The slow phase of the reaction of the enzyme with

iodoacetamide must reflect general alkylation of other groups, presumably including some of those groups that react with mercurials. Its velocity is higher in the reduced enzyme, after the rapid reaction is complete, than it is in the oxidized enzyme (Fig. 3). This indicates that steric changes must be caused in the enzyme, either by reduction or by the rapid alkylation reaction. The velocity of the slow reaction increases with increasing pH.

There is at present little evidence as to how a reactive thiol is involved in the catalytic reaction of xanthine oxidase. Fridovich & Handler (1958) postulated a metal-sulphur bond in the oxidized enzyme that, on uptake of a single electron supplied by the substrate, splits to the reduced metal and a thiol group. Attractive as such a mechanism may be, direct evidence for it seems to be lacking at the present time. More recently, Rajagopalan & Handler (1964) suggested that a molybdenum mercaptide group was present in the active centre of the closely related enzyme aldehyde oxidase. On the other hand, lipoyl dehydrogenase has been reported to contain an essential disulphide group in the oxidized enzyme that is reduced by two electrons from the substrate to a pair of thiol groups (Massey & Gibson, 1964). Such a mechanism, if it operated in xanthine oxidase, would require 2 thiol groups to appear on reduction (or 4 thiol groups, if 2 active centres/mol. are assumed). The fact that we find close to 1 thiol group/mol. argues against this, but the possibility that 2 thiol groups/mol. are involved is not completely excluded by our results and hence we are unable to distinguish between these mechanisms. A related possibility is that a disulphide group in the resting enzyme is split on reduction to give 1 free thiol group, the second sulphur atom then reacting with some other group in the enzyme, perhaps, for example, with quinquivalent molybdenum. If this secondary intramolecular reaction should be incomplete at high pH values, it would explain the variation of ΔI_1 with pH. It should be pointed out that the disulphide link postulated here must, from stoichiometric considerations, be distinct from the disulphide groups proposed by Brumby *et al.* (1965) and which they suppose to be related to the iron and 'labile sulphide' of xanthine oxidase. Finally, the possibility cannot be excluded that the thiol group arises not as a direct consequence of reduction, as is implied in the above mechanisms, but as a result of steric changes in the enzyme.

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