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The Chemistry of Xanthine Oxidase

10. THE INHIBITION OF THE BOVINE ENZYME BY PURINE 6-ALDEHYDE*

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A number of workers have reported on the powerful inhibition of xanthine oxidase by 2-amino-4-hydroxypteridine 6-aldehyde (Hofstee, 1949; Lowry, Bessey & Crawford, 1949; Kalckar, Kjeldgaard & Klenow, 1950; Byers, 1952). Since this compound contains two potential substrate groups, namely the pteridine nucleus and the aldehyde group, it was of interest to study an analogous compound containing the purine ring system and an aldehyde group. The preparation of purine 6-aldehyde (Giner-Sorolla, Zimmerman & Bendich, 1959) prompted us to examine the effects of this compound on the aerobic oxidation of xanthine by highly purified bovine milk xanthine oxidase. The results presented here show that the purine aldehyde is a powerful inhibitor of this enzyme, but the kinetics of the reactions are unusual.

MATERIALS

The purine 6-aldehyde hydrochloride hydrate was a gift from Dr A. Giner-Sorolla of the Sloan-Kettering Institute, New York. It was stated to be 95% pure and the concentrations given in this paper have been calculated on that basis. A stock solution was prepared by dissolving 10.3 mg. in water to 5 ml. This was stored at 4°, the resulting precipitate being redissolved (by warming to room temperature) before diluting a portion to a suitable concentration for use in the following experiments. The purine aldehyde was found to be stable for about 1 month under these conditions.

Pyrophosphate buffers have been used throughout and were prepared by mixing 50 mM solutions of tetrasodium pyrophosphate (British Drug Houses Ltd., A.R. grade) and disodium dihydrogen pyrophosphate (British Drug Houses Ltd., laboratory grade) to give the required pH.

All water was glass-distilled and then deionized by a mixed-bed ion-exchange column.

Xanthine (Roche Products Ltd.) was stored at room temperature as a 10 mM solution in 20 mM-NaOH.

Xanthine oxidase, prepared from buttermilk by the method of Gilbert & Bergel (1964), was used as the eluate (stage AM5) having a ratio E_{280}/E_{450} 5.3 and, originally, a ratio activity/ $E_{450}^{1\text{ cm}}$ 95 (these ratios correspond to the 'PFR' and 'AFR' values respectively of Avis, Bergel & Bray, 1955). During several months' storage at 4° some denaturation occurred and the enzyme was therefore occasionally centrifuged and passed through a Sephadex G-25 column (Pharmacia, Uppsala, Sweden; cf. Porath & Flodin, 1959) to remove free flavin. This process also removed an inhibitor, presumably that reported by Bray (1959). The ratio activity/ $E_{450}^{1\text{ cm}}$ was 35 at the time these experiments were carried out.

METHODS

Except where stated otherwise, the rate of oxidation of xanthine was measured by determining the rate of increase of extinction at 295 m μ over the initial 1-4 min. of the reaction. A Unicam SP.500 spectrophotometer with thermostatically-controlled cell housing (23.5 $\pm$ 0.2°) was used for these measurements, all buffer solutions being pre-incubated in the apparatus before the addition of enzyme, inhibitor and substrate. The standard (optimum) xanthine concentration was 0.1 mM.

* Part 9: Gilbert & Bergel (1964).

In the anaerobic experiments, a special cuvette of the Thunberg type was used, the enzyme being pipetted into the body and the purine aldehyde or xanthine, or both, into the side arm. Moist nitrogen was then bubbled through the enzyme solution and over the inhibitor or substrate for about 15 min. Extinction measurements were made before and at intervals after mixing of the solutions.

In a number of assays, the rate of oxidation of the xanthine increased slightly during the initial stages of the reaction, reaching a steady value after an interval that was dependent on the inhibitor concentration and the preincubation period. This time, however, did not exceed 2.5 min. for the highest concentration of inhibitor (see Fig. 4). In all cases, only the steady-rate values have been used for calculation. The ratio of the rate obtained in the presence of the inhibitor to that in its absence (under otherwise identical conditions) is referred to as the fractional activity (α).

RESULTS

Although the degree of inhibition was unaffected by the time in which the substrate (xanthine) and purine aldehyde were in contact, preliminary experiments showed that the reaction of the enzyme with the inhibitor was slow. For this reason the dependence of the fractional activity on the purine aldehyde concentration was initially determined with an arbitrary preincubation period of 2 min. (Fig. 1, curve A). Inhibition was detectable even when the inhibitor concentration was of the same order of magnitude as that of the enzyme (about $10\mu\text{M}$). The effect of varying the preincubation period was then checked at three different inhibitor concentrations covering a 100-fold range (Fig. 2). The Figure shows that the 2 min. preincubation period adopted in the first experiment was sufficient for equilibrium to be attained only when the purine aldehyde concentrations exceeded about $2\mu\text{M}$. To illustrate this point more clearly, the three minimum (or equilibrium) fractional activities corresponding to the three inhibitor concentrations (determined from Fig. 2) have been plotted as curve B in Fig. 1. Under these conditions, 50% inhibition could be achieved with purine aldehyde concentration (i_{50}) $0.2\mu\text{M}$, i.e. 1/500 that of the substrate concentration. This value increased by only 15% when the xanthine concentration was increased by a factor 2.5.

Fig. 3 illustrates the proportionality between the uninhibited rate and the enzyme concentration. With a 2 min. preincubation period, a similar variation was observed for the inhibited rate only at the higher enzyme concentrations; a decrease in the amount of xanthine oxidase, however, resulted in a divergence toward higher rates. The net effect was that the fractional activity decreased from unity toward a constant value as the volume of enzyme was increased.

To observe the effect of the substrate on the rate of association of the inhibitor and enzyme, these

two latter reactants were added simultaneously to a solution of xanthine. The rate of approach to equilibrium (see Fig. 4) was considerably less than when the enzyme and inhibitor were allowed to

Fig. 1. Dependence of the fractional activity on the purine aldehyde concentration. $\circ$ (curve A), $10\mu\text{l}$. of enzyme and $0.25\mu\text{l}$. portions of inhibitor were added to 2.5 ml . portions of 50 mM -pyrophosphate buffer, pH 8.1. After 2 min. incubation at 23° , $25\mu\text{l}$. of 10 mM -xanthine was added and the rate of oxidation of the latter determined (see text). $\triangle$ (curve B), conditions as for curve A but the incubation period was extended until equilibrium was reached. Data from Fig. 2.

Fig. 2. Effect of incubation period on the fractional activity. Enzyme ($10\mu\text{l}$. amounts) and purine aldehyde (at an appropriate concentration) were incubated at 23.5° with 2.5 ml . of 50 mM -pyrophosphate buffer, pH 8.1. After a convenient time ($0.25\text{--}15\text{ min.}$), $25\mu\text{l}$. of 10 mM -xanthine was added and the rate of oxidation of the latter determined. The process was repeated a number of times for each inhibitor concentration, the incubation period being varied in each case. Final purine aldehyde concentrations: $\circ$, $39\mu\text{M}$; $\blacktriangle$, $0.39\mu\text{M}$; $\square$, $3.9\mu\text{M}$.

Fig. 3. Effect of enzyme concentration on the inhibited and uninhibited rates and fractional activity. 9.8 mM-Purine aldehyde (1 μ l.) and 2–20 μ l. portions of enzyme were added to 2.5 ml. amounts of 50 mM-pyrophosphate buffer, pH 8.1. After 2 min. incubation at 23.5°, 25 μ l. of 10 mM-xanthine was added and the rate of oxidation of the latter determined. Control experiments were carried out similarly with omission of the inhibitor. Left ordinate: $\circ$, uninhibited rate; $\square$, inhibited rate. Right ordinate: $\triangle$, fractional activity.

react in the absence of substrate. Thus, as can be seen from Fig. 2, with 3.9 μ M-purine aldehyde alone equilibrium was reached in about 1 min. and resulted in approximately 80 % inhibition, whereas in the presence of xanthine equilibrium was not achieved even after 7 min. with 7.8 μ M-inhibitor.

The data presented in Fig. 5 show that the rate of approach to equilibrium was virtually independent of pH over the range 8.1–6.5 but decreased greatly as the acidity was increased. Thus at pH 5.9, the $t_{\frac{1}{2}}$ value (i.e. the time taken for the fractional activity to fall by one-half the maximum amount) is approximately twice that for the reactions occurring at the other pH values. This Figure also shows how the pH affects the equilibrium fractional activity. From these and other curves the inhibition was found to be maximal at pH 7 (Fig. 6).

Experiments carried out to see if the purine aldehyde was oxidized by the enzyme did not give unequivocal results. Incubation of the compound with xanthine oxidase (in the absence of xanthine) resulted in a change in the spectrum of the inhibitor but only after several hours. Since qualitatively similar results were obtained when the enzyme was omitted, and in view of the instability of the latter, it is difficult to interpret these observations. More positive evidence on this aspect came from an examination of the effect of purine aldehyde on the spectrum of xanthine oxidase under anaerobic conditions. All substrates that have been examined in this way reduce the enzyme with

Fig. 4. Effect of the substrate on the rate of attainment of equilibrium. 9.3 mM-Purine aldehyde (2 μ l.) and 10 μ l. of enzyme were pipetted simultaneously into a mixture of 2.5 ml. of 50 mM-pyrophosphate buffer, pH 8.0, and 25 μ l. of 10 mM-xanthine, and the rate of oxidation of the latter was followed ($\bullet$). In the control experiment ($\circ$), the purine aldehyde was omitted. Given for comparison is the assay curve ($\triangle$) in which the purine aldehyde (3.9 μ M) was preincubated with the enzyme for 2 min. before the addition of xanthine.

Fig. 5. Effect of pH on the rate of attainment of equilibrium. Enzyme (10 μ l.) and 10 μ l. of 9.8 mM-purine aldehyde were incubated at 23.5° with 2.5 ml. of 50 mM-pyrophosphate buffer of the appropriate pH. After a convenient time (0.5–6 min.) 25 μ l. of 10 mM-xanthine was added and the rate of oxidation of the latter determined. The process was repeated a number of times for each pH value but the incubation period was varied in each case. $\triangle$, pH 8.1; $\square$, pH 7.4; $\bullet$, pH 5.9.

a consequential general decrease in absorption within the range 300–700 $m\mu$ (see, e.g., Gilbert, 1963*b*). As can be seen from Table 1, in contrast with xanthine, purine aldehyde had negligible effect on the 450 $m\mu$ extinction of the enzyme; moreover it prevented the reduction of the enzyme by xanthine.

DISCUSSION

In discussing the inhibition results presented it is essential to bear in mind the complexity of the uninhibited reactions. For an insight into this aspect the reader is referred to the review article by Bray (1963) and the more recent paper by Gilbert (1963*b*). In particular, attention is drawn to the fact that no satisfactory rate equation exists which takes into account both substrates (xanthine and oxygen) and substrate inhibition.

It seems reasonable to conclude from the anaerobic experiment (Table 1) that the purine aldehyde is not rapidly oxidized by the enzyme. The observed results cannot therefore be due to the formation of a product of such a reaction and it is sufficient to consider only those processes in which the purine aldehyde is directly involved. In view of the complexity of the kinetics observed in the present system (see below), it is not possible by means of Lineweaver–Burk plots to determine clearly whether the purine aldehyde acted competitively or non-competitively with the xanthine. The increase in the rate of oxidation of xanthine noted during the first few seconds of certain assays (see Methods and Fig. 4) suggests a substrate-induced displacement of the inhibitor from a complex with the enzyme, and thus favours a true competitive mechanism. Two other observations are also in agreement with a competitive mechanism: (a) the influence of the substrate on the rate of attainment of equilibrium between enzyme, inhibitor and the enzyme–inhibitor complex (Fig. 4); (b) the increase in i_{50} with increasing substrate concentration.

When Michaelis-type kinetics are obeyed by the inhibitor, the reaction order with respect to this compound, i.e. the value of 'n' in the equilibrium:

may be obtained by determining the slope of the linear plot of $\log(1-\alpha)/\alpha$ against $\log i$ (i , concn. of inhibitor). The data of Fig. 1 have been replotted in this form in Fig. 7 and it can be seen that the slope of curve *B* (equilibrated system) is 0.52 and the slope of curve *A* approaches this value at high inhibitor concentrations (i.e. under conditions where the 2 min. preincubation is sufficient for equilibrium). The data of Fig. 2 would also appear

Fig. 6. Variation of the equilibrium fractional activity with pH. Enzyme (10 μ l.) and 10 μ l. of 0.98 mM-purine aldehyde were incubated at 23.5° with 2.5 ml. of 50 mM-pyrophosphate buffer of the appropriate pH. After 10 min. 25 μ l. of 10 mM-xanthine was added and the rate of oxidation of the latter determined. (Compare with Fig. 5.)

Table 1. *Effect of xanthine and purine aldehyde on the 450 $m\mu$ absorption of the enzyme under anaerobic conditions*

Expt. A: 0.1 ml. of 9.8 mM-purine aldehyde was added anaerobically to 3.0 ml. of enzyme ($E_{450}^{1\text{cm.}} = 0.7$). Expt. B: 0.1 ml. of 10 mM-xanthine was added anaerobically to 3.0 ml. of enzyme containing purine aldehyde (310 μ M, preincubated for 10 min.). Expt. C: 0.1 ml. of 10 mM-xanthine was added anaerobically to 3.0 ml. of enzyme.

Expt.	Time (min.)	$E_{450}^{1\text{cm.}}$ (% of initial value)
A	0	100
	0.5	100
	19	99
	38	97
	82	96
	120	96
B	180	98
	0	100
	0.5	100
	3	100
	7	99
	12	100
C	0	100
	0.5	84
	1	83
	4	77
	35	56

to be consistent with a fractional order. Thus it can be shown (cf. Goldstein, 1944) that the half-period ($t_{\frac{1}{2}}$) for the approach to equilibrium is given by the equation:

$$t_{\frac{1}{2}} = \ln 2 / (k_1 \cdot i^n + k_{-1})$$

where k_{+1} and k_{-1} are the forward and reverse rate constants for the reaction (see above). For powerful inhibitions of the type described, $k_{-1} \ll k_{+1} \cdot i^n$ and therefore the half-period should be inversely proportional to the n th power of the inhibitor concentration, i.e. $t_{\frac{1}{2}} \propto i^{-n}$. Table 2 shows that the reaction between xanthine oxidase and purine aldehyde obeys this relationship, provided that $n = 0.5$.

Since inhibition can be detected when the purine aldehyde concentration is comparable with that of the enzyme, it is necessary to consider the possibility that the kinetics deviate from the simple Michaelis type. If one takes into account the proportion of inhibitor bound to the enzyme (Strauss & Goldstein, 1943; Goldstein, 1944) it may be shown that, for the fully competitive inhibitor, the fractional activity is related to the inhibitor concentration by the equation:

$$i = \left\{ K_i \cdot \left(1 + \frac{s}{K_s} \right) \left(\frac{1-\alpha}{\alpha} \right) \right\}^{\frac{1}{n}} + n e_0 (1-\alpha)$$

where e_0 and s are the enzyme and substrate concentrations respectively, and K_s and K_i are the apparent Michaelis constants for the substrate and inhibitor respectively. [The derivation of this equation is similar to that given by Strauss & Goldstein (1943), who, however, defined the fractional activity in a different way. It has been assumed that the substrate reaction approximated to simple Michaelis-Menten kinetics.] By using the following values: $n = 0.5$, $i_{50} = 0.2 \mu\text{M}$ (curve B, Fig. 1), $s = 100 \mu\text{M}$, $K_s = 1 \mu\text{M}$ (Lowry *et al.* 1949; Bray, 1959) and $e_0 = 10 \mu\text{M}$ (estimated from E_{450} of the enzyme; cf. Bray, Malmström & Vänngård, 1959), a curve may be obtained which fits extremely close to that of curve B, Fig. 1. The contribution of the term $n e_0 (1-\alpha)$ (which represents the fraction of the inhibitor bound to the enzyme) is, however, small. It therefore seems that the powerful inhibitory action of the purine aldehyde on xanthine oxidation is not the result of an exceptionally low Michaelis constant, but is principally due to a

mode of action which results in a fractional order with respect to this component.

The fractional order seems to indicate a multi-stage reaction, but the mechanism is not clear. Abnormal kinetics would be expected if: (1) the reaction involved two molecules of enzyme each having one reactive site; (2) one inhibitor molecule blocked two sites on one enzyme molecule; (3) the enzyme reacted with a dimeric form of the inhibitor; (4) the combination of the enzyme and inhibitor resulted in a multiple-point attachment of the latter; (5) a complete change in the mechanism of the oxidative reaction occurred.

Mechanism (1) may be dismissed on the basis of several arguments: thus the dependence of the fractional activity on the enzyme concentration (Fig. 3) is of the opposite form to that required by mechanism (1) and can be simply explained on the basis of the inability of the system to reach equilibrium under some conditions. Similarly, it can be shown that mechanism (3) would not result in kinetics of the observed form, and in any case there is no evidence for the existence of a dimer of the inhibitor. A dimeric enzyme is in fact a special case of mechanism (2) but, since we are only concerned with reactions involving active enzyme centres, this latter mechanism would seem to require that the two enzymic sites involved must both be fully active. This is unlikely especially as it appears that the ratio of inactive to active sites

Fig. 7. Dependence of fractional activity on the purine aldehyde concentration. A replot of the data of Fig. 1: $\circ$ (curve A), 2 min. preincubation period; $\triangle$ (curve B), equilibrated system.

Table 2. Relationship between the half-period for the reaction ($t_{\frac{1}{2}}$) and the inhibitor concentration

Data presented in this Table have been obtained from Fig. 2.

Concn. of inhibitor (i)	$t_{\frac{1}{2}}$ (min.)	$t_{\frac{1}{2}}$ (relative)	i^{-n} (relative)	
			$n = 1$	$n = 0.5$
39 μM	2.0	10.0	100	10.0
0.39 μM	0.7	3.5	10	3.2
3.9 μM	0.2	1.0	1	1.0

(or molecules) was about 3 to 1. If mechanism (2) is correct, then it follows either that the two active sites are so close together that one purine aldehyde molecule can sterically block both, or that the two sites influence each other in some way so that the binding of a purine aldehyde molecule at one centre makes the second unavailable to another molecule of either substrate or inhibitor. It is also necessary to postulate that the second stage of the reaction be relatively rapid since otherwise an increase in the degree of inhibition would have been detected as the second site became blocked. Unless the blocking of the second site were extremely fast compared with the rate at which a second purine aldehyde molecule could be bound, one might also expect the order of the reaction (with respect to the inhibitor) to increase with increasing purine aldehyde concentration.

The most likely explanation for the fractional order would seem to be either (4) or (5). Goldstein (1952) has stated that a slow approach to equilibrium is indicative of the formation of a complex in which the inhibitor is strongly bound. The data are thus not inconsistent with a multiple-point attachment of the inhibitor to the enzyme (e.g. via the purine nucleus and the aldehyde group). An examination of the inhibitory properties of the corresponding 2- and 8-formylpurines might substantiate this view. On the other hand, since it has been suggested (Fridovich & Handler, 1959) that xanthine oxidase can be reoxidized by at least two different mechanisms (via the flavin and via the metal cofactors), it seems feasible that the binding of certain compounds could lead to a change in the overall oxidative process (e.g. a change in the rate-determining step).

The pH data are also difficult to interpret in view of the complexity of the kinetics. The rapid change in the rate of approach to equilibrium at pH values below about 6.5 (Fig. 5) would seem to indicate that a group within, or closely adjacent to, the active site has pK in the region 5.5, since the purine aldehyde has pK values well separated from this (pK_a 2.4, pK_b 8.8; Giner-Sorolla *et al.* 1959). A similar pH-dependency has been observed for the reaction of the enzyme with NADH and with *p*-chloromercuribenzenesulphonic acid (Gilbert, 1963*a*, *c*). At this stage, however, it is not possible to attribute these results to the ionization of any particular group.

SUMMARY

1. The powerful inhibitory action *in vitro* of purine 6-aldehyde on the aerobic oxidation of

xanthine by bovine milk xanthine oxidase has been studied under various conditions.

2. The onset of inhibition is slow, the rate being dependent on the concentration of the reactants, the presence or absence of the substrate and also the pH.

3. Inhibition is maximal at pH 7.0 under the conditions used.

4. The order of the reaction with respect to the purine aldehyde is fractional.

5. The observations are consistent with the inhibitor being reversibly and strongly bound at the same site as the substrate and with a group at the active site having pK in the region 5.5–6.0.

6. The unusual kinetics are tentatively ascribed to multi-point attachment of the inhibitor to the enzyme or to a change in the overall mechanism of xanthine oxidation.

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