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HUMAN AND BOVINE XANTHINE OXIDASES

INHIBITION STUDIES WITH OXIPURINOL

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Abstract.—Oxipurinol inhibited human xanthine oxidase and bovine xanthine oxidases by very similar mechanisms. It bound to an electronically reduced form of human xanthine oxidase in a manner similar to that previously discerned from its interactions with the bovine enzyme [review article: Spector, *Biochem. Pharmac.* **26**, 355 (1977)]. Xanthine was a good source for the reducing equivalents because it did not compete with oxipurinol for binding to reduced enzyme. The inhibition of the rate of urate production progressively increased with time. Studies of the effect of the concentration of oxipurinol on the rate constant of the development of this inhibition revealed that a complex was rapidly formed between oxipurinol and reduced bovine or human xanthine oxidases (K_D of about 8 μM). At 37° these complexes were converted to stable complexes at a maximum rate of about 1.6 min^{-1}. The rate constant was highly temperature dependent with an energy of activation of 30 kcal/mole (cf. 13 kcal/mole for the energy of activation for catalysis). These data support the earlier conclusions that the formation of stable complexes probably reflects a massive rearrangement of the initial complexes. The isolated oxipurinol–xanthine oxidase complexes spontaneously reverted to active enzyme with a rate constant of 0.02 min^{-1} at 37°. The energy of activation for the "reactivation" was similar to that for the formation of the stable complexes. The rates of "reactivation" could be stimulated by high concentrations of xanthine: 2.4-fold at 50 μM and 3.4-fold at 100 μM. The constant for the overall inhibition by oxipurinol was approximately 100 nM with both enzymes.

Allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine) is a clinically useful antihyperuricemic agent. Its primary effect is mediated by inhibiting xanthine oxidase, the enzyme responsible for the synthesis of uric acid. Allopurinol's unconventional mechanism of inhibition has been the subject of several investigations [1–6] and review articles [7, 8]. Basically, allopurinol is a "suicide" substrate for the enzyme. The oxidized product, oxipurinol (4,6-dihydroxypyrazolo[3,4-*d*]pyrimidine), binds tightly to the electronically reduced enzyme and thereby accounts for the inhibition. Following the original observation of the inhibition of human xanthine oxidase [1], the detailed mechanism studies have focused on bovine xanthine oxidase [2–6]. In the present study, several new approaches have been used to further characterize the interactions of oxipurinol with bovine xanthine oxidase. Comparative studies with highly purified human xanthine oxidase are also presented.

MATERIALS AND METHODS

Xanthine oxidase

Human liver xanthine oxidase was purified by ion exchange and affinity chromatography [9]. It had a 280/450 nm absorbance ratio of 5.2 and a specific activity of 3.8 μmoles/min/mg at 37°. Bovine xanthine oxidase was purchased from Boehringer Mannheim.

Standard assay

Standard reaction mixtures contained 50 μM xanthine in 50 mM potassium phosphate buffer at pH 6.8 that contained 0.3 mM EDTA and 0.1 mg bovine serum albumin per ml (Buffer A). Reactions were initiated with xanthine oxidase and were maintained at 37° with an electronically controlled temperature regulator. The production of uric acid was monitored at 292 nm (change in extinction = 9.75 cm^{-1}, mM^{-1}) with a Gilford 250 recording spectrophotometer. Studies with 2-aminopurine as substrate were monitored at 260 nm (change in extinction = 9.1 cm^{-1} mM^{-1}).

Kinetic constants

K_m and V_{max} were determined from digitalized data [nmoles product (P) and seconds elapsed (T)] collected during the entire course of the reaction. The data were analyzed according to the integrated form of the Michaelis–Menten equation with the computer program described earlier for the analysis of the reactions catalyzed by adenosine deaminase [10]. Inactivation rate constants (k'_{inact}) were calculated from similar digitalized data by a computer program that iteratively determined the amount of product that theoretically would have been produced if 100% inactivation had occurred (P_f) and the pseudo first-order rate constant (k'_{inact}) from the slope of plots of $\ln(P_f - P)$ versus T [11]. Secondary analysis of the relationship of k'_{inact} to the concentration of oxipurinol was performed according the computer program "HYPER" described by Cleland [12]. First-order reactivation rate constants for

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the dissociation of the isolated oxipurinol–xanthine oxidase complex were calculated from the slope of plots of $\ln(1 - v_1/v_0)$ versus the time of incubation. The inhibited velocities, v_1, were determined at various times during the incubation. To neutralize any variations between samples, v_0 values were determined from autologous samples that had reactivated completely. The velocities of the uninhibited controls remained constant during these incubations. The v_1 values for reactivation studies with 50 and 100 μM xanthine present were calculated over various segments of the accelerating progress curve for the production of uric acid (prior to significant substrate depletion). This was facilitated by a computer program that utilized digitalized data similar to that described above.

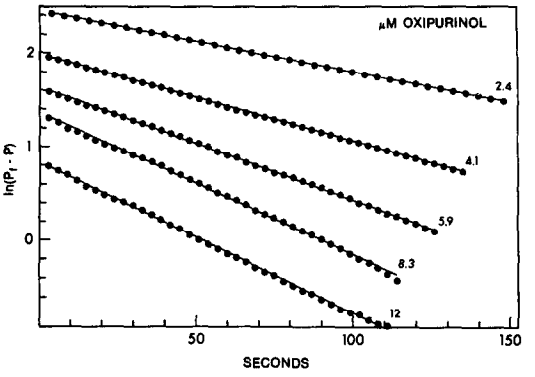

Fig. 2. Pseudo first-order "inactivation" of human xanthine oxidase by oxipurinol. The digitalized data of Fig. 1 were processed as described in Materials and Methods.

RESULTS

"Inactivation"

Time dependence. The development of the potent inhibition of human xanthine oxidase by oxipurinol is demonstrated in Fig. 1. Oxipurinol caused the rate of production of uric acid to decelerate as the reaction proceeded. Furthermore, as previously observed with bovine xanthine oxidase [5], the rate of the development of inhibition of the human enzyme was dependent upon the concentration of oxipurinol. Progressive inhibition did not occur during preincubations in the absence of xanthine. Although the inhibition by oxipurinol was not irreversible, the inhibited complex was adequately stable during the assays to justify treating oxipurinol as an apparent inactivator. Therefore, in order to further study the process of progressive inhibition, data similar to those of Fig. 1 were collected at different concentrations of xanthine. "Inactivation" rate

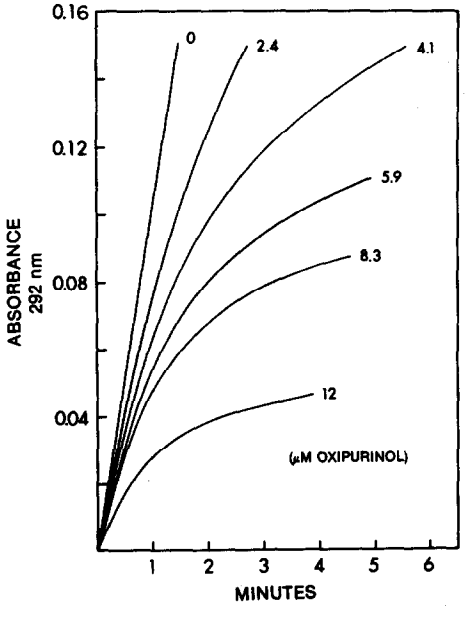

Fig. 1. Effect of oxipurinol on the production of uric acid by human xanthine oxidase. The initial concentration of xanthine was 30 μM.

constants (k'_{inact}) were then calculated from the transformed data as described in Materials and Methods and exemplified in Fig. 2. Replots of k'_{inact} versus [oxipurinol] in the double-reciprocal form are presented in Fig. 3, A and B.

The replots for both enzymes show a positive intercept on the Y-axis. This indicates that the rate of "inactivation" is saturable and that the formation of the "inactivated" (stable) species is preceded by the formation of a rapidly reversible (initial) complex [13]. The k'_{inact} values determined in the presence of 15 and 28 μM xanthine were practically identical for both enzymes. However, in the presence of 50 μM xanthine, the k'_{inact} values of the bovine enzyme were slightly slower than those determined at 15 and 28 μM, while those of the human enzyme in the presence of 50 μM xanthine were slightly faster than those in the presence of 14 or 28 μM xanthine. Although these differences are small, they were reproducible.

The dependence of the k'_{inact} values on the concentration of oxipurinol over the range where the k'_{inact} values are independent of the concentration of xanthine can be quantitated [13]. The dissociation constant, K_D, for oxipurinol from the initially formed complex and the true first-order rate constant (k_{inact}) for the conversion of this complex into the stable complex were calculated from the negative reciprocal of the x-axis and the reciprocal of the y-axis intercepts respectively. The K_D values were 8.6 and 7.2 μM for oxipurinol with the human and bovine enzymes respectively. The corresponding k_{inact} values were 1.8 and 1.4 min^{-1}. The values determined in the presence of 50 μM xanthine were 26 μM and 4.3 min^{-1} for human xanthine oxidase and 24 μM and 2.1 min^{-1} for bovine xanthine oxidase. These constants and others are summarized in Table 1.

To further explore the species dependence on the relationship of the concentration of xanthine to the k'_{inact} values, highly purified xanthine oxidase was obtained from mouse liver and intestine by a method identical to that used to purify human xanthine oxidase. The k'_{inact} values obtained in the presence of 25 and 50 μM xanthine were virtually identical. Their dependency upon the concentration of oxipurinol was also very similar to that observed with

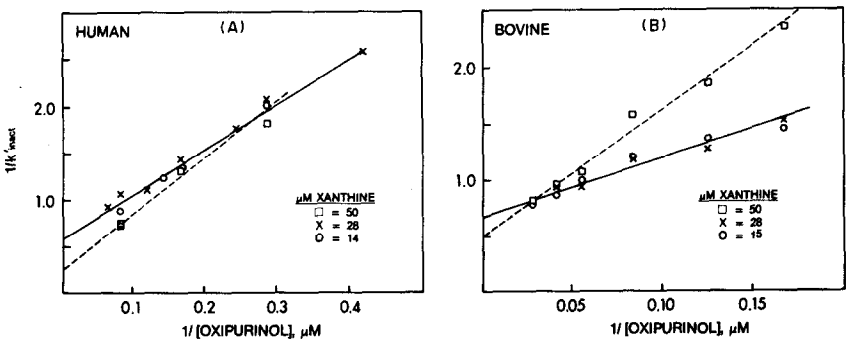

Fig. 3. Double-reciprocal plots of the rate of development of inhibition (k'_{inact}) versus the concentration of oxipurinol. The initial concentrations of xanthine and oxipurinol are indicated in the figure. Examples of how the data were obtained are presented in Figs. 1 and 2. The solid lines are drawn to the fit of the combined data obtained at 14 and 28 μM with human (A) and 15 and 28 μM with bovine (B) xanthine oxidases. The dashed lines are drawn to fit the data obtained at 50 μM xanthine.

human and bovine xanthine oxidases (K_D = 7.5 μM; k_{inact} = 1.8 min^{-1}).

Temperature dependence. The effect of temperature on the rate of formation of the stable complex was studied to determine the energy requirement of this process. The k_{inact} values were calculated from decelerating reactions in the presence of 50 μM xanthine and 24 μM oxipurinol at various temperatures. The Arrhenius plot for the data obtained with human xanthine oxidase is presented in Fig. 4. The energy of activation of this process was 31 kcal/mol. The value for bovine xanthine oxidase was 28 kcal/mole. For comparative purposes the effect of temperature on the V_{max} of the oxidation of xanthine (catalysis) was also studied (Fig. 4). The energy of activation for the oxidation of xanthine was determined to be 13 kcal/mole for xanthine oxidase from either source. This value is similar to the one (14.5 kcal/mole) previously reported by Massey *et al.* [14] at pH 8.3 with bovine xanthine oxidase. The K_M values for xanthine showed only small changes over the temperature range studied, 37 to 18°. These values increased from 4 to 7 μM and 5 to 7.5 μM for bovine and human xanthine oxidases respectively.

"Reactivation"

Spontaneous reactivation. Earlier studies with bovine xanthine oxidase have shown that oxipurinol

Table 1. Summary of constants

	Enzyme source	
	Human	Bovine
Xanthine		
K_m	5 μM	4 μM
V_{max}	100 (relative)	100 (relative)
Energy of activation	13 kcal/mole	13 kcal/mole
Allopurinol*		
K_I	2 μM	4 μM
V_{max}	58 (relative)	94 (relative)
Oxipurinol		
Overall inhibition constant		
K_I	85 nM	100 nM
Initial binding to reduced enzyme		
K_D (at 15 or 28 μM xanthine)	8.6 μM	7.2 μM
Formation of stable complex ("Inactivation")		
Rate constant (37°)	1.8 min^{-1}	1.4 min^{-1}
Energy of activation	31 kcal/mole	28 kcal/mole
Breakdown of stable complex ("Reactivation")		
Rate constant (37°)		
No xanthine	0.018 min^{-1}	0.20 min^{-1}
50 μM Xanthine	0.044 min^{-1}	0.45 min^{-1}
100 μM Xanthine	0.062 min^{-1}	0.064 min^{-1}
Energy of activation	25 kcal/mole	27 kcal/mole

* From Ref. 9.

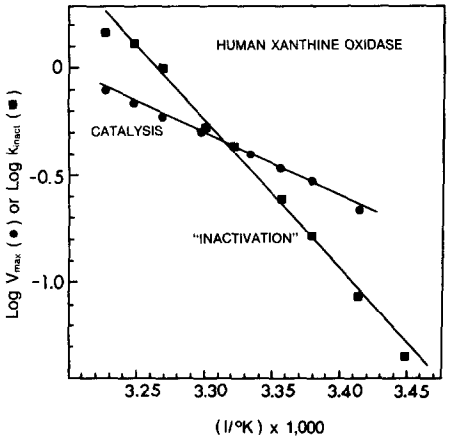

Fig. 4. Arrhenius plots comparing the effect of temperature on catalysis and "inactivation" of human xanthine oxidase.

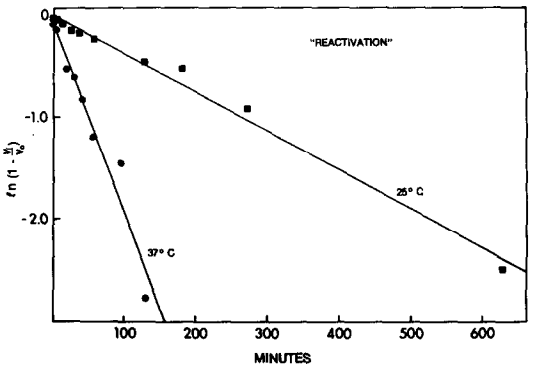

Fig. 5. Effect of temperature on the spontaneous reactivation of human xanthine oxidase

forms its inhibitory complex with the molybdenum cofactor in the electronically reduced form, Mo^{IV} [3, 4]. Reducing equivalents can be donated from substrates such as xanthine or allopurinol or from a chemical reductant such as dithionite (see Ref. 8 for review). To prepare and isolate the stable oxipurinol-reduced enzyme complex, 20 mM dithionite and 110 μM oxipurinol were incubated with human xanthine oxidase in Buffer A with additional bovine serum albumin (1.5 mg/ml total) under house vacuum for 20 min at room temperature. The complexed enzyme (200 μl) was chilled to 4° and then isolated by rapid desalting without dilution by sequential passage through two 0.5 × 6 cm Sephadex G-25 columns at 4° [15]. The columns were pre-equilibrated with 2 mg/ml of bovine serum albumin to prevent non-specific binding. Control incubations were performed without oxipurinol.

Human xanthine oxidase treated and isolated in this manner was inhibited by greater than 95%. When maintained at 4°, it remained in the inhibited state for many hours and only slowly regained activity; the $T_{1/2}$ was about 30 hr. The enzyme was "reactivated" much faster when incubated at warmer temperatures. Rate constants for the "reactivation" were 0.0036 min^{-1} ($T_{1/2}$ = 190 min) at 25° and 0.018 min^{-1} ($T_{1/2}$ = 39 min) at 37° (Fig. 5). Identical experiments with bovine xanthine oxidase revealed that its rates of spontaneous reactivation were very similar to those of the human enzyme. These rate constants were 0.0034 and 0.020 min^{-1} at 25 and 37° respectively.

The energy of activation for the "reactivation" process was calculated from the rate constants at 25 and 37°. The values were 25 and 27 kcal/mole for human and bovine xanthine oxidases respectively.

Stimulation of "reactivation" by xanthine. Previous studies [4] have shown that xanthine accelerated the "reactivation" of bovine xanthine oxidase. The present studies confirm these observations. At 37°, 50 and 100 μM xanthine caused the rate constant to increase from 0.020 to 0.045 and 0.064 min^{-1} respectively. A similar effect was detected with human xanthine oxidase where the spontaneous rate of 0.018 min^{-1} was increased to 0.044 min^{-1} by 50 μM xanthine and to 0.062 min^{-1} by 100 μM xanthine.

Since the velocities of uninhibited reactions at 50 and 100 μM xanthine were within 5% of each other, it appeared that the effect of xanthine was related to its concentration and not to catalysis. Therefore, it seemed unlikely that the effect was related to the production of superoxide anion or hydrogen peroxide. In support of this contention, neither superoxide dismutase nor catalase was capable of nullifying the stimulating effect of xanthine on "reactivating" bovine xanthine oxidase. Identical rate constants were obtained in the presence and absence of excess superoxide dismutase or catalase in "reactivation" studies with 50 μM xanthine. Furthermore, 2-aminopurine, an alternative substrate with a comparable K_M (8 μM) and a V_{max} of only 23% that of xanthine [9], had the same stimulating effect as did xanthine.

DISCUSSION

The present study was an in-depth investigation of the mechanisms by which oxipurinol inhibits xanthine oxidase isolated from human and bovine sources. It served to further characterize these unusual interactions. It also established that the mechanisms for the enzyme from the two sources are very similar. It was demonstrated previously that the species of bovine xanthine oxidase which is susceptible to inhibition by oxipurinol is the electronically reduced enzyme [3, 4]. The same conclusion can now be drawn for the human enzyme based on the following observations: (1) no "inactivation" occurred in the absence of electron donors; (2) "inactivation" did occur in the presence of electron donors such as the substrates xanthine and allopurinol [9] or the chemical reductant dithionite; (3) no "inactivation" occurred in the presence of an artificial electron acceptor that can directly reoxidize the molybdenum of the enzyme [9].

The studies of the effects of substrate and inhibitor on the rates of the development of inhibition have provided useful information. The positive intercept of the double-reciprocal plots of k'_{inact} versus concentration of oxipurinol (Fig. 3, A and B) indicates that an initial complex between oxipurinol and xanthine oxidase is formed prior to the formation of a

stable complex. Williams and Bray [5] had reached the same conclusion from studies of the extrapolated initial velocities with the bovine enzyme. However, the present studies are not in agreement with their assumption of simple competition between xanthine and oxipurinol for this initial binding to reduced enzyme. With human (Fig. 3A), bovine (Fig. 3B), and mouse (data not shown) xanthine oxidases, the rates of "inactivation" were relatively independent of the concentration of xanthine. Thus, although xanthine was required to initiate the "inactivation" process by donating reducing equivalents, it did not effectively compete with oxipurinol for binding to the electronically reduced enzyme. It had been concluded from electron paramagnetic resonance studies [16] that xanthine binds to reduced bovine xanthine oxidase. It appears either that its affinity towards reduced enzyme is considerably weaker than its K_m value and/or that it is binding at a locus that is not identical to the locus for oxipurinol binding. The former conclusion supports the report of Hille and Stewart [17] who have titrated reduced bovine xanthine oxidase with xanthine. They found the binding to proceed in two steps with dissociation constants for xanthine of 125 and 25 μM respectively.

The studies that demonstrate the effect of xanthine on the rate constants for the "reactivation" of the oxipurinol–xanthine oxidase complex may shed light on the relationship between xanthine and oxipurinol. Earlier studies have demonstrated that oxipurinol-"inactivated" enzyme may be "reactivated" at three distinct rates: spontaneous "reactivation" is the slowest [1, 3]; direct reoxidation of the reduced molybdenum [3, 18, 19] by artificial electron acceptors causes the rapid release of oxipurinol; and cycling of the enzyme in the presence of xanthine results in an intermediate rate of "reactivation" [4]. It was postulated that, in the latter case, the stimulation of the spontaneous rate of "reactivation" may be due to the reoxidation of the molybdenum by the superoxide anion or hydrogen peroxide byproducts of the reaction [8]. However, the present studies suggest that the effect of xanthine is related to its concentration and not to its catalysis. A rough calculation reveals that approximately 70 μM xanthine produces half-maximum stimulation of the spontaneous rate of "reactivation". This is approximately 10-fold its K_m value. Furthermore, the double desalting and the additional retention of unbound oxipurinol by Sephadex G-25 [3, 4] ensure that only stoichiometric amounts of oxipurinol were present with the isolated complex. Moreover, because the K_D for the initial binding of oxipurinol is in the micromolar range and because free enzyme should rapidly reoxidize, once it dissociated, oxipurinol would not be capable of recombining under these conditions. Therefore, the stimulation of the "reactivation" by xanthine cannot be due to the prevention of recombination by competition with oxipurinol for binding to the same locus. It is more likely that xanthine can bind to the reduced xanthine oxidase–oxipurinol complex and thereby promote the release of oxipurinol.

Spontaneous "reactivation" of the complex occurs with rate constants of about 0.02 min^{-1} at 37° and 0.0035 min^{-1} at 25° for xanthine oxidase from both sources. The values at 25° compare favorably with the value of 0.002 min^{-1} determined at this temperature by Massey *et al.* [3]. All relevant constants are summarized in Table 1.

In agreement with an earlier study with bovine xanthine oxidase [4], the k'_{inact} values of both human and bovine were affected dramatically by temperature. The energy of activation values for the formation and breakdown of the stable oxipurinol–xanthine oxidase complexes were about 30 kcal/mole. These values are greater than twice the values for catalysis and probably represent massive internal rearrangements of the enzyme–oxipurinol complexes. Earlier stopped-flow studies by Massey *et al.* [3] and electron paramagnetic resonance studies by Williams and Bray [5] had detected changes that correlate with the progressive onset of inhibition and support the hypothesis that the formation of a stable complex is the result of a conformational change of the initial complex. The nature of the chemical coordination of oxipurinol to the molybdenum of the enzyme has been the subject of two recent reports [6, 20].

An overall K_I for the inhibition of xanthine oxidase by oxipurinol can be calculated from:

$$K_I = [K_D]\,[k_{inact}/(k_{reactivation} + k_{inact})].$$

This constant accounts for the dissociation constant from the initial complex as well as the rates of formation and breakdown of the stable complex. At 37°, using the values of 8.6 and 7.2 μM for the K_D of oxipurinol from reduced human and bovine xanthine oxidases, respectively, the K_I values are 85 and 100 nM respectively. When the constants determined from the "inactivation" data collected in the presence of 50 μM xanthine are used, the K_I values are 160 nM with human and 230 nM with bovine xanthine oxidase. These values for oxipurinol are considerably higher than the value previously obtained by a different method as reported by Spector and Johns [4]. However, re-examination of the calculations in that report reveals that the inhibitor concentrations prior to dilution into the assay mixture should have been used for the determination of the overall K_I. With this consideration, recalculation of the original data produces a value closer to the present ones. The value of about 100 nM is probably a reasonable estimate of the overall potency of oxipurinol and is consistent with its clinical potency (review article: Ref. 8).

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