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On the Mechanism of the Two-Way Oxidation of 2-Chloropurine by Mammalian Xanthine Oxidase

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2-Chloropurine is attacked by mammalian xanthine oxidase simultaneously at C-6 and C-8. The ratio of the two rates (=1) is insensitive to variations of pH or temperature. Experiments with 2-chloropurine as inhibitor indicate instantaneous association of this purine with the active center of the enzyme. It is concluded that the rate-determining step, viz., conversion of ES → (ES)*, is common to both pathways and that only in the subsequent interaction with water does the route fork.

I. Introduction

It is generally recognized that enzyme-catalyzed reactions involve activated states of both substrate and active center. In the activated complex of the two participants, commonly designated as (ES)*, bond lengths and bond angles may be changed and electrons may be redistributed to facilitate the cleavage of certain linkages and the formation of new ones. In those cases, where alternative pathways are available, the process of activation also determines the direction of the enzymatic reaction by favoring one out of several isomeric or tautomeric structures. For example, in the oxidation of purines by xanthine oxidase (XO), a substituent at C-6 usually directs attack to position 8 (1–3). The only exception is hypoxanthine, in which oxidation takes place at C-2, because of a special activation process, involving tautomerization (4). Other striking illustrations of the relationship between fine structure and mode of activation were found in the aminopurine series. For example, 2-amino- and 2-methylaminopurine are attacked at C-6, whereas the dimethylamino derivative is oxidized at C-8 (5). It was concluded that because of the absence of hydrogen in the amino substituent, the mode of attachment of 2-dimethylaminopurine must be different from that of the other two congeners.

These examples demonstrate clearly that special features in the structure of the substrate are responsible for the unidirectional reaction, observed in all cases. It was, therefore, most surprising that enzymatic oxidation of 2-chloropurine proceeds simultaneously and at equal speed along two pathways, as shown in Fig. 1 (6). This led to the idea that a single substrate may be capable of forming two different ES-complexes. The present paper is devoted to the study of this possibility.

According to Gutfreund and Sturtevant (7), oxidation by XO involves the following reaction steps:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \qquad (a)$$

$$ES \underset{k_4}{\overset{k_3}{\rightleftarrows}} (ES)^* \qquad (b)$$

$$(ES)^* + H_2O \underset{k_6}{\overset{k_5}{\rightleftarrows}} EH_2 + S{-}O \qquad (c)$$

$$EH_2 + O_2 \rightarrow E + H_2O_2 \qquad (d)$$

† Part of a Ph.D. thesis, submitted to the Faculty of Science, The Hebrew University, Jerusalem, Israel.

where E is the enzyme in the oxidized state, EH_2 is the enzyme in the reduced state, S is the substrate in the reduced state, and S—O is the substrate in the oxidized state.

Step (d) is identical for all oxidations catalyzed by XO, whether fast or slow. Therefore, this step can certainly not be rate determining for such sluggish reactions as the conversion of 2-chloropurine, with a rate of 1.5% relative to xanthine oxidation.

rate constants. Therefore, complete equality of the over-all rates along route A and B in Fig. 1 can be explained most simply, if the rate-determining step is common to both pathways. Three possibilities must be considered: (1) A *single* ES-complex is formed from XO and 2-chloropurine, and this step is much slower than all subsequent reactions; (2) the ES-complex, common to pathway A and B, is transformed into two different activated complexes in the rate-determining step; and (3) *two*

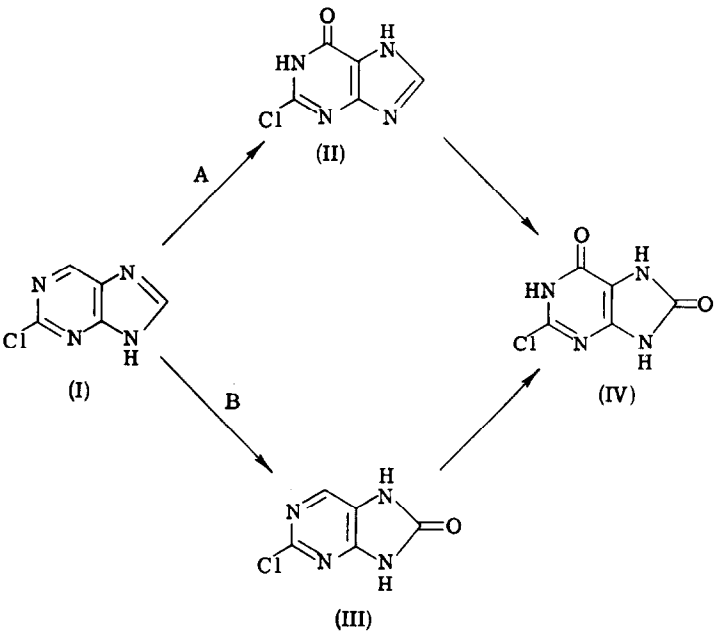

FIG. 1. Enzymatic oxidation of 2-chloropurine.

The intimate mechanism of step (c) has not yet been established. In a previous paper (6), the hypothesis was advanced that the water molecule participates in two fragments: H^+ is bound to a negatively charged group of the active surface, while OH^- attacks a CH group of the purine ring, releasing a hydride ion which is transferred to the enzyme. This specification of step (c) is analogous to the mechanism proposed recently for alcohol dehydrogenase (8) and could explain the decrease of enzymatic activity on the acid side.

Steps (a)–(c) are characterized by six *different* ES-complexes are formed. [This requires either that all rate constants, characteristic for steps (a)–(c), be equal, or, less probable, that the combination of these three reactions with individually different rates gives the same over-all velocity for both routes.]

It is possible to distinguish between these alternatives by systematic variation of the reaction conditions. For example, if 2-chloropurine forms a single ES-complex, alterations of pH or changes of temperature should not modify the ratio v_2/v_3, observed for the two simultaneous reactions under standard conditions.

II. Experimental Procedure

Highly purified milk xanthine oxidase,‡ at a dilution of 1:7500, produced under standard conditions (pH 8.0; 28°C) 1 μg/ml/min of uric acid, with $6.5 \times 10^{-5} M$ xanthine as substrate.

In all experiments, 10 units/ml of catalase (Worthington) were added, i.e., an enzyme concentration sufficient to decompose 10 mg/ml/min of H_2O_2, when the latter is approximately $1.5 \times 10^{-3} M$.

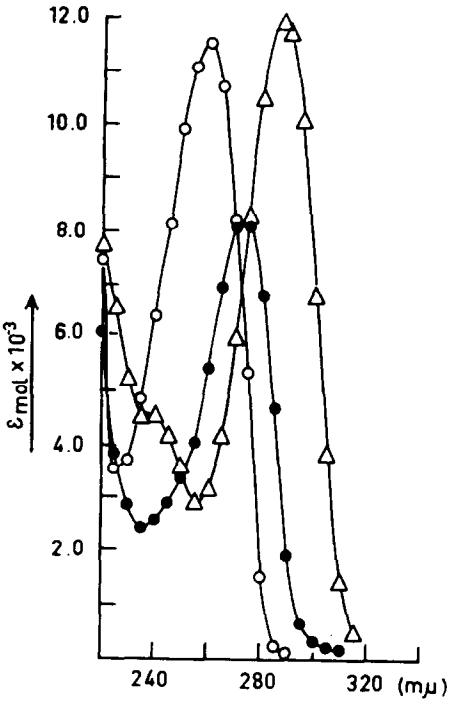

Fig. 2. Ultraviolet absorption spectra of: (●) 2-chloropurine (I); (○) 6-hydroxy-2-chloropurine (II); and (△) 8-hydroxy-2-chloropurine (III), at pH 7.0 (0.1 *M* phosphate buffer).

Kinetic measurements were performed with the aid of a Beckman UV spectrophotometer, equipped with a thermospacer. Three rates were measured simultaneously, based on the use of isosbestic points (Fig. 2): (1) rate of consumption of the substrate (v_1), by following the increase of optical density (OD) at the isosbestic point of the 6-hydroxy (II) and 8-hydroxy (III) derivatives: (2) rate of formation of II, characterized by an increase of OD at the isosbestic point of I and III; and (3) rate of formation of III, expressed as increase of OD at the isosbestic point of I and II near 300 mμ (6).

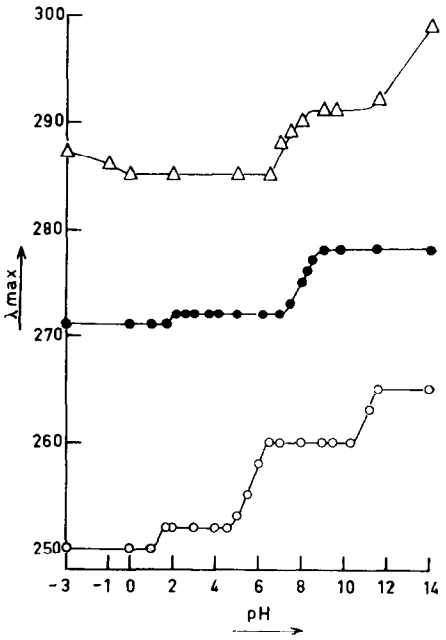

Fig. 3. Absorption maxima as function of pH. Key: (●) 2-Chloropurine (I); (○) 6-hydroxy-2-chloropurine (II); and (△) 8-hydroxy-2-chloropurine (III).

TABLE 1
Isosbestic Points as Function of pH

pH	1[a]	2[b]	3[c]
6.5	232	251	300
7.0	234	251	300
8.0	235	255	308
9.0	235	256	308
9.4	235	256	308

Note. All wavelengths in millimicrons.

[a] Isosbestic point of II and III, at which disappearance of 2-chloropurine (= v_1) was measured.

[b] Isosbestic point of I and III, for determination of v_2, characteristic for the formation of the 6-hydroxy derivative (II).

[c] Isosbestic point of I and II, at which formation of III (= v_3) was evaluated.

Since the position of the isosbestic points

‡ This enzyme was the gift of Professor F. Bergel and Dr. D. A. Gilbert of the Chester Beatty Institute of Cancer Research, London, England.

is a function of pH, it was necessary to determine the spectra of the three purines, involved in pathway A and B, at all pH values used. The variation of λ_{max} with pH is shown in Fig. 3. The wavelengths used for rate determinations at each pH are summarized in Table 1.

The following buffers were used: pH 6.5, 7.0, and 8.0—0.1 *M* phosphate; pH 9.0—0.1 *M* boric acid-borax; pH 9.4—0.1 *M* borax-NaOH.

The temperature dependence of the enzymic rates was measured at pH 8.0. The substrate concentration in all experiments was 6.5×10^{-5} *M*, producing a maximum velocity of oxidation at all pH values.

III. Results

A. *Effect of pH on the Two Simultaneous Reactions of 2-Chloropurine*

The results of the measurements over the feasible pH range from 6.5 to 9.5 are given in Fig. 4, which demonstrates the following points. (a) All three reactions measured show the same pH dependence, characterized by an asymmetrically bell-shaped curve. (b) For all three reactions, the pH optimum lies at about 8.2, corresponding to our previous findings that the pH optimum of XO is independent of the substrate used (9). However, the shape of the pH-activity curve varies from one substate to the other.

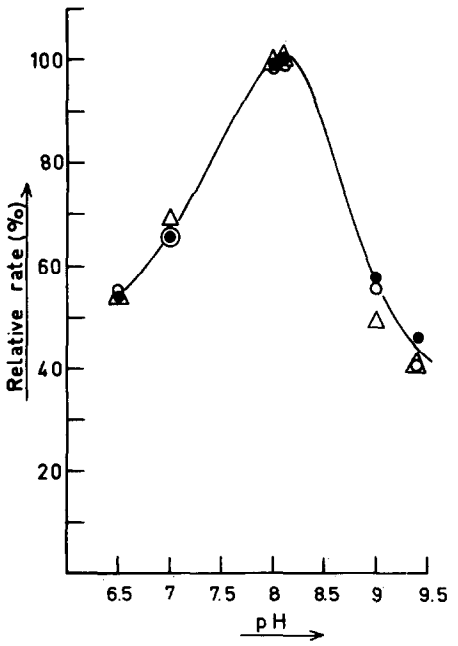

Fig. 4. Relative enzymatic rates as function of pH. Key: (●) Rate of disappearance of I; (○) rate of formation of II; and (△) rate of formation of III. For each reaction the highest rate (at pH 8.2) was taken as 100%. Substrate concentration, 10^{-4} *M*; XO, 1:1000; catalase, 1:5000; temperature, 28°C. Buffers, see Section II.

B. *Influence of Temperature Changes on the Simultaneous Reactions*

The Arrhenius plot in Fig. 5 shows again that no dissociation of rates occurs within the temperature range available for enzyme reactions, i.e., from +5 to +50°C.

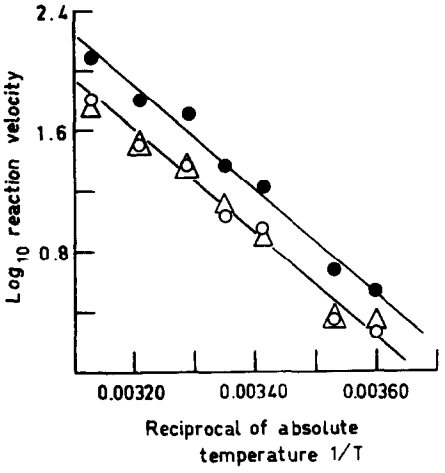

Fig. 5. Logarithm of reaction velocities as function of reciprocal of absolute temperature. Key: (●) 2-Chloropurine (I); (○) 6-hydroxy-2-chloropurine (II); and (△) 8-hydroxy-2-chloropurine (III).

The curves in Fig. 5 exhibit identical slopes, from which the activation energy has been calculated as 15500 cal/mole. It should be noted that the intercepts of the two lines in Fig. 5 reflect the fact that the rate of disappearance of 2-chloropurine possesses always twice the value of v_2 or v_3.

C. *Attempts to Determine the Ratio k_2/k_1*

The experiments described so far support the conclusion that the two simultaneous

oxidations of 2-chloropurine must start along a common path, i.e., they involve the same ES-complex. The forking of the reaction path can thus occur at two points: either at the conversion of ES to the activated complex (ES)* or at step (c). If formation of the ES-complex were, indeed, the only step common to pathways A and B, then it should also be the rate-determining step, in order to explain the results of Figs. 4 and 5. Some idea about the rate of association of enzyme and substrate may be gained by using the latter as inhibitor. Indeed, 2-chloropurine was found to reduce the rate of oxidation of a number of other purines tested effectively already at concentrations of 1–2 μg/ml. However, true equilibration between enzyme and inhibitor could not be established, as preincubation of these two components is already accompanied by some oxidation to II and III, both of which exhibit entirely different affinities to the active center. Therefore, measurements could only be performed by adding inhibitor and substrate *simultaneously* and extrapolating the competitive inhibition observed to zero time. Such experiments were, however, inconclusive, as the degree of inhibition varied from one substrate to the other and thus was merely a measure of the different affinities of the latter, as shown in Table 2. Nevertheless,

TABLE 2
INHIBITORY EFFECT OF 2-CHLOROPURINE ON THE ENZYMATIC OXIDATION OF VARIOUS SUBSTRATES BY XO

Substrate	Inhibition (%)
Xanthine	20
1-Methyl-2-hydroxypurine	45
3-Methyl-2-hydroxypurine	28
9-Methyl-2-hydroxypurine	77
6,8-Dihydroxypurine	23

Note. XO and catalase, 1:5000; 2-chloropurine, 6.5×10^{-6} *M*; all substrates, 6.5×10^{-5} *M*; pH 8.0; 28°C. A mixture of inhibitor and substrate was added to the enzymes at zero time. The initial rates obtained were compared with the rates in the absence of inhibitor.

it was observed that the initial reaction rates in the system enzyme + inhibitor + substrate were always linear, suggesting that equilibrium between XO and 2-chloropurine is established *very rapidly*. It is, therefore, improbable that formation of the ES complex is the rate-determining step for the conversion of 2-chloropurine. On the other hand, the K_m value of 1.3×10^{-5} *M*, derived from Fig. 6 by the method

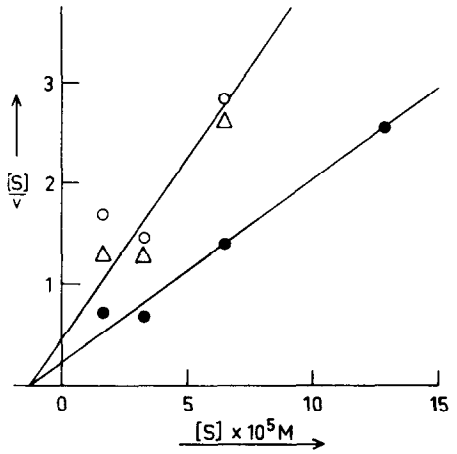

FIG. 6. Determination of Michaelis-Menten constant according to Dixon (10). (Intercept on *X*-axis = $-K_M$.) Note that the intercepts of the two curves on the *X*-axis coincide. On the *Y*-axis, they differ because V_{max} of either pathway A or B is only one-half of V_{max} of the disappearance of I, i.e., the curve for disappearance of I has one-half the *Y*-intercept of the upper line.

of Dixon (10), is very small and close to that of adenine ($K_m = 1.2 \times 10^{-5}$ *M*) (1). Therefore, either step (b) or (c) must be rate determining. However, since we postulate that the latter must be common for both pathways, there remains only one possibility, viz., that the transformation ES → (ES)* is still shared by both simultaneous reactions and is the slowest step; only in the subsequent reaction with water does branching of the reaction take place.

IV. DISCUSSION

The observations reported suggest that 2-chloropurine forms with XO a single complex, in which two different positions in the purine ring become activated. As the ratio between the two oxidation products is insensitive to changes of external conditions (pH or temperature), it is most

probably determined by electron distribution within the $(ES)^*$-complex. Nevertheless it may appear strange that interaction of the activated form with OH^- (or H_2O) in step (c) should not be influenced by variation of ambient conditions. This may be ascribed to either (or both) of the following reasons: (1) the formation of $(ES)^*$ is rate determining and so slow that variations in the velocity of the very rapid step (c) cannot be detected; and (2) a water molecule is included in the activated complex and thus does not participate in changes of the external medium.

It now appears possible that in all reactions, catalyzed by XO, the step $ES \rightarrow (ES)^*$ is rate determining. It would, therefore, be of great interest to inquire whether the activation energy of other substrates of XO is close to the value found for 2-chloropurine. Such studies may shed light on the problem whether or not always the same groups of the active center participate in the attachment of a substrate.

The probability to encounter twin pathways is greatest with very slow enzymatic reactions. This is demonstrated, e.g., by experiments in the pteridine series, where only the most tardy reactions use branched routes (11). Apparently, when the oxidation proceeds rapidly, it is insensitive to delicate electronic influences of substituents and thus employs only a solitary track. If, however, the reaction is sluggish, inductive and mesomeric effects become very important for the activation of certain positions, which in fast processes cannot compete with the main point of attack.

Finally it should be recognized that the equality of v_2 and v_3 in the case of 2-chloropurine is accidental and that other substrates may be found which use simultaneously two pathways, but at *unequal* rates. In such a case again external conditions should have no effect on the ratio of v_2 to v_3.

Acknowledgment

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