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XANTHINE OXIDASE

STUDY OF THE ENZYME-CATALYZED OXIDATION OF HYPOXANTHINE THROUGH THE CHEMILUMINESCENCE OF LUMINOL

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SUMMARY

1. Some characteristics of the curves of light *versus* time obtained by action of xanthine oxidase upon hypoxanthine in the presence of O_2 and luminol are described.

2. The shape of these curves differs from those described for enzyme–substrate compounds according to the Briggs–Haldane kinetics and from that of experimental curves of reaction rate *versus* time during the enzymic oxidation of hypoxanthine.

3. The emission curves are compared with theoretical ones representing the transient accumulation of unstable intermediates originating from an enzyme–substrate compound, and their resemblance is shown.

4. A hypothetical sequence of reactions is proposed which fits the experimental kinetic data.

INTRODUCTION

The application of chemiluminescence in studying the course of the enzymic oxidation of hypoxanthine by xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2) has provided a new tool for the investigation of the mechanism of this reaction. TOTTER *et al.*[1,2] showed the resemblance of the enzymatically induced chemiluminescence of luminol (6-amino-2,3-dihydro-1,4-phthalazinedione) to curves of "enzyme–substrate compound" concentration *versus* time. A more complex mechanism was found when lucigenin was used[3–5].

Further findings, however, led us to reexamine the explanation of the emission with luminol. At high substrate concentrations the maximum emission intensity is not reached at the very beginning of the reaction but after a sometimes significant time interval[2]. In our work, when hypoxanthine was used, this shift in the maximum always replaced the expected plateau corresponding to the zero-order situation[6]. This suggests the possibility that the time-course of some intermediate might not follow that of the whole enzymic reaction.

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MATERIALS AND METHODS

The xanthine oxidase was prepared in our laboratory according to the method of HORECKER AND HEPPEL[7]. The obtained samples catalyzed the production of 0.51–0.72 μmole of uric acid per min and per mg of protein from xanthine, in 0.1 M pyrophosphate buffer (pH 8.3) at room temperature (20°). The protein content of these samples as measured by its absorbance at 280 nm varied from 10 to 13 mg/ml. The $A_{280\ nm}/A_{450\ nm}$ ratios[8] ranged from 6.0 to 7.5. The determinations of enzyme activity were made in a Beckman D.U. spectrophotometer, taking the readings of absorbance at regular time intervals, at $\lambda = 295$ nm as a measure of uric acid production. An extinction coefficient difference at this wavelength between xanthine and uric acid of 9.5 $mM^{-1} \cdot cm^{-1}$ was applied to these values for the calculation of enzyme units[9]. In the case of prolonged reactions, these were carried out under continuous bubbling of O_2, in order to avoid its exhaustion in the solution.

The chemiluminescent reactions were studied in a Model 520 M Photovolt multiplier photometer, fitted with a special device which made possible the commencement of the reaction by injection of the enzyme into a dark compartment containing the tube with the mixture of reactants. O_2 was bubbled and a recorder was adapted to the photometer.

The following reactants were used: hypoxanthine from Sigma Chemical Co.; xanthine and EDTA·$2H_2O$ (disodium salt) from Merck; luminol from Fluka and catalase (bovine) from N.B. Co.; all other reactants were of analytical grade.

Enzyme concentration in each individual experiment will be referred to in International Units (I.U.) as defined by the Enzyme Commission of the International Union of Biochemistry[10], except for temperature conditions. Consequently, we define a unit as the amount of enzyme which causes the production of 1 μmole of uric acid per min from xanthine, in 0.1 M pyrophosphate buffer (pH 8.3) under aerobic conditions at 20°.

All experiments included in this paper are representative of a minimum of three series with identical qualitative results. The theoretical curves were computed as described in the legend to the corresponding figure.

RESULTS

Course of the reaction at various initial concentrations of enzyme and substrate

Fig. 1 shows three curves from a series of luminescence records at various initial concentrations of hypoxanthine. Their areas increase linearly with increasing substrate concentration, as could be expected from curves of enzyme–substrate complexes. However, they show specific properties which are noted below. Fig. 2 presents a family of curves in which the variable is the concentration of enzyme.

The agreement of K_m values calculated from chemiluminescent and spectrophotometric techniques was already stated in the above-mentioned paper[2]. We generally obtained compatible results, although our values for K_m were higher, ranging from 0.1 to 0.3 mM (hypoxanthine of pH 10.0).

In Fig. 3 the time-course of an individual enzymic reaction, as followed by readings of uric acid production, is compared with that of the chemiluminescence of luminol under similar conditions. Maximum values of reaction velocity and light intensity are reached at different times.

Spceific effects upon the enzymic and the chemiluminescent reactions

The specific inhibition of chemiluminescence by uric acid is shown in Fig. 4. Although light emission was strongly inhibited, there was no appreciable effect upon the enzymic reaction measured by uric acid production. A similar inhibition of chemiluminescence is caused by catalase, 2-mercaptoethanol[11] and cysteine[12]. The enhancing effect of CN^- upon chemiluminescence was previously reported[2,13].

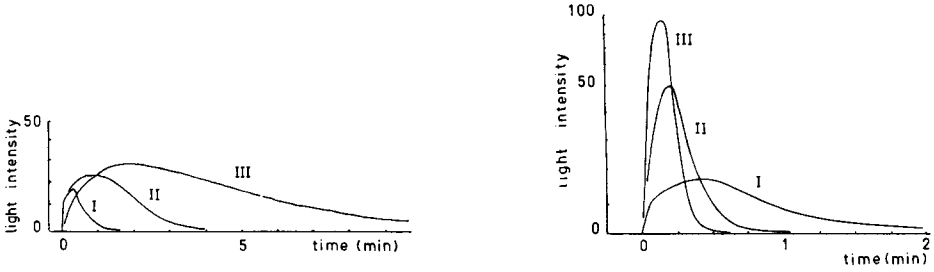

Fig. 1. Curves of chemiluminescent emission at various hypoxanthine concentrations. The xanthine oxidase was diluted in each test tube to approx. 0.8 I.U./ml. The respective concentrations of hypoxanthine were, in mM: Curve I, 0.41; Curve II, 1.65; Curve III, 5.0. Other reactants were: 0.5 mM luminol, 62.5 μM EDTA and 25 mM carbonate–bicarbonate buffer (pH 10.0). The reactions were carried out under O_2 bubbling as described under MATERIALS AND METHODS. Light intensity was measured in photometer scale units.

Fig. 2. Curves of light *vs.* time obtained with xanthine oxidase, hypoxanthine, luminol and O_2, varying enzyme concentrations. The original sample of enzyme was diluted, from Curve I to Curve III, to the following concentrations, in I.U./ml: 0.16, 0.49, and 0.81, respectively. Other reactants were: 0.1 mM hypoxanthine, 0.5 mM luminol, 25 mM carbonate–bicarbonate buffer (pH 10.0). The experiment was carried out at 17°. Light intensity is given in photometer scale units.

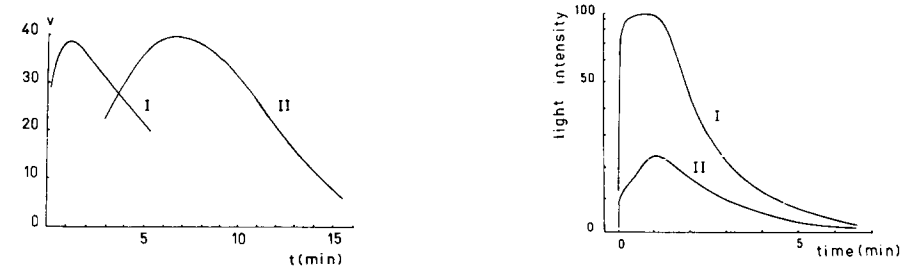

Fig. 3. Compared curves of reaction velocity *vs.* time in the enzymic oxidation of hypoxanthine. I. By spectrophotometrically measuring uric acid production. II. By recording the chemiluminescence of luminol. The reaction was carried out in 25 mM carbonate–bicarbonate buffer (pH 10.0). The concentration of the reactants was: 1.25 mM hypoxanthine; 0.5 mM luminol (included only in Reaction II); the enzyme was at a final concentration of 0.14 I.U./ml and both reactions were carried out under bubbling of O_2. Light intensity is given in photometer scale units $\times$ 0.1. The production of uric acid was followed by reading the absorbance at 320 nm every 30 sec; the values so obtained were plotted in a graph, from which those of velocity were obtained as the derivatives of the curve taken at regular time intervals. Values of v are given in $\Delta A/\Delta t \times 600$.

Fig. 4. Effect of uric acid on the chemiluminescence obtained by action of xanthine oxidase upon hypoxanthine in the presence of luminol and O_2. Curve I, without uric acid; Curve II, with uric acid. The concentration of hypoxanthine was in both cases 69 μM; the same concentration of uric acid was included in the reaction mixture of Curve II. Other reactants were 0.5 mM luminol, 12.5 mM carbonate–bicarbonate buffer (pH 10.0), and 0.04 I.U./ml of xanthine oxidase. Spectrophotometric controls showed no significative effect on enzyme activity, whereas uric acid also inhibited the chemiluminescence of luminol induced by alkaline persulfate.

The curve of luminescence corresponding to an inhibited enzymic reaction is illustrated in Fig. 5, obtained by using guanine together with hypoxanthine. Here, the main effect of the inhibitor is to "stretch" the curve, the last portion of which declines more slowly in the inhibited case.

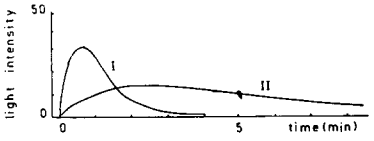

Fig. 5. Curves of chemiluminescent emission of luminol with xanthine oxidase, hypoxanthine and O_2, showing the inhibition of the enzymic reaction by guanine. Curve I, without guanine; Curve II, with guanine. The concentrations of the reactants were: 0.5 mM luminol, 1.25 mM hypoxanthine, 0.825 mM guanine (only in the reaction mixture of Curve II); 0.125 mM EDTA, and 25 mM carbonate–bicarbonate buffer (pH 10.0). The concentration of the enzyme was 0.95 I.U./ml. Light is given as galvanometer deflections × 0.1.

DISCUSSION

The fact that the maximum light intensities in Fig. 1 are not the initial ones and that the chemiluminescence does not present a true zero order even at high substrate concentrations indicates that the kinetics of such curves differs from the simple Michaelis–Menten or Briggs–Haldane mechanisms[6,14]. The delay in the attainment of a maximum value is not to be expected from the reported kinetic data, since the K_m values show a great affinity for the combination of the enzyme with its substrates and O_2[15,16].

We have already reported some other peculiarities of the enzymatically induced chemiluminescence. Variations in the initial concentrations of enzyme and substrate affect the shape and the duration of the curves of chemiluminescence as already shown (Figs. 1 and 2). This effect may be visualized by variations in the "half times" or by measuring the values of a "first-order constant" of the final portion of these curves. The latter were directly related to the enzyme concentration and inversely to the initial concentration of hypoxanthine[17]. As a practical consequence, we can explore the effects on the enzymic and on the chemiluminescent steps by examining those on the time-course of the reaction and on light intensity, respectively (see Figs. 4 and 5).

Curves of a shape similar to those of chemiluminescence may reflect the level of an unstable product developed from one of the complexes during the enzyme-catalyzed reaction and consumed in a subsequent step. Considering the enzymic reaction, we can abstract two consecutive steps in which an intermediate is formed and subsequently disappears:

$$\text{Compound A} \xrightarrow{k_a} I \qquad (1)$$

$$I \xrightarrow{k_b} \text{Compound B} \qquad (2)$$

where I represents the intermediate.

Fig. 6 shows theoretical concentration curves of an enzyme–substrate compound, plotted *versus* time, and of a hypothetical intermediate derived from the former by applying the rate equations corresponding to Steps 1 and 2. One can see that, when values of I concentration are calculated taking k_a slightly greater than k_b,

the curve of I concentrations has an ascendent phase during the time in which ES concentration remains nearly constant (Curve III, Fig. 6; *cf.* with Fig. 1).

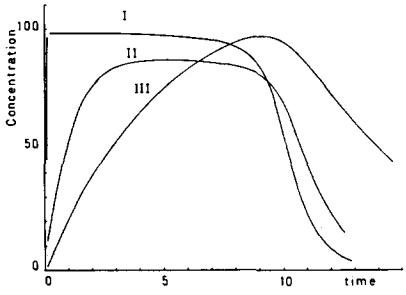

Fig. 6. Theoretical concentration curves of an enzyme–substrate compound (Curve I) and of an intermediate produced from such a compound (Curves II and III). Curve I was calculated from the following values: $E_0 = 100$; $S_0 = 1000$; $k_1 = k_2 = 0.0001$; $k_3 = 0.01$, by repeating the following sequence of operations: $[E]_t = [E]_0 - [ES]_{t-1}$; $[S]_t = [S]_0 - [ES]_{t-1} - [P]_{t-1}$; $[ES_a] = k_1[E]_t[S]_t + [ES]_{t-1}$; $[ES_b] = [ES_a] - k_2[ES_a]$; $[ES]_t = [ES_b] - k_3[ES_b]$; $[P]_t = [P]_{t-1} + k_3[ES_b]$. In this curve the successive values of $[ES]_t$ were graphed, including 100 calculated values each arbitrary unit of time on the graph. Curves II and III were calculated from one of each 10 calculated values of $[ES]_t$ and making $[I]_t = [I]_{t-1} + k_a[ES]_t - k_b([I]_{t-1} + k_a[ES]_t)$. The values assumed for the constants in Curve II were $k_a = k_b = 0.1$ and, in Curve III, $k_a = 0.1$ and $k_b = 0.02$.

The question arises as to whether we are dealing with one of the forms or complexes of the enzyme[15,18] or with a rather different compound, such as a free radical[2,11,19,20] or an excited form of luminol. The data in Fig. 4 would indicate that production of the intermediate occurs during a step different from that of uric acid. These results are compatible with the various mechanisms proposed to date for reactions of xanthine oxidase[15,16] in which the production of uric acid and the reduction of O_2 occur at different steps. However, further conclusions could be drawn from the described findings. The independence of the time-course of the luminescent curves with respect to luminol concentration and to the factors which specifically affect light intensity and its dependence on the concentrations of enzyme and substrate[17] strongly suggest that this intermediate does not correspond to the luminescent reactions and that it accumulates in a previous rate-limiting step. An attempt to explain this dependence may be made assuming a sequence of reactions like the following:

$$EX_1 \xrightarrow{k_1} EX_2 \tag{3}$$

$$EX_2 + S \xrightarrow{k_2} EX_3 \tag{4}$$

$$EX_1 \xrightarrow{k_3} I \tag{5}$$

$$I + EX_2 \xrightarrow{k_4} EX_4 \tag{6}$$

Here, EX_1 to EX_4 represent the compounds corresponding to different stages of the enzymic reaction, and I is the intermediate the concentration of which light intensity depends upon.

A roughly approximated expression for $d[I]/dt$ may be made assuming $k_4[I] \ll k_2[S]$ and a steady state for $[ES_2]$:

$$\frac{d[I]}{dt} = k_3[EX_1] - k_4[I]\frac{k_1[EX_1]}{k_2[S]} \tag{7}$$

If I behaves like the intermediate of Curve III in Fig. 6, as the reaction approaches its end, $d(I)/dt$ will tend to $-k_4[I]\,k_1[EX_1]/k_2[S]$. This sequence is presented only as a model which can fit a kinetics similar to that of the enzymatically induced chemiluminescence, if $[EX_1]$ is made independent of $[S]_0$. It is difficult to imagine such a sequence in the enzymic cycle of reactions, but it could indicate that a low-molecular-weight intermediate acts as a carrier between two forms or complexes of the enzyme during its catalytic action. A similar role was assigned to oxygen radicals in reduction of cytochrome *c* by xanthine oxidase[20], but in our case it seems that it is not the same compound that acts on luminol. We cannot go further with the present data; more work is needed to clarify these facts and the role of additional factors, such as substrate and product inhibitions, in determining the shape of the experimental curves.

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