ISOLATION AND PROPERTIES OF THE ENZYME WHICH CATALYZES THE OXIDATION OF FORMYCIN B

Sir:

In a previous paper[1] we reported that a variety of organisms were able to convert formycin B to oxoformycin B, analogs of inosine and xanthosine respectively[2,3]. No enzymic reaction has ever been known which catalyzes the oxidation of inosine to xanthosine at the nucleoside level. In this respect, the mechanism of oxidation of formycin B *in vivo* was of particular interest and an attempt was made to isolate the enzyme from rabbit liver and to study its properties.

Assay of the enzymic activity:

The complete reaction mixture contained, in a final volume of 1.0 ml, ^{3}H-formycin B at 0.02 mM (2.7×10^{-2} μC), potassium phosphate at 0.05 M, pH 7.1 and 0.1 ml of enzyme solution. The reaction mixture devoid of ^{3}H-formycin B was preincubated for 2 minutes prior to initiation of the reaction by adding the substrate. In particular experiments, 0.1 ml of aqueous solution of a candidate compound which may affect the reaction was included. The solution was added to the assay system together with ^{3}H-formycin B solution at zero time. The reaction was performed at 37°C for 15 minutes and terminated by addition of 1 ml of 10 % perchloric acid to the mixture. Denatured protein was removed by centrifugation and the supernatant was neutralized to faint green of bromthymol blue with KOH solution. Potassium perchlorate was removed by centrifugation and the supernatant was passed through a column of 0.2 ml wet carbon followed by washing with 3 ml of water. From the carbon column, ^{3}H-formycin B and ^{3}H-oxoformycin B were eluted with a solvent system of isopropanol – water – 28 % aqueous ammonia (100 : 96 : 4, by volume). The eluent was evaporated *in vacuo* to dryness, the residue was dissolved in 0.2 ml of water, charged on a column (0.9×2 cm) of Dowex 50W-X2 (H^+) resin and eluted with 3.8 ml of water. Under these conditions, ^{3}H-oxoformycin B was quantitatively released from the resin which still retained ^{3}H-formycin B. The latter compound could be eluted with 0.2 N NH_4OH. Radioactivities of these two fractions were measured with PPO-dioxane in a Beckman Liquid Scintillation System. Good separation of ^{3}H-oxoformycin B from ^{3}H-formycin B under these conditions was confirmed by a paper chromatography in a solvent system of butanol – acetic acid – water (2 : 1 : 1, by volume). The Rf's of formycin B and oxoformycin B were 0.33 and 0.46 respectively. The reaction rate is expressed as % conversion of formycin B to oxoformycin B based on the following equation:

$$\frac{\dfrac{\text{cpm of oxoformycin B}}{0.58}}{\text{cpm of unreacted formycin B}+\dfrac{\text{cpm of oxoformycin B}}{0.58}}\times100\ \%$$

Note: Specific radioactivity of oxoformycin B is 58 % of that of formycin B.

No side-reaction was observed even when the crude extract was used as enzyme solution. The amount of enzyme in the reaction mixture was limited to a range where the reaction rate was proportional to the enzyme concentration. The reaction rate was proportional to the period of incubation time up to 15 minutes.

Isolation procedure:

A preliminary study revealed that the enzyme, localized in liver, kidney cortex, kidney medulla and spleen occurred in a decreasing order, based on the activity per tissue weight. No appreciable activity was observed in serum or blood cells. In an isolation procedure, 785 g of wet liver slices were taken from 5 rabbits with an average weight of 3 kg. All the procedures were performed in a cold room where the temperature was maintained below 2°C. The liver slices were homogenized with 5 volumes 0.25 M sucrose solution in an electric mixer at a moderate speed for 3 minutes. The juice thus obtained was centrifuged at 9,000 r.p.m. ($7,000\times g$) for 10 minutes. The supernatant was combined with a washing which was obtained by suspending the sediment in 300 ml of 0.25 M sucrose and spinning it at 9,000 r.p.m. ($7,000\times g$). The total $7,000\times g$ supernatant was centrifuged again at 11,300 r.p.m. ($10,300\times g$) for 10 minutes to give

Table 1. Inhibition of formycin B oxidation by various compounds

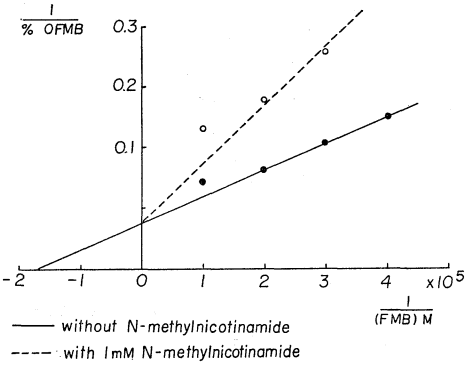
Possible inhibitor	Concentration (mM)	Inhibition (%)
None		0
N-Methylnicotinamide	1.0	58
	0.1	38
	0.01	7
Inosine	0.1	9
Allopurinol	0.1	2
Hypoxanthine	0.1	−7

4,200 ml of a slightly turbid supernatant. This fraction was used as the starting material and referred to as "crude extract". The enzymic activity of freshly prepared crude extract was 57 international units per 1 mg of protein (bovine serum albumin equivalent). To 4,200 ml of the crude extract, 12.6 g of protamine sulfate was added and the solution was left standing for 3 hours followed by centrifugation at 9,500 r.p.m. for 10 minutes to obtain 3,900 ml of supernatant. To the supernatant, after buffering with 39 ml of 5 M potassium phosphate at pH 7.8, 563 g of ammonium sulfate was added in small portions under rapid mixing to achieve 25 % saturation with the salt. The mixture was left standing overnight and centrifuged at 15,000 r.p.m. for 10 minutes to remove the precipitate which carried only a small amount of the total enzyme. In a similar way as described above, 513 g of ammonium sulfate was added to 4,100 ml of the supernatant to achieve 45 % saturation. The precipitate was collected by centrifugation, and was completely dissolved in 0.05 M potassium phosphate buffer at pH 7.8 to give 120 ml of a solution, designated as "ammonium sulfate fraction". This fraction was used as enzyme solution unless otherwise stated. These purification procedures resulted in 6-fold purification of the enzyme over the crude extract, while the recovery of total enzymic activity in the ammonium sulfate fraction was 92 %.

Enzyme properties:

Since the enzyme was found constitutively in rabbit liver, it was of great interest to know the normal function of this enzyme. Various metabolites which could be the normal substrate for the enzyme were examined for their possible inhibition of the oxidation of formycin B. As shown in Table 1, only N-methylnicotinamide, a substrate for aldehyde oxidase[4] (1.2.3.1.), inhibited the reaction at fairly low concentrations while inosine, hypoxanthine and allopurinol[5] did not affect the reaction. These observations strongly suggest that aldehyde oxidase is the enzyme responsible of the oxidation of formycin B in liver. On the contrary, xanthine oxidase (1.2.3.2.) should be ruled out because neither hypoxanthine nor allopurinol inhibited the reaction. Furthermore, as we reported earlier[1], a purified preparation of xanthine oxidase did not catalyze the oxidation of formycin B.

Fig. 1. The effect of substrate concentration on the reaction velocity in the presence or absence of N-methylnicotinamide.



Recently we were informed that Dr. R. E. PARKS, Jr. of Brown University, Providence, Rhod Island, U.S.A. and his co-workers found that aldehyde oxidase activity paralleled with the reactivity with formycin B at all stages of purification. Their findings would be conclusive evidence for the above observations. Kinetic studies were made on the formycin B-oxidation reaction. As shown in Fig. 1, the effect of substrate concentration on the reaction velocity was determined in the presence or absence of a fixed amount of N-methylnicotinamide. From the LINEWEAVER-BURK plots, apparent *Km* of formycin B with the enzyme and *Ki* of N-methylnicotinamide against formycin B-oxidation were calculated to be 5×10^{-6} M and 7×10^{-4} M respectively. The competitive behavior of formycin B to N-methylnicotinamide was confirmed by a separate experiment in which the enzyme was incubated with the inhibitor 30 seconds before addition

of ^{3}H-formycin B, the substrate. Even under these conditions, the extent of inhibition was essentially the same as that obtained by the usual way. The relation between pH and reaction velocity was studied in 0.05 M potassium phosphate. The highest velocity was observed at pH 7.0 while at pH 6.0 and 8.3 the velocity was reduced to 91 % and 45 % of the optimum value respectively. When the reaction was performed in 0.05 M tris-HCl, pH 7.0, instead of potassium phosphate, the reaction rate was reduced to only 24 % of the value observed in phosphate buffer at the same pH. However, the optimal reaction rate was achieved in tris buffer if as little as 5 mM phosphate was present in the reaction mixture. Since phosphate is not a substrate of the reaction, it is likely that the enzyme is activated by phosphate.

The enzymic activity of the ammonium sulfate fraction could be retained without appreciable loss as long as 40 days when its pH was adjusted to 7.8 and the fraction was frozen. The enzyme was activated by 11 % upon exposure to pH 8.2 at 45°C for 10 minutes. It is uncertain whether this characteristic is intrinsic to the nature of the enzyme because of the low purity of the enzyme preparation.

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