Biochimica et Biophysica Acta, 293 (1973) 105–110

BBA 66786

EFFECT OF PURINE ANALOGUES ON IMP-PYROPHOSPHORYLASE

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(Received July 13th, 1972)

SUMMARY

IMP pyrophosphorylase was purified 35 times from an extract of beef liver by a modification of the method of Lukens and Herrington. It was found that increasing the temperature of the heating phases gives a better preparation.

The kinetic constants of the enzyme were determined, K_m (hypoxanthine) = $1.0 \cdot 10^{-5}$ M, K_m (5-phosphoribosyl-1-pyrophosphate) = $5.5 \cdot 10^{-5}$ M. The effect of purine analogues was determined by measuring the conversion of [^{14}C]hypoxanthine to [^{14}C]IMP. 41 substances were examined and 4 competitive inhibitors; 6-mercaptopurine, 2-amino-6-mercaptopurine, 4-hydroxy-6-aminopyrazolo (4,3d)pyrimidine and isoguanine, were found.

INTRODUCTION

IMP pyrophosphorylase (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) catalyzes the transfer of the ribose-5-phosphate moiety of 5-phosphoribosyl-1-pyrophosphate to hypoxanthine (Kornberg *et al.*[1]).

Hypoxanthine + 5-phosphoribosyl-1-pyrophosphate $\rightleftharpoons$ inosine 5-monophosphate (IMP) + + PP_i.

Nyhan and Lesh[2] and others[3–6] found that some patients with an excessive production of uric acid had decreased IMP pyrophosphorylase activity and an increased purine biosynthesis *de novo* in liver, brain cells and skin fibroblasts.

In order to study the possibility of inducing the symptoms of the Nyhan-Lesh syndrome in experimental animals various substances were tested with regard to their ability to inhibit IMP pyrophosphorylase *in vitro*. The inhibition of IMP pyrophosphorylase by 6-mercaptopurine, 2-amino-6-mercaptopurine and 8-azaguanine had been shown earlier by Carter[7], Atkinson and Murray[8], Miech *et al.*[9] and Way and Parks[10] The present report deals with the effect of 32 hypoxanthine and guanine analogues and 10 purine analogues on the IMP pyrophosphorylase from beef liver.

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EXPERIMENTAL PROCEDURE

Materials

[8-^{14}C]Hypoxanthine (9.6 Ci/mole, the Radiochemical Centre, Amersham, England), phosphorylribose-1-pyrophosphate, inosine monophosphate, hypoxanthine, 2-hydroxypurine, 6-amino-2-hydroxypurine (isoguanine), 6-chloro-2-aminopurine, 6-hydroxy-2-acetylaminopurine, guanine-7-*N*-oxide·HCl, 8-bromguanine, 4-hydroxypyrazolo(3,4d)pyrimidine (allopurinol), 8-azaxanthine, 7-methylguanine, xanthine, adenine and inosine (Sigma Chemical Co., St. Louis, USA). 6-Mercaptopurine, 2-amino-6-mercaptopurine, 2-mercapto-6-hydroxypurine, 2,8-dimercapto-6-hydroxypurine, 2,6-dimercapto-7-methylpurine, 8-azahypoxanthine, 8-azaguanine, 1-methylguanine and uric acid (Fluka A.G., Bucks, Switzerland). 6-Benzylthiopurine, 2-aminopurine and purine (Schuchardt, Munich, Germany). 6-Chloropurine (N.B.C., Cliveland, USA). Guanine (La Roche, Basel, Switzerland). 4-Hydroxy-6-aminopyrazolo (4,3d)pyrimidine·CH_3SO_3H, 4-methyl-6-methylaminopyrazolo (4,3d)pyrimidine, 4-methyl-6-guanidylpyrazolo (4,3d)pyrimidine, 3,4-dimethyl-6-aminopyrazolo (4,3d)-pyrimidine, 4-ethyl-6-aminopyrazolo (4,3d)pyrimidine, 6-methyl-8-aminothiazolo (4,5d)pyrimidine and 6-methyl-2,8-diaminothiazolo (4,5d)pyrimidine were gifts from F. L. Rose, ICI Ltd., Manchester, England. 2,6-Dimercaptopurine was a gift from J. A. Montgomery, Southern Research Institute, Birmingham, Alabama. 6-Mercapto-2-hydroxypurine, 6-mercapto-2-methylaminopurine and 6-mercapto-2-amino-9-methylpurine were gifts from Prof. J. F. Henderson, University of Alberta Cancer Research Unit, Edmonton, Alberta, Canada. 3-Deoxyinosine, 6-methylthiopurine and 9-methylhypoxanthine were gifts from K. Overgaard-Hansen, Biochemical Institute B, University of Copenhagen. 6-(1-Methyl-4-nitro-5-imidazolyl)thiopurine (Scanmeda, Copenhagen, Denmark). Xanthine oxidase (EC 1.2.3.2) was prepared from milk according to the method of Klenow and Emberland[11].

The IMP pyrophosphorylase was prepared from beef liver by a slight modification of the procedure of Lukens and Herrington[12]. The enzyme solution was heated during preparation Steps 1 and 2; 10 °C higher than indicated by the authors[12]. In this way the conversion of [^{14}C]hypoxanthine to inosine was reduced from 30% to 0% of the satured [^{14}C]hypoxanthine. No xanthine oxidase[13], purine nucleoside phosphorylase (EC 2.4.2.1)[13] or adenine phosphoribosyltransferase (EC 2.4.2.7) activity was found in any of the preparations. The final enzyme preparation was purified 35-fold and was stored at −18 °C in small aliquots. Immediately before use an aliquot was thawed and diluted in 0.033 M Tris–HCl buffer (pH 7.4) and under these conditions no loss of activity was measured during 4 months. Under the assay conditions described below and at a saturating concentration of substrate the enzyme unit was 0.11 μmole hypoxanthine per min. The specific activity of the enzyme was 6.1 units per mg protein.

Methods

Assays were carried out essentially as described by Kornberg *et al.*[1]. Protein was determined by the method of Warburg and Christian[14]. The hypoxanthine concentration was measured by enzymatic differential spectrophotometry using xanthine oxidase as described by Kalckar[13].

To the reaction mixture containing $MgCl_2$ (5.0 mM), Tris–HCl buffer (25 mM, adjusted to pH 7.4), enzyme (30 μg protein per ml of the reaction mixture), analogue

(62.5 μM) and [8-^{14}C]hypoxanthine in three different concentrations (0.1 mM, 0.3 mM and 0.6 mM) 5-phosphoribosyl-1-pyrophosphate (0.15 mM) was added to start the reaction (final volume of the reaction mixture, 0.6 ml). Aliquots (0.2 ml) were removed at various times and pipetted into 30 μl 2 M perchloric acid in ice. Each mixture was centrifuged and adjusted to pH 7 with 1 M KOH. 50 μl from each sample were applied to Whatman No. 40 paper together with non-radioactive IMP and developed in sodium acetate–alcohol buffer (pH 7.5) for 17 h. The IMP spot was localized by examining the chromatograms in ultraviolet light. The spots were cut out and put into a 10-ml scintillation solution (4 g PPO and 50 mg POPOP per l toluene) and counted in a Packard model 3324 Tricarb liquid-scintillation spectrometer system.

Enzyme kinetics

Inhibition was studied by measuring the effect of non-radioactive compounds on the enzymatic conversion of [^{14}C]hypoxanthine to [^{14}C]IMP. For each inhibitor initial velocities were determined at 3 different concentrations of [^{14}C]hypoxanthine. Corresponding experiments were carried out without inhibitor. Each experiment was seen 2 or 3 times. When inhibition was observed inhibition constants were calculated from the slopes of the lines (K'_m) in a system where the initial velocity (v, μmoles IMP per 5 min) is plotted against initial velocity divided by substrate concentration ($v/[S]$, S = hypoxanthine (μM)). The inhibitor constants are calculated according to the Eqn (ref. 15)

$$K'_m = K_m \left(1 + \frac{I}{K_i}\right)$$

RESULTS

19 determinations of the K_m value for hypoxanthine were made, each of these was carried out together with 4–6 experiments each with different analogues. Michaelis constants for the substrates of the enzyme were found to be similar to those previously found by Lukens and Herrington[12]. K_m (hypoxanthine) = $1.0 \cdot 10^{-5}$ M; K_m (5-phosphoribosyl-1-pyrophosphate) = $5.5 \cdot 10^{-5}$ M. The K_m value for guanine was determined in the same way as for the analogues. The K_m value was found to be $1.6 \cdot 10^{-5}$ M.

The analogues were chosen to cover a greatly varied area of chemical structures and consist partly of compounds with normal purine rings, but altered sidegroups, especially in Positions 1, 7, 8 and 9 (Table I, Group A) and partly of compounds with an altered ringsystem instead of the purine ring (Table I, Group B), pyrazolopyrimidines, thiazolopyrimidines and triazolopurines.

In addition some derivates (Table I, Group C) and the purine nucleosides inosine and β-D-3-deoxyinosine (Table I, Group D) have been examined. Finally the activity of a few adenine analogues was determined. Table I shows the range of the K'_m values of the 41 hypoxanthine and guanine analogues and derivatives. In this table it is seen that significant inhibition (the Wilcoxon, Mann-Whitney rank sum test, $p = 0.01$) of IMP-pyrophosphorylase was seen with only 4 substances, all of which were competitive inhibitors (Fig. 1). The other analogues which showed no

TABLE I

K'_m VALUES FOR HYPOXANTHINE AND GUANINE ANALOGUES AND OTHER COMPOUNDS FOR BEEF LIVER IMP PYROPHOSPHORYLASE

Reaction mixture conditions are as described in the text.

Group	*Analogue*	*Range* $K'_m \times 10^{-6}$ *(M)*
A. Hypoxanthine and guanine analogues where the sidegroups are changed	6-Mercaptopurine	58.5–80.1
	6-Methylthiopurine	8.1– 8.5
	6-Benzylthiopurine	8.1– 8.6
	Azathioprine	7.3– 7.2
	9-Methylhypoxanthine	8.1– 8.5
	2-Hydroxypurine	8.5–10.6
	Guanine	49.7–51.5
	2-Amino-6-mercaptopurine	49.6–91.7
	6-Mercapto-2-hydroxypurine	7.8–10.5
	2,6-Dimercaptopurine	8.1– 9.8
	2-Mercapto-6-hydroxypurine	7.5–10.7
	2,8-Dimercapto-6--hydroxypurine	8.4–13.9
	6-Amino-2-hydroxypurine	15.4–25.0
	6-Chloro-2-aminopurine	8.2– 9.5
	2-Aminopurine	7.5–10.6
	6-Hydroxy-2-acetyl-aminopurine	9.3–11.1
	6-Mercapto-2-methyl--aminopurine	9.5–10.6
	2,6-Dimercapto-7--methylpurine	7.4– 8.2
	Guanine-7-*N*-oxide·HCl	7.8– 9.0
	8-Bromoguanine	6.2– 7.7
	6-Mercapto-2-amino-9--methylpurine	9.5–10.5
B. Hypoxanthine and guanine analogues where the ring system is changed	4-Hydroxypyrazolo (3,4d) pyrimidine (allopurinol)	6.6– 8.4
	4-Hydroxy-6-aminopyrazolo (4,3d) pyrimidine	35.2–37.0
	4-Methyl-6-aminopyrazolo (4,3d) pyrimidine	9.6–11.3
	4-Methyl-6-methylamino-pyrazolo (4,3d) pyrimidine	9.3–11.7
	4-Methyl-6-guanidyl pyrazolo (4,3d) pyrimidine	9.6–11.2
	3,4-Dimethyl-6-aminopy-razolo (4,3d) pyrimidine	7.5–10.7
	4-Ethyl-6-aminopyrazolo (4,3d) pyrimidine	7.4–12.1
	6-Methyl-8-aminothiazolo (4,5d) pyrimidine	8.5–10.1
	6-Methyl-2,8-diamino thiazolo (4,5d) pyrimidine	9.4–11.1
	8-Azahypoxanthine	9.3–10.5
	8-Azaguanine	9.4–10.6
	8-Azaxanthine	6.0– 7.6
C. Derivates of hypoxanthine and guanine	1-Methylguanine·HCl	9.0– 9.7
	7-Methylguanine	7.8– 7.9
	Xanthine	12.2–13.5
	Uric acid	8.8– 9.0
D. Adenine and analogues of adenine	Adenine	6.8– 7.6
	Purine	8.6– 8.7
	6-Chloropurine	6.8– 8.1
	6-Methylpurine	9.8–11.8
E. Nucleoside	Inosine	8.0–13.4
	3-Deoxyinosine	8.0–10.8
Experiments without inhibitor		7.0–13.0

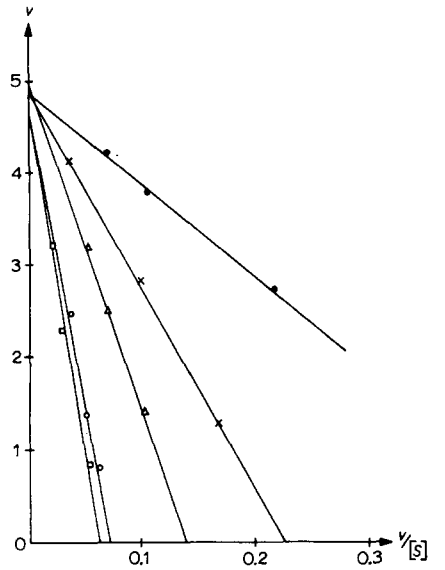

Fig. 1. Initial velocites (v, μmoles IMP synthesized per 5 min) are plotted against initial velocity divided by the substrate concentration ($v/[S]$, S = hypoxanthine (μM)). Inhibitors: □—□, 6-mercaptopurine; ○—○, 2-amino-6-mercaptopurine; △—△, 4-hydroxy-6-aminopyrazolo (4,3d) pyrimidine; ×—×, isoguanine; ●—●, control *i.e.* no inhibitor.

TABLE II

INHIBITION CONSTANTS FOR THE ENZYME CALCULATED FOR THE FOUR SIGNIFICANT INHIBITORS AS DESCRIBED IN THE TEXT

Inhibitor	K_i *(M)*
6-Mercaptopurine	$9.6 \cdot 10^{-6}$
2-Amino-6-mercaptopurine	$1.1 \cdot 10^{-5}$
4-Hydroxy-6-aminopyrazolo (4,3d) pyrimidine	$2.5 \cdot 10^{-5}$
Isoguanine	$6.1 \cdot 10^{-5}$

significant difference in K'_m values could be non-competitive inhibitors. However, this was not the case, as can be seen by plotting the experiments as previously described. There was no significant ($P > 0.05$) difference between the initial velocities (v) in the analogue experiment and the corresponding experiment without analogue. The K_i values calculated for the 4 inhibitors are given in Table II.

DISCUSSION

The formation of IMP from hypoxanthine and 5-phosphoribosyl-1-pyrophosphate catalyzed by IMP pyrophosphorylase purified from beef liver, was used as a screening system to test the inhibition of the enzyme by hypoxanthine and guanine analogues and derivatives. In mammals it has not been possible to separate the IMP pyrophosphorylase activity towards hypoxanthine and guanine[4,16], a fact which has been confirmed in this study.

Only a few substances show significant inhibition of the enzyme, indicating that the structural demand of the enzyme is very specific. As confirmed in this study 6-mercaptopurine and 2-amino-6-mercaptopurine have been found to be competitive inhibitors of IMP pyrophosphorylase[7,8,17,18]. Some new inhibitors have also been found, namely 4-hydroxy-2-aminopyrazolo(4,3d)pyrimidine, $K_i = 2.5 \cdot 10^{-5}$ M and isoguanine, $K_i = 6.1 \cdot 10^{-5}$ M. On the other hand, no inhibition of the enzymatic conversion of hypoxanthine to IMP was observed with 8-azaguanine, azathioprine, 4-hydroxypyrazolo (3,4d)pyrimidine and xanthine which has been reported by Kelley *et al.*[3,19] and Anderson and Law[20].

The best inhibitor of the IMP pyrophosphorylase *in vivo* which at the same time could elevate the hypoxanthine and uric acid concentration, should be a non-competitive rather than a competitive inhibitor. A competitive inhibitor could be converted to the nucleotide which might inhibit purine biosynthesis *de novo* and in that way reduce the production of hypoxanthine and uric acid[4,21]. At the same time the organism will lose 5-phosphoribosyl-1-pyrophosphate which is a limiting substrate for the 5-phosphoribosyl-1-pyrophosphate amidotransferase (EC 2.4.2.14) reaction, the first step in purine biosynthesis *de novo*[22].

The effect of 41 hypoxanthine and guanine analogues and derivatives on IMP pyrophosphorylase activity have been examined in this paper. None of these compounds were non-competitive inhibitors. The best inhibitor was 6-mercaptopurine, $K_i = 9.6 \cdot 10^{-6}$ M, but this substance would not be the inhibitor of choice *in vivo* for the reason mentioned above. A new competitive inhibitor 4-hydroxy-6-aminopyrazolo (4,3d)pyrimidine, which is almost as effective as 6-mercaptopurine has been found.

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