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PROPERTIES OF 5′-PHOSPHORIBOSYLPYROPHOSPHATE AMIDOTRANSFERASE IN VIRUS INDUCED MURINE LEUKEMIA

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SUMMARY

Virus-induced 5′-phosphoribosylpyrophosphate amidotransferase (ribosylamine 5′-phosphate:pyrophosphate phosphoribosyltransferase (glutamate amidating), (EC 2.4.2.14), the first enzyme in purine biosynthesis, was isolated from spleens of mice infected with Friend leukemia virus, purified and certain of its properties were studied. The enzyme, which catalyzes the synthesis of 5′-phosphoribosylamine has a requirement for two substrates: 5′-phosphoribosyl pyrophosphate as the phosphoribosyl donor, and for a nitrogen containing compound as a source of the amino group of 5′-phosphoribosylamine. Ribose 5-phosphate could not replace 5′-phosphoribosyl pyrophosphate. Nitrogen containing compounds found to be substrates were NH_4Cl, $(NH_4)_2SO_4$ and glutamine. The enzyme was sensitive to inhibition by purine ribonucleotides. Inhibition was competitive with 5′-ribosyl pyrophosphate and glutamine, but non-competitive with respect to NH_4Cl.

INTRODUCTION

Although *PP*-ribose-*P* amidotransferase (ribosylamine 5′-phosphate:pyrophosphate phosphoribosyltransferase (glutamate amidating, EC 2.4.2.14) has been studied in avian and bacterial systems, little information on the mammalian enzyme is available[1–3]. In a preliminary publication we reported some of the properties of a *PP*-ribose-*P* amidotransferase present in cell-free extracts of spleens of mice with virus-induced leukemia[4]. The activity of this enzyme increased considerably during the first week after infection with Friend leukemia virus and declined gradually thereafter.

The study of *PP*-ribose-*P* amidotransferase in mammalian tissues was initially hampered by the instability of the enzyme and by its relatively low activity in mammalian cells, the object of the present studies was to purify the mammalian enzyme and to investigate some of its properties. Advantage was taken of the fact

that enzyme activity increases early in the course of virus induced murine leukemia. Therefore preparations were made approx. 1 week after infection with Friend leukemia virus when the spleens were enlarged and enzyme activity was at its peak.

MATERIALS AND METHODS

Chemicals

Purines and purine nucleotides were obtained from the Nutritional Biochemical Corporation, amino acids from Calbiochem., DEAE-cellulose as DE-52 from Whatman, Sephadex G-25 from Pharmacia.

Preparation of animals

Random bred female Swiss mice, weighing 18–20 g obtained from Taconic Farms were infected with Friend leukemia virus by intra-peritoneal injection of 0.2 ml of virus filtrate obtained from a 20% homogenate of leukemic spleens as previously described[5]. Mice were killed by cervical compression, the spleens removed, placed on ice and homogenized as quickly as possible as described in RESULTS.

Enzyme assay

The assay used was that originally described by HARTMAN, LEVENBERG AND BUCHANAN[6] as adapted by NIERLICH AND MAGASANIK[3]. Briefly, it is based on the following reactions:

Step 1: *PP*-Ribose-*P* + glutamine → 5′-phosphoribosylamine + PP_i (1)

5′-Phosphoribosylamine + glycine + ATP ⇄ 5′-phosphoribosylglycineamide + ADP + P_i (2)

Step 2: 5′-phosphoribosylglycineamide + IMP + H_2O → 5′-phosphoribosylformylglycineamide + 5′-phosphoribosyl-5-amino-4-imidazolecarboxamide (3)

The 5′-phosphoribosyl-5-amino-4-imidazolecarboxamide formed in the coupled assay is measured colorimetrically by the method of BRATTON AND MARSHALL (see ref. 3).

Reaction 1 is catalyzed by *PP*-ribose-*P* amidotransferase, Reaction 2 by *P*-ribosylglycineamide synthetase. A partially purified fraction of *P*-ribosylglycineamide synthetase prepared from chicken livers by the method of HARTMAN was therefore added to the reaction mixture[7]. Since Reaction 3 requires transformylating enzymes and cofactors, these were supplied by adding a 15–30% ethanol fraction of chicken liver prepared as described[8]. The enzyme assay was carried out in 2 steps: Step 1: the reaction mixture (0.4 ml) containing 0.2 μmole *PP*-ribose-*P*, 4 μmole NH_4Cl, 0.8 μmole $MgCl_2$, 3.5 μmole glycine, 0.4 μmole ATP, 30 μmole Tris–HCl buffer (pH 8.9) and 0.02 ml of a standardized preparation of *P*-ribosylglycineamide synthetase was incubated for 5 min at 37°. Step 2: reactions carried out in Step 1 were stopped by the addition of 15 μmoles EDTA with 2.5 μmoles IMP (0.1 ml), the latter served to initiate Step 2 of the assay. After the addition of 0.1 ml (2.5 mg) of a standardized 15–30% ethanol fraction of chicken liver homogenate dissolved in 0.025 M potassium phosphate buffer (pH 7.5), incubation was continued for 30 min at 37°. The reaction was stopped by the addition of 0.1 ml of a solution of 15% trichloroacetic acid in 1 M HCl followed by 0.05 ml acetic anhydride. After centrifugation, 5′-phosphoribosyl-5-amino-4-imidazolecarboxamide formed during the second

step of the assay was determined by the method of BRATTON AND MARSHALL (see ref. 3). 6.9 nmoles of 5-amino-4-imidazolecarboxamide standard give an absorbance reading of 0.10 at 540 mμ. Proteins were measured spectrophotometrically[9].

RESULTS

Purification of PP-ribose-P amidotransferase

88 female Swiss mice were sacrificed 8 days after they had been infected with Friend leukemia virus by intraperitoneal injection of 0.2 ml of virus filtrate. The spleens weighed 97 g. All steps were carried out at 4° and all buffers contained 10^{-3} M 2-mercaptoethanol and 10^{-4} M EDTA. Spleens were homogenized with a Virtis homogenizer in 0.1 M ammonium citrate buffer (pH 5.0), 1.0 ml/g spleen. The homogenate was centrifuged for 60 min at 100 000 × *g* and the precipitate discarded (Table I).

TABLE I

PURIFICATION OF *PP*-RIBOSE-*P* AMIDOTRANSFERASE FROM SPLEENS OF LEUKEMIC MICE

Activity is expressed as nmoles *P*-ribosylamine formed per min per mg protein under standard assay conditions.

Stage and procedure	*Protein (mg/ml)*	*Activity (nmoles/mg)*	*Relative activity*	*Yield*
1. Soluble extract	37	1.24	1	100
2. Heat-stable fraction	23	1.91	1.5	52*
2a. Heat-stable fraction stored at −18° for 48 h	21	0.65	0.5	17
3. $(NH_4)_2SO_4$ fraction	4.8	2.70	2.2	6*
4. DEAE-cellulose chromatography	1.1	23.6	19.0	12*

* These stages of purification were carried out with only a part of the preceding enzyme fraction. The recovery reported has been corrected for sample size.

The pH of the supernatant fraction (Stage 1) was adjusted to 7.5 by the gradual addition of 1.0 M tribasic potassium phosphate buffer (to 104 ml supernatant 6 ml buffer were added), placed in a water bath (70°) and heated to 60° under constant stirring. As soon as the temperature of the supernatant fraction reached 60° it was placed on ice and allowed to cool to 4°. After centrifugation the precipitate was discarded. The heat-stable supernatant fraction (Stage 2), was adjusted to pH 5.4 by the addition of 1 M monobasic potassium phosphate buffer. (To 21 ml of supernatant, 8 ml of buffer were added). This solution was precipitated by the addition of 8 g of solid $(NH_4)_2SO_4$ (27.8 g/100 ml, 45% satn.), the precipitate collected by centrifugation and dissolved in 15 ml 0.035 M potassium phosphate buffer (pH 7.5). This solution (Stage 3) was dialyzed overnight against the same buffer. 9.9 ml of the dialyzed solution (Stage 3) were applied to a DEAE-cellulose column (2 cm × 22 cm) which was previously equilibrated with 0.035 M potassium phosphate buffer (pH 7.5). The enzyme was eluted from the column with potassium phosphate buffer (pH 6.5), by creating a linear gradient of increasing salt concentration. Two connecting identical cylindrical chambers were used, the reservoir chambers contained 100 ml 0.8 M potassium phosphate buffer; the mixing chamber, 100 ml 0.05 M potassium phosphate buffer. The eluate was collected in fractions of approx. 5 ml. After 140 ml of colorless

eluate had been collected, the bulk of the enzyme activity emerged in two consecutive fractions of the column eluate which had a light brown color (Stage 4).

Enzyme stability

The enzyme at Stage 2 proved to be very unstable and lost approximately one-half of its activity during overnight dialysis at 4° as well as during 48-h storage at −18°. Desalting of this fraction by passage through a Sephadex G-25 column caused a similar loss of activity. The enzyme fractions obtained after DEAE-cellulose column chromatography (Stage 4) lost their activity very rapidly when stored at 4° or when kept in an ice bucket. Storage for 4–5 h could lead to a loss of 50% of the enzyme activity. No appreciable loss of enzyme activity was observed however, when the enzyme was stored at −18° for several days; the enzyme could withstand freezing and thawing. By thawing the enzyme fractions just prior to incubation and refreezing them immediately after use, it was possible to preserve their activity.

TABLE II

EFFECT OF DIFFERENT NITROGEN CONTAINING SUBSTRATES ON *PP*-RIBOSE-*P* AMIDOTRANSFERASE ACTIVITY

The conditions of assay were as previously described in MATERIALS AND METHODS. 0.11 mg enzyme Stage 4 was added. In all cases 0.01 M nitrogen containing substrate was added as indicated.

Substrate added	*P-ribosylamine formed (nmoles/min)*
None	0.40
Glutamine	2.50
NH_4Cl	3.10
$(NH_4)_2SO_4$	3.10
Asparagine	0.50
Aspartate	0.74
Carbamyl phosphate	0.78

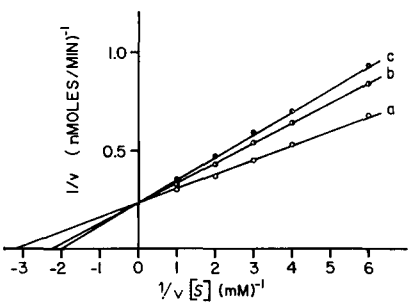
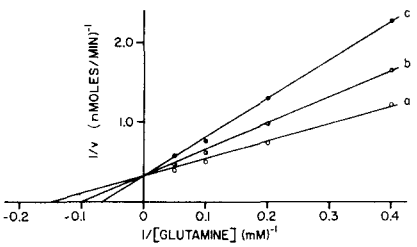


Fig. 1. Effect of *PP*-ribose-*P*-concentration [*S*] and the addition of AMP on the activity of *PP*-ribose-*P* amidotransferase. Double reciprocal plot of velocity and *PP*-ribose-*P* concentration ([*S*]) as indicated. Curve a, determinations were done under standard conditions as described in MATERIALS AND METHODS; Curve b, conditions as described with the addition of 10^{-3} M AMP; Curve c, conditions as above, but with the addition of $2 \cdot 10^{-3}$ M AMP. 0.12 mg enzyme Stage 4 were incubated.

Substrate specificity

The enzyme required *PP*-ribose-*P*; ribose-5-*P* could not be substituted for *PP*-ribose-*P*. Several nitrogen containing compounds were tested to see whether they could serve as substrates. Enzyme at Stage 4 could use glutamine, NH_4Cl and $(NH_4)_2SO_4$, while carbamyl phosphate, aspartate and asparagine were not suitable substrates (Table II).



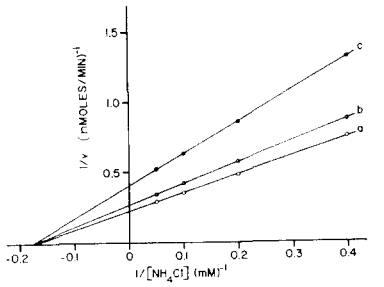


Fig. 2. Effect of glutamine concentration and the addition of AMP or GMP on the activity of *PP*-ribose-*P* amidotransferase. Double reciprocal plot of velocity and glutamine concentration as indicated. Curve a, assays were carried out as described in MATERIALS AND METHODS; Curve b, conditions as described with the addition of $2 \cdot 10^{-3}$ M GMP; Curve c, conditions as above but with the addition of $2 \cdot 10^{-3}$ M AMP. 0.12 mg enzyme Stage 4 was used.

Fig. 3. Effect of NH_4Cl concentration and the addition of AMP or GMP on the activity of *PP*-ribose-*P* amidotransferase. Double reciprocal plot of velocity *versus* NH_4Cl concentration as indicated. Curve a, standard conditions as described in MATERIALS AND METHODS; Curve b, conditions as described, but with the addition of $2 \cdot 10^{-3}$ M GMP; Curve c, conditions as above, but with the addition of $2 \cdot 10^{-3}$ M AMP. 0.12 mg enzyme Stage 4 was incubated.

TABLE III

COMPARISON OF REACTION RATES USING GLUTAMINE AND NH_4Cl AS SUBSTRATES AT DIFFERENT STAGES OF ENZYME PURIFICATION

Assays were carried out as described in MATERIALS AND METHODS using 0.1 ml enzyme (stage as indicated). Substrates were added as indicated at a concn. of 0.01 M.

Stage of purification	*Substrate (nmoles P-ribosylamine formed/min)*		*Activity ratio*
	Glutamine	*NH_4Cl*	
1	4.0	4.6	0.87
2	4.1	4.4	0.93
3	1.1	1.3	0.85
4	2.1	2.6	0.81

Enzyme constants

The K_m for *PP*-ribose-*P* was $3.3 \cdot 10^{-4}$ M (Fig. 1). The K_m for glutamine and for ammonium chloride did not differ significantly from each other; that for glutamine was $7 \cdot 10^{-3}$ M, that for NH_4Cl was $6 \cdot 10^{-3}$ M (Figs. 2, 3). Enzyme activity with NH_4Cl as substrate exceeded that with glutamine at all stages of enzyme purification (Table III).

Substrate inhibition

NH_4Cl was not inhibitory at concentrations ranging from 10^{-3} M to $5 \cdot 10^{-2}$ M. Glutamine was inhibitory at concentrations above $2.5 \cdot 10^{-2}$ M (Fig. 4).

Requirements

Mg^{2+} was required for the reaction. EDTA at a concentration of 0.035 M inhibited the reaction completely.

Inhibition by purines and their derivatives

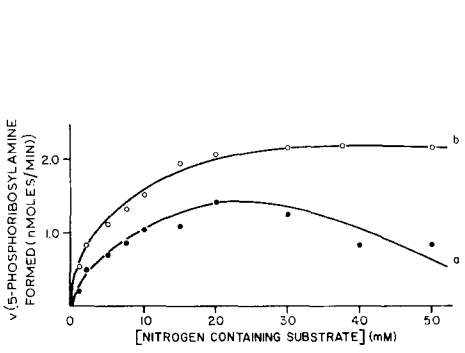
The enzyme responded to inhibition by purine ribonucleotides at various stages of purification (Table IV). Enzyme at Stage 1 was most sensitive to feedback inhibition. Sensitivity to inhibition by end-products of the pathway diminished, but was not abolished with increasing purification. If enzyme fractions following precipitation by $(NH_4)_2SO_4$ (Stage 3), were dialyzed against distilled water, they became less sensitive to end-product inhibition. During dialysis against distilled water the enzyme precipitated and recovery of the activity required extraction with 0.025 M potassium phosphate buffer (pH 7.3). This procedure also caused a significant loss in enzyme activity, as much as 50–70% of the initial activity could be lost, and precipitation at low ionic strength was therefore omitted in our purification procedure.

TABLE IV

INHIBITION OF *PP*-RIBOSE-*P* AMIDOTRANSFERASE ACTIVITY BY PURINES AND THEIR DERIVATIVES

Conditions of assay were as described in MATERIALS AND METHODS with the exception that *PP*-ribose-*P* concn. was $5 \cdot 10^{-4}$ M and that of glutamine was 10^{-2} M. Purines and their derivatives were added in the concentrations indicated.

Enzyme fraction added	*Inhibitor added*	*Concn. (μmoles/ml)*	*Inhibition (%)*
Stage 1	None	0	0
	Adenine	1	70
		2	80
	AMP	1	50
		2	58
		4	70
	Guanine	1	37
		4	34
	GMP	1	10
		4	40
Stage 2	None	0	0
	AMP	2	32
Stage 4	None	0	0
	AMP	1	15
		2	30
		4	50
	GMP	1	11
		4	32



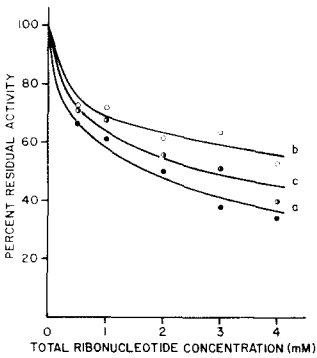


Fig. 4. Effect of two nitrogen containing substrates on *PP*-ribose-*P* amidotransferase activity. Curve a, determinations were done under standard conditions with glutamine as substrate at the concentrations indicated; Curve b, conditions as above, but with NH_4Cl as substrate at the concentrations as indicated.

Fig. 5. Effect of purine ribonucleotides on *PP*-ribose-*P* amidotransferase activity. The effect of AMP (Curve a), GMP (Curve b) and an equimolar mixture of AMP and GMP (Curve c) is charted. The inhibitors were added at the concentrations indicated on the abscissa. Assay conditions were as described in MATERIALS AND METHODS. 0.5 mg enzyme Stage 3 were incubated per assay.

Inhibition by adenine and AMP was consistently more potent than inhibition by guanine and GMP. The inhibitory effect of AMP and GMP, incubated under standard conditions in the same assay system, was additive; the inhibitory effect was no greater than that observed with equimolar concentrations of either inhibitor alone (Table IV, Fig. 5). In contrast to their effect on the avian and microbial enzyme, AMP and GMP did not act synergistically on the murine enzyme; the addition of one inhibitor did not promote the effect of the other[2,3]. Inhibition with AMP or GMP was competitive with respect to glutamine and non-competitive with respect to NH_4Cl (Figs. 2, 3). Inhibition by AMP was competitive with respect to *PP*-ribose-*P* (Fig. 1).

DISCUSSION

We have previously reported that *PP*-ribose-*P* amidotransferase is induced in the spleens of mice soon after infection with a leukemia virus. This enzyme is sensitive to regulatory controls similar to those which inhibit the activity of the enzyme derived from bird livers and from microorganisms[2,3]. The reaction catalyzed by *PP*-ribose-*P* amidotransferase need not be the only source of *P*-ribosylamine in the cell. We have established that *P*-ribosylamine synthesis from ribose-5-*P* and ammonia is enzymatic, and that the enzyme which catalyzes the reaction is sensitive to inhibition by purines and their derivatives[10]. The biological significance of this enzyme which catalyzes an alternative first step in purine biosynthesis, has yet to be established in mammalian systems, although there is some evidence that it exists in spleens of leukemic mice and in Ehrlich ascites cells[10,11].

PP-Ribose-*P* amidotransferase is believed to be the regulatory enzyme of *de novo* purine biosynthesis. Its inactivation or derepression is thought to play an important role in causing an acceleration of *de novo* purine biosynthesis in gout[12] and

in some forms of leukemia[4]. The results of our investigation support this hypothesis. Although our studies of the properties of the mammalian enzyme have been limited by the instability of the enzyme and its relatively low activity in mammalian tissues, it was possible to partially purify the enzyme (Table I).

The properties of the enzyme isolated from spleens of mice infected with leukemia virus were found to be similar to those of the avian and bacterial enzymes in several respects. The extreme instability of the murine enzyme is a property which it shares with the microbial enzyme; attempts to purify the microbial enzyme resulted in only a 6-fold increase in specific activity[3].

Enzymes from all three sources can use only *PP*-ribose-*P*. However, they differ in their requirements for a nitrogen containing substrate. The murine enzyme can use glutamine as well as ammonia as substrate; its affinity for either of these substrates is similar (Figs. 2, 3). The enzyme derived from chicken liver was reported to have a much greater affinity for glutamine than for ammonium chloride[1].

The possibility that two independent *PP*-ribose-*P* amidotransferases may exist, one using glutamine, and the other ammonia, cannot be ruled out by our data. Enzyme activity with ammonium chloride as substrate exceeds that with glutamine at all stages of enzyme purification, but the ratio of the reaction rates remains similar (Table III). It is therefore less likely that we are dealing with two different enzymes.

Certain of the factors which exert control over the activity of avian and bacterial *PP*-ribose-*P* amidotransferase are also operative in the enzyme we have isolated. Purine bases and their derivatives inhibit the activity of the mammalian enzyme. However, the mode of inhibition by AMP and by GMP with respect to the two nitrogen containing substrates is different. AMP and GMP compete directly with glutamine, but inhibition with respect to NH_4Cl is non-competitive (Figs. 2, 3). These findings are consistent with the assumption that glutamine and NH_4Cl interact differently with the enzyme. Glutamine and NH_4Cl could bind to two independent enzymes or to separate sites of the same enzyme, or they could share a common binding site to which they are attached in a different manner.

AMP and GMP acted synergistically upon avian and bacterial *PP*-ribose-*P* amidotransferase[2,3]. Their effect upon the murine enzyme however, was merely additive, and therefore, no more than one binding site for these inhibitors need exist, although the data do not exclude multiple binding sites for these inhibitors (Fig. 5).

The present investigations are consistent with the view that *P*-ribosylamine synthesis, catalyzed by *PP*-ribose-*P* amidotransferase, is an important site for the regulation of *de novo* purine synthesis in neoplastic mammalian tissues. These results are in accordance with our studies on the regulation of this enzyme *in vivo* in virus-induced murine leukemia[4].

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