Properties of 5-Phosphoribosyl-1-pyrophosphate Amidotransferase from Human Lymphoblasts*

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SUMMARY

5-Phosphoribosyl-1-pyrophosphate amidotransferase was assayed in human lymphoblasts maintained in tissue culture, and some of the properties of this enzyme that catalyzes the first committed step of de novo purine biosynthesis were determined. Cell-free preparations of the enzyme catalyze the phosphoribosylpyrophosphate-dependent conversion of 75 to 85 mmoles of the cosubstrate glutamine to glutamic acid per hour per mg of protein. The enzyme exhibits a dependence for magnesium ion, is stable to heating at 60° for 15 min, and exhibits a pH optimum from 6.8 to 7.4. Substrate velocity curves for phosphoribosylpyrophosphate are sigmoidal, whereas glutamine shows hyperbolic kinetics. Analysis of the data for phosphoribosylpyrophosphate by the Hill equation yields an interaction coefficient of 2, and a half-maximal velocity, $V_m$, of 0.25 mM. The $K_m$ for glutamine at saturating phosphoribosylpyrophosphate concentrations is 1.6 mM. Phosphoribosylpyrophosphate amidotransferase is subject to feedback inhibition by adenylc and guanylic acids, the ultimate products of de novo purine biosynthesis. The concentrations of AMP and GMP which inhibit enzyme activity by 50% are 1.8 and 0.5 mM, respectively. Mixtures of the two nucleotides show additive inhibition, and it is proposed that only one type of inhibitory site exists on the enzyme. Lineweaver-Burk double reciprocal plots show GMP to be a competitive inhibitor with respect to phosphoribosylpyrophosphate. Presence of the nucleotide abolishes the cooperative interaction of phosphoribosylpyrophosphate with the enzyme.

The first reaction unique to purine biosynthesis is catalyzed by 5-phosphoribosyl-1-pyrophosphate (PP-ribose-P) amidotransferase (EC 2.4.2.14). The enzyme catalyzes the amida
tion, by the amide nitrogen of PP-ribose-P to yield $\beta$-phosphoribosylamine: PP-ribose-P + glutamine + $\text{H}_2\text{O}\rightarrow \text{Mg}^{2+} \beta$-phosphoribosylamine + glutamic acid $\text{PP}_i$.

The purine biosynthetic pathway is regulated in part by inhibition of PP-ribose-P amidotransferase by the purine 5'-ribonucleotide products of the pathway. Studies by Rottman and Guarino (2), Nierlich and Magasanik (3), and Shiio and Ishii (4) in bacterial systems, and studies in yeast by Nagy (5) have demonstrated the sensitivity of the PP-ribose-P amidotransferase enzyme to inhibition by purine ribonucleotides. Similarly, end product regulation of de novo purine biosynthesis has been demonstrated in partially purified and in homogenous avian liver preparations by Wyngaarden and associates (6-9). PP-Ribose-P amidotransferase from mammalian sources had not been as extensively investigated. Caskey et al. (7) briefly characterized the rat liver enzyme and demonstrated its sensitivity to ribonucleotide inhibition. More recently, mammalian tumor enzyme has been investigated in partially purified extracts of cultured adenocarcinoma 755 cells by Hill and Bennett (10), in crude extracts from murine spleen infected with Friend leukemia virus by Reem and Friend (11), and in hepatoma tissue culture cells by Martin (12).

The study of the early steps of de novo purine biosynthesis in human cells and end product regulation by purine ribonucleotides has been limited to indirect assays in whole cells based on the accumulation of the third intermediate metabolite of de novo purine biosynthesis, $\alpha$-N-formylglycinamidribonucleotide in cells exposed to azaserine (13, 14). The purpose of this investigation has been to assay and elucidate the properties of PP-ribose-P amidotransferase in cell-free extracts of human origin. Human splenic lymphoblasts, maintained in cell culture, have been utilized for this purpose.

EXPERIMENTAL PROCEDURE

Materials—L-[U-14C]Glutamine (127 mCi per mmole) was obtained from Schwarz-BioResearch, stored frozen at $-20^\circ$ in aqueous solution, and purified at weekly intervals before use by passage through a column of Dowex 1 $\times$ 2 (chloride) resin. PP-Ribose-P (dimagnesium salt) was purchased from P & L laboratories, and its purity was determined by converting limiting amounts of PP-ribose-P to AMP with excess [8-14C]adenine and purified red blood cell adenine phosphoribosyltransferase (15). Preparations were generally 60 to 65% pure. L-Glutamine, L-glutamic acid (sodium salt), and disodium salts of

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adenyllic and guanylic acid were obtained from Sigma; cellulose thin layer sheets (no. 6065) were from Eastman Kodak, and DEAE-cellulose paper discs (DE-81) were from Whatman. Eagle's minimal essential media was obtained from Grand Island Biological. Other chemicals were reagent grade and purchased commercially.

**Origin and Maintenance of Cells**—The Wil 2 line of diploid human lymphoblasts, obtained as an explant from a nonmalignant splenic biopsy, was provided by Dr. R. A. Lerner, Scripps Clinic and Research Foundation, La Jolla, Calif. Suspension cultures of the lymphoblasts were grown at 37° in a glycerol shaker in stoppered Erlenmeyer flasks containing Eagle's minimal essential medium supplemented with twice the usual concentration of vitamins, 10% fetal calf serum, antibiotic (100 units of penicillin, 100 µg of streptomycin per ml of medium), and an antimycotic (0.25 µg of fungizone per ml of medium). Cells grown to a density of 0.8 × 10^6 to 1.2 × 10^6 cells per ml of medium were used for the experiments described.

**Preparation of Cell-free Extracts of Lymphoblasts**—Lymphoblasts harvested by centrifugation at 800 × g for 5 min were washed twice in 0.9% NaCl, followed by a single washing in 0.29 M sucrose-0.01 M Tris-HCl (final pH 7.0). Between washings the cell suspension was packed by centrifugation at 800 × g for 5 min. After the final wash, the cells were suspended in the Tris-sucrose buffer at a density of approximately 10^6 cells per ml, and disrupted by freeze-thawing three times in liquid nitrogen. Cell debris and mitochondria were collected by centrifugation at 39,000 × g for 30 min at 4° and discarded. The supernatant fraction which contained the PP-ribose-P amidotransferase was free of any mitochondrial phosphatase-dependent glutaminase activity (16).

**Assay of PP-ribose-P Amidotransferase Activity**—The assay of PP-ribose-P amidotransferase activity was based on the PP-ribose-P dependent hydrolysis of glutamine to glutamic acid. Generally 50 µl of the cell-free supernatant fractions were incubated in total reaction volumes of 100 µl containing 2.0 mM PP-ribose-P, 4.0 mM L-[U-14C]glutamine (specific activity 2 µCi per µmole), 5 mM magnesium chloride, and 50 mM Tris-HCl (pH 6.8). A duplicate reaction mixture containing all of the above additions except that water replaced PP-ribose-P was used to determine the amount of phosphate-independent glutaminase (17) activity present in the extracts. PP-Ribose-P amidotransferase activity was calculated from the difference in the glutamic acid formed with and without added PP-ribose-P. Reactions were initiated by the addition of glutamine, carried out at 37° for 30 min or as indicated, and terminated by the addition of 200 µl of 95% ethanol at 0°. Precipitated protein was collected by centrifugation at 1500 × g for 5 min. Glutamic acid was separated from glutamine either by thin layer chromatography on cellulose, as described by Wood et al. (18), or on DEAE-cellulose discs, as described by Martin (12). In the thin layer method, 3 or 5 µl of the ethanol-treated supernatant was applied to the cellulose sheets, previously spotted with 3 µl of a marker/carrier solution containing 0.5 µg each of glutamine and glutamic acid per ml, and developed for 45 min in a solvent system of chloroform-methanol-17% ammonium hydroxide (2:1:1 by volume). The Rf values for glutamic acid and glutamine were 0.26 and 0.47, respectively. The amino acids were localized with ninhydrin, as previously described (18). (When DEAE discs were employed, the reaction mixture was diluted to a volume of 500 µl with 95% ethanol, and 50-µl volumes were applied to the discs.) Glutamine was eluted from the discs with glass-distilled water within 30 min. After aspiration of excess water on a sintered glass plate, the DEAE discs were dried under an infrared lamp. Samples were counted in a Beckman LS 225 scintillation counter. New England Nuclear Liquifluor in toluene (100 ml Liquifluor to 2.37 liters of toluene) was used as scintillant, and counting efficiencies were about 80%.

**Protein Determination**—Protein content was measured by the method of Lowry et al. (19).

**RESULTS**

**General**—The specific activity of lymphoblast PP-ribose-P amidotransferase from human lymphoblasts was between 75 and 85 nmoles per hour per mg of protein. Contaminating glutaminase activity varied between 20 and 30 nmoles per hour per mg of protein. Dialysis had no effect on PP-ribose-P amidotransferase activity, and the enzyme was stable to heating at 60° for at least 15 min. Storage of the cell-free extract at 4° or −20° overnight resulted in a greater than 50% loss of enzyme activity.

Fig. 1 shows the relationship between enzyme activity and protein concentration (A) and incubation time (B). Under standard assay conditions, the reaction was linear for at least 60 min and over a protein concentration range of 100 to 800 µg. Assays generally contained 500 µg of protein and were carried out for 30 min.

**pH Optimum**—Optimal pH for PP-ribose-P amidotransferase
TABLE I

Effect of magnesium on PP-ribose-P amidotransferase activity

Reactions were carried out as described under "Experimental Procedure" with the exception that the sodium salt of PP-ribose-P replaced the magnesium form, and final magnesium concentrations were as indicated in the Table. Sodium PP-ribose-P was prepared by passage of a solution (0.25 ml) containing 50 µmoles of the dimagnesium salt of PP-ribose-P over a column (0.5 × 5 cm) of Chelex 100 chelating resin, which had been washed free of excess sodium chloride with water. Water was used to elute the PP-ribose-P which was recovered in greater than 97% yield in the final 2 ml of a 2.5-ml elution volume. Final magnesium concentration was less than 0.002 µmoles of Mgz+ per pmole of PP-ribose-P, as determined by atomic absorption spectrophotometry. Enzyme extract was dialyzed for 2 hours against two buffer changes of 0.01 M Tris (pH 6.8) prior to assay. Relative activity is expressed as percentage of maximal activity found at the indicated PP-ribose-P concentration.

| PP-Ribose-P concentration | Magnesium concentration | Relative activity |
|---------------------------|-------------------------|------------------|
| mM                        | mM                      | % Maximum        |
| 4                         | 40                      | 84               |
| 2                         | 10                      | 90               |
| 1                         | 5                       | 98               |
|                           | 2.5                     | 66               |
|                           | 1                       | 77               |
|                           | 0.5                     | 66               |
| 0.25                      | 5                       | 79               |
|                           | 2.5                     | 80               |
|                           | 1.25                    | 95               |
|                           | 0.625                   | 100              |
|                           | 0.25                    | 95               |

activity was from 6.8 to 7.4 with Tris-HCl buffer. A similar pH profile was observed with phosphate buffer, although maximal activity was 20 to 30% less than that found with Tris-HCl buffer. Assays were carried out at pH 6.8, since glutaminase activity increased with increasing pH (optimum pH 8.6).

Effect of Magnesium—Table I illustrates the dependence of PP-ribose-P amidotransferase activity on magnesium ion. At the three PP-ribose-P concentrations shown, maximal enzyme activity was achieved at magnesium concentrations 2.5 to 5 times that of the corresponding PP-ribose-P concentration. When no magnesium was added to the reaction mixture which contained 4 mM PP-ribose-P, the enzyme retained 48% of maximal activity. This residual activity may have been the result of trace amounts of the cation in the reaction mixture or bound to the enzyme. The effect of other divalent cations on enzyme activity was not determined.

Effect of Substrate Concentration—Fig. 2 shows the relationship between the activity of PP-ribose-P amidotransferase and the PP-ribose-P concentration. The substrate velocity curve is sigmoidal in nature, and the Lineweaver-Burk plot is concave upwards, rather than linear. The glutamine concentration was saturating at 4 mM, and magnesium concentrations were optimal for the PP-ribose-P concentrations indicated. To investigate further the apparent cooperative binding of PP-ribose-P to multiple sites on the enzyme, the substrate velocity data were replotted according to the equation proposed by Hill (20): log v/(Vmax - v) = n log [s] - log K, where v is velocity, Vmax is maximum velocity, K is a constant reflecting the affinity of the enzyme for substrate, and n is a measure of cooperativeness among the binding sites. Fig. 3A shows that the results of a plot of log [v/Vmax - v] as a function of log PP-ribose-P concentration was a straight line with a slope, n, of 2. Values of n greater than 1 in other enzymes have been interpreted to indicate multiple and interacting substrate binding sites on an enzyme molecule (21, 22). Contrasted to the results found with PP-ribose-P, the glutamine substrate versus velocity curve is hyperbolic, and a double
FIG. 4. Dependence of PP-ribose-P amidotransferase activity on glutamine concentration. Standard assays were carried out with the exception that glutamine concentration was varied as indicated. Control reaction mixtures containing water instead of PP-ribose-P were run at each glutamine concentration to determine glutaminase activity. Total glutamic acid formed in any reaction mixture never exceeded 15% of the initial glutamine concentration. Protein concentration was 500 μg, and incubation time 30 min. Inset shows double reciprocal plot of reaction rate and glutamine concentration.

FIG. 5. Inhibition of PP-ribose-P amidotransferase activity as a function of purine ribonucleotide concentration. Reactions were performed as described under “Experimental Procedure,” with the exception that AMP (○) or GMP (△), or both (□), were added to the reaction mixtures in the concentrations indicated and PP-ribose-P concentration was 10 mM. Equimolar concentrations of AMP and GMP were used when both nucleotides were present. Reactions were made up in ice, initiated by the addition of enzyme (450 μg) and carried out for 30 min at 37°. The remaining activity ($V_{	ext{r}}$) is expressed on the ordinate as a percentage of the activity in a control sample ($V_0$), which was assayed under identical conditions except for the absence of inhibitors.

The effect of GMP on the double reciprocal plot of velocity versus PP-ribose-P concentration is shown in Fig. 6. The cooperative binding of the substrate is apparently eliminated by GMP, since the nonlinear concave upwards plot was converted to a straight line in the presence of the nucleotide. A Hill plot of the data showed that GMP increased the apparent $K_m$ of PP-ribose-P without alteration in the $V_{	ext{max}}$ value. It is not known whether this apparently competitive inhibition results from the direct competition of GMP and PP-ribose-P for the catalytic site or is the result of GMP binding to an allosteric site.

**Discussion**

The results of the studies just described show the presence and some of the properties of PP-ribose-P amidotransferase in nonmalignant diploid human cells. PP-Ribose-P amidotransferase has been extensively investigated from other nonhuman sources,
and it is of interest to compare the properties of the lymphoblast enzyme with the previously published results.

The sigmoidal kinetics observed when PP-ribose-P concentration was varied have been observed in enzyme preparations from yeast (5) and from adenocarcinoma 755 cells (10). PP-ribose-P amidotransferase from Aerobacter aerogenes (3), Bacillus subtilis (2, 4), and rat liver (7) have all shown typical Michaelis-Menten kinetics for PP-ribose-P. The substrate velocity pattern observed with pig liver enzyme is dependent on the purity of the enzyme preparation employed. Partially purified enzyme (6, 7) exhibits normal Michaelis-Menten kinetics, whereas a more purified preparation exhibits sigmoidal kinetics (9). Non-cooperative binding of glutamine has been a consistent finding in all systems studied.

With respect to PP-ribose-P, the lymphoblast enzyme has an interaction coefficient, $n$, of 2 as compared to values of 1.9 for the adenocarcinoma enzyme (10) and 1.7 to 2.5 for the yeast enzyme (5). The data describing interaction coefficients 2 and 1 for PP-ribose-P and glutamine, respectively, correct our previously reported results (1) which were in error because of an incorrect analysis of the $r_\max - V$ versus substrate plots.

The magnesium salt of PP-ribose-P presumably is the substrate for the PP-ribose-P amidotransferase catalyzed reaction. It is of less than optimal ratio of magnesium to PP-ribose-P exists as the substrate concentration is decreased, the velocity of the reaction may fall off at a rate faster than predicted. The slope on the double reciprocal plot would therefore be concave upwards rather than linear, and cooperative binding of substrate would be assumed. The apparent cooperative binding of PP-ribose-P to PP-ribose-P amidotransferase in the present study cannot, however, be explained by this mechanism. The optimal ratio of magnesium to PP-ribose-P over the concentration range of PP-ribose-P shown in Fig. 2 is between 2.5 and 5 to 1 (Table I), and these ratios were maintained during the kinetic studies. It is of interest that the cooperative binding of PP-ribose-P to PP-ribose-P amidotransferase can be abolished by heating the enzyme to 60$^\circ$C for 15 min.1 Although the heat treatment has little or no effect on total enzyme activity at saturating PP-ribose-P concentrations, heating does convert the double reciprocal plot of velocity $versus$ PP-ribose-P concentration to typical Michaelis-Menten kinetics.

PP-ribose-P amidotransferase from human cells is sensitive to inhibition by purine nucleotides. Interests differences in the nature of purine nucleotide inhibition of PP-ribose-P amidotransferase are observed when the results of the present study are compared with previously published studies. Of relevance to PP-ribose-P, the lymphoblast enzyme has an interaction coefficient, $n$, of 2 as compared to values of 1.9 for the adenocarcinoma enzyme (10) and 1.7 to 2.5 for the yeast enzyme (5). The data describing interaction coefficients 2 and 1 for PP-ribose-P and glutamine, respectively, correct our previously reported results (1) which were in error because of an incorrect analysis of the $r_\max - V$ versus substrate plots.

The data in Fig. 4 indicate that PP-ribose-P amidotransferase from human cells is sensitive to inhibition by both 6-aminopurine and 6-hydroxypurine nucleotides. The data further indicate that there is probably a single nucleotide binding site and that it possesses greater affinity for GMP than AMP. There is also specificity for purine ribonucleotides, since pyrimidine ribonucleotides at concentrations as high as 5 mM failed to inhibit the enzyme. Further studies in a more purified enzyme preparation under a variety of assay conditions may be necessary to evaluate fully the apparent lack of synergistic inhibition.

The absence of two qualitatively distinct inhibitory sites for 6-aminopurine and 6-hydroxypurine ribonucleotides in the human enzyme is similar to the results found in adenocarcinoma cells and yeast, and in contradiction to the findings in the other systems discussed. Aerobacter, Bacillus, and pigeon liver PP-ribose-P amidotransferase all show greater than additive inhibition in the presence of both GMP and AMP. The $K_m$ values for AMP and GMP found with the lymphoblast enzyme, 1.8 and 0.5 mM, respectively, are comparable to values found in the bacterial systems (3, 4). Studies by Wyngaarden and Ashton (6) and Rowe et al. (9) have shown that the pigeon liver enzyme's sensitivity to purine nucleotide inhibition varies as much as 30-fold with the state of purification and the age of the enzyme preparation. The $K_m$ values for AMP and GMP in the most sensitive enzyme preparations from pigeon liver were 0.09 mM. Adenocarcinoma 755 PP-ribose-P amidotransferase was relatively insensitive to inhibition by adenine or guanine nucleotides. A 2.33 mM concentration of AMP and a 3.33 mM concentration of GMP inhibited the enzyme by only 30 and 40%, respectively. Other nucleotides were more effective inhibitors, however. A 0.09 mM concentration of 6-methylthiopurine ribonucleotide inhibited the adenocarcinoma enzyme by 50%.

Although the liver is presumably the main site of purine biosynthesis from small molecules, it has been previously shown that extracellular human tissue is capable of de novo purine biosynthesis. Rosenboom et al. (12) and Henderson et al. (13) have shown that human fibroblasts maintained in tissue culture accumulate formylglycinamide ribonucleotide, the third intermediate of de novo synthesis, when the cells are exposed to azaserine. These investigators have also shown that the cell's accumulation of formylglycinamide ribonucleotide is diminished when adenine or guanine is added to the incubation media. These studies and clinical studies which demonstrated an inhibition of $[1-14C]$glycine incorporation into urinary uric acid in human subjects by the purine precursor 4-amino-5-imidazolecarboxamide (23) and by adenine (24) provide evidence for feedback control of an early step in de novo purine biosynthesis in man. The ability to study PP-ribose-P amidotransferase from human cells in tissue culture should aid in further understanding of the factors involved in the regulation of de novo purine bio-

1 Wood, A. W., and Seegmiller, J. E., unpublished observations.
synthesis and in the elucidation of the biochemical defects in individuals with aberrations in purine synthesis.

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