Purification and Properties of Phosphoribosyl Pyrophosphate Synthetase from Rat Liver*

(Received for publication, May 17, 1973)

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SUMMARY

Phosphoribosyl pyrophosphate synthetase has been purified extensively from rat liver. Gel filtration studies indicate that the enzyme is a complex of large molecular weight. On electron microscopy, stacking of enzyme molecules into long, linear aggregates is observed. The subunit molecular weight is 40,500, as measured by disc gel electrophoresis in sodium dodecyl sulfate. MgATP appears to be the required form of ATP as substrate, and excess free Mg2+ stimulates the reaction at low MgATP concentrations. The enzyme is highly specific for ribose 5-phosphate, and substrate inhibition occurs at ribose 5-phosphate concentrations above 1.5 mM. Like phosphoribosyl pyrophosphate synthetases from other sources, the rat liver enzyme requires inorganic phosphate for catalytic activity, but is not inactivated by dialysis against phosphate-free buffer. In the presence of optimal levels of phosphate and free Mg2+, the apparent K_m is 0.22 mM for MgATP and 0.29 mM for ribose 5-phosphate.

Phosphoribosyl pyrophosphate is an important intermediate in the biosynthetic pathways for purine, pyrimidine, and pyridine nucleotides, and in bacteria, for the amino acids histidine and tryptophan (1). The formation of PRPP from ribose-5-P and ATP is catalyzed by PRPP synthetase (EC 2.7.6.1). The enzyme from various bacterial (1, 2) and animal (3-7) sources has been studied and found to be inhibited by certain nucleotides, particularly by ADP. These findings have suggested that the enzyme has an important degree of regulatory control, as is expected of an enzyme catalyzing the formation of an intermediate involved in diverse biosynthetic pathways. The studies reported in this paper described certain properties of highly purified PRPP synthetase from rat liver.

* This work was supported in part by Research Grant Ca 13980.01 from the United States Public Health Service.
† Operated by the University of Chicago for the United States Atomic Energy Commission; formerly Argonne Cancer Research Hospital.
‡ The abbreviation used is: PRPP, 5-phosphoribosyl 1-pyrophosphate.

MATERIALS AND METHODS

Sodium ribose-5-P, [8-14C]hypoxanthine, IMP, and ammonium sulfate were purchased from Schwarz-Mann, Orangeburg, N.Y. Streptomycin sulfate was obtained from Nutritional Biochemical Corp., Cleveland, O., and all sugars from Sigma Chemical Co., St. Louis, Mo. Magnesium PRPP and all nucleotides were obtained from P-L Biochemicals, Milwaukee, Wis. New England Nuclear Corp., Boston, Mass., provided the [32P]phosphoric acid used to prepare [γ-32P]ATP by the method of Glynne and Chappell (8). Bovine serum albumin and Agarose A-1.5m were obtained from Calbiochem, San Diego, Calif. Blue dextran 2000 was the product of Pharmacia, Uppsala, Sweden.

Assays

During purification of the enzyme, activity was followed by measuring the PRPP-dependent conversion of [8-14C]hypoxanthine to IMP in the presence of hypoxanthine guanine phosphoribosyltransferase, purified 113-fold from human erythrocytes by the method of Krenitsky et al. (9). The incubation mixture contained the following in a final volume of 70 μl: 1400 nmoles of potassium phosphate (pH 7.6); 350 nmoles of MgSO4; 70 nmoles of Na2EDTA; 50 nmoles of ribose-5-P; 55 nmoles of ATP; 250 nmoles of [8-14C]hypoxanthine (2.0 μCi per pmole); 2.5 μg of hypoxanthine-guanine phosphoribosyltransferase (137 nmoles of IMP formed per min per mg); and enzyme fraction sufficient to give a linear relationship between reaction velocity and protein concentration. Incubation was carried out for 30 min at 37°, and the reaction was stopped by placing the samples in a boiling water bath for 30 s. After the addition of 10 μl of 0.1 M IMP, a 50-μl aliquot of each sample was spotted on a 2.4-cm DEAE-paper disc (Whatman DE 81). The discs were washed with 50 ml of water and 10 ml of absolute ethanol, dried, immersed in counting fluid (Aquasol, New England Nuclear), and counted in a Packard Tri-Carb liquid scintillation counter. Under assay conditions, over 95% of IMP is retained on the disc, while hypoxanthine is not retained at all.

For all other studies, except as noted below, activity was measured by an adaptation of the charcoal binding assay of Switzer (1). Samples contained the following in a final volume of 0.5 ml: 50 μmoles of potassium phosphate (pH 8.0); 5 μmoles of MgCl2; 1 μmole of ribose-5-P; 0.7 μmole of ATP; 0.05 μmole of EDTA: 0.05 mg of bovine albumin; [γ-32P]ATP to give a specific activity of 100 to 200 cpm per pmole; and enzyme as
specification. After incubation at 37°C for 30 min, the reaction was terminated by the addition of 0.5 ml of 5% perchloric acid. The samples were centrifuged for 10 min, and 0.2 ml of acid-washed Norit A charcoal (30% v/v) was added with vigorous mixing. After 10 to 15 min, 0.2 ml of a carrier solution of 5 mg per ml of bovine albumin in 50 mM sodium pyrophosphate (pH 7.0), was added. The samples were centrifuged at 2,000 × g for 10 min, and 0.6 ml of the charcoal-free supernatant was removed for assay. Since ATPase activity in crude enzyme preparations also releases radioactivity that is not adsorbed by charcoal, this assay was not used with the less purified enzyme. All kinetic experiments reported below used only purified enzyme, free of nonspecific ATPase activity.

The reaction rate is linear with time over a 90-min period. For both assays, 1 unit of activity is defined as 1 nmole of PRPP formed per min. Protein concentration was determined by the Lowry method (10).

**Enzyme Purification**

**Acetone Powder Fraction**—Acetone powder was prepared from the livers of adult male Sprague-Dawley rats (11). Forty-eight grams of powder were extracted in a 20-volume excess of Enzyme Buffer, which consisted of 50 mM potassium phosphate (pH 7.6); 2.5 mM mercaptoethanol; 0.1 mM Na2EDTA; 6 mM MgCl2; 0.3 mM ATP; and 0.3 mM ribose-5-P. The presence of substrates was found to enhance the stability of the enzyme during purification and storage. After stirring for 2 hours at 4°C, the extract was centrifuged at 10,000 × g for 20 min and the supernatant recovered. All subsequent steps were carried out at 4°C.

**Acid Precipitation**—The acetone powder fraction was brought to pH 5.8 by the dropwise addition of 1.2 M acetic acid and the precipitate removed by centrifugation as above. The supernatant was similarly brought to pH 5.3 and again centrifuged. The acid precipitate was dissolved in one-tenth the original volume of Enzyme Buffer, and was stirred overnight. The acid precipitation fraction was clarified by centrifugation at 10,000 × g for 10 min.

**Streptomycin Precipitation**—The precipitate fraction was brought to pH 6.0 with 1.2 M acetic acid, and 0.2 volume of 10% streptomycin sulfate was added quickly with constant stirring. The mixture was stirred for 20 min, centrifuged, and the supernatant discarded. The precipitate was taken up in 1 ml of Enzyme Buffer as that used for the acid precipitate. Smaller volumes led to poor recovery of activity.

**Ammonium Sulfate Precipitation and Heating at 55°C**—It was found useful to increase the protein concentration of the streptomycin fraction prior to the heat step. After dilution to a protein concentration of 1 mg per ml by the addition of Enzyme Buffer, the fraction was brought to 25% saturation with a saturated solution of ammonium sulfate. The precipitate was discarded after centrifugation and the supernatant brought to 80% saturation. After stirring for 20 min and centrifugation, the precipitate was dissolved in one-tenth the original volume with Enzyme Buffer. This material was placed in a constant temperature water bath at 55°C for 10 min with continuous stirring. The sample was then chilled, the precipitate removed by centrifugation, and dialysis carried out overnight against a 50-volume excess of Enzyme Buffer.

**Agarose Gel Filtration**—The dialyzed 55°C heat fraction was concentrated to a volume of 5 ml or less in an Amicon ultrafiltration cell using a PM 30 membrane. The concentrated enzyme was applied to a column of Agarose A-1.5m, 2.5 × 58 cm (bed volume 275 ml), eluted with Enzyme Buffer, and 4-ml fractions were collected. Active fractions were pooled as described above and concentrated by ultrafiltration.

**Polyacrylamide Gel Electrophoresis**

Electrophoresis on 5% acrylamide gels (pH 8.8) was performed as previously described (12). Component molecular weights were estimated by electrophoresis in sodium dodecyl sulfate according to the method of Weber and Osborn (13).

**Electron Microscopy**

Samples of purified enzyme were diluted to 37 μg of protein per ml in Enzyme Buffer. Drops were allowed to stand for 3 min on copper grids covered with carbon-coated collodion membranes. The droplets were removed by touching to filter paper, and were replaced with several changes of 1% aqueous uranyl acetate. After the last droplet of uranyl acetate had been removed, the grids were allowed to dry in air. They were examined and photographed at × 60,000 in a Siemens 101 electron microscope at 80 kv accelerating voltage.

**RESULTS**

**Enzyme Purification and Stability** A representative example of the purification results is shown in Table I. After elution from the agarse column, the major activity appears in the void volume, with a steep shoulder overlapping the appearance of the first measurable protein peak (see Fig. 1). Only those fractions eluting before this protein peak are combined as the agarse column fraction.

The purification procedure was carried out several different times, with final recovery as high as 20% and purification up to 3400-fold, depending mainly on the specific activity of the original acetone powder fraction. The purified enzyme retains 50% of its original activity after 2 months in Enzyme Buffer at 4°C. Storage at −20°C results in more rapid loss of activity.

When the purified enzyme is assayed at low protein concentrations, a loss of specific activity results. A similar inactivation of erythrocyte PRPP synthetase has recently been noted (14). The loss of activity of the dilute rat liver enzyme can be prevented by the addition of albumin, EDTA, or sulfhydryl compounds to the assay. This property of the enzyme is discussed more fully in the accompanying article (15).

**Electron Microscopy**—The electron microscopic appearance of the purified enzyme is shown in Fig. 2, where the characteristic features of the molecule are detailed. PRPP synthetase mole-

**Table I**

Purification of rat liver PRPP synthetase

Activity is measured by the hypoxanthine-guanine phosphoribosyltransferase assay, as described under "Materials and Methods." One unit of activity is defined as 1 nmole of PRPP formed per min under stated conditions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total</th>
<th>Units/ml</th>
<th>% Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone powder extract</td>
<td>1,900</td>
<td>24,900</td>
<td>1.2</td>
<td>53.0</td>
<td>10.2</td>
</tr>
<tr>
<td>Acid precipitate</td>
<td>192</td>
<td>13,200</td>
<td>12.5</td>
<td>48.8</td>
<td>45.4</td>
</tr>
<tr>
<td>Streptomycin precipitate</td>
<td>200</td>
<td>12,160</td>
<td>56.3</td>
<td>45.4</td>
<td>48.8</td>
</tr>
<tr>
<td>55°C heat</td>
<td>35</td>
<td>4,750</td>
<td>222.0</td>
<td>10.1</td>
<td>182.0</td>
</tr>
<tr>
<td>Agarose gel filtration</td>
<td>5</td>
<td>2,440</td>
<td>1,250</td>
<td>9.8</td>
<td>1,020</td>
</tr>
</tbody>
</table>
FIG. 1. Elution of PRPP synthetase activity from Agarose A-1.5m. □—□, absorbance; ⋅—⋅, cpm above background, [3H]-IMP, hypoxanthine-guanine phosphoribosyltransferase assay; ○—○, blue dextran elution, $A_{660}$.

FIG. 2. Electron micrographs of purified PRPP synthetase. Magnification: × 228,000; inset, × 342,000.

Clusters are stacked 7 nm apart (on center) in linear arrays of varying lengths. Individual molecules (shown by the arrows) appear to be composed of a ring with three asymmetrically attached protuberances which give the molecule the shape of an isosceles triangle, the base of which is longer than its two sides. The base of the triangle measures 14 nm, the sides 11 nm, and the hole in the ring 3 nm.


Disaggregation of Enzyme—Attempts were made to alter the state of aggregation of the enzyme by removal of phosphate or substrates (present in the buffer during purification) by dialysis. As judged by sucrose density gradient centrifugation, no change in the aggregation of the enzyme resulted from these procedures.

When the purified enzyme was treated with 1 M NaCl and reapplied to Agarose A-1.5m gel columns, a single peak of activity eluted after the void volume, indicating dissociation of the enzyme into a lower molecular weight form. The active fractions were pooled and concentrated, and the NaCl was removed by dialysis against Enzyme Buffer. When this material was eluted from the agarose column, the activity again appeared in the void volume. Thus, the dissociation of the enzyme by high salt concentrations is reversible.

Polyacrylamide Gel Electrophoresis—On standard 5% acrylamide gels, a discrete major protein band is accompanied by three to five minor bands of greater mobility, which were estimated to consist of less than 10% of the total stainable material on the gels. Similarly, the major band identified after gel electrophoresis in 6 M urea and in sodium dodecyl sulfate comprised greater than 90% of the stainable protein.

The position of the major band after pretreatment and electrophoresis in sodium dodecyl sulfate corresponds to a molecular weight of 40,500. These results are shown in Fig. 3.

pH Optimum—A broad pH optimum from 8.0 to 8.8 for the PRPP synthetase reaction is shown in Fig. 4. Over 70% of the activity remains at pH 7.0.

Substrate Specificity—Table II, which lists the ability of various nucleotides to replace ATP as pyrophosphoryl donor in PRPP synthetase reaction, shows that only dATF can fully replace ATP at the concentrations shown.

Various analogues were tested as pyrophosphoryl acceptors in place of ribose-5-P. No activity could be detected in the presence of 0.1 or 0.4 mM levels of ribose, ribose-1-P, deoxyribose-5-P, or glucose-6-P.

Divalent Cation Requirement—PRPP synthetase requires divalent cation for activity; the greatest activity is seen with Mg$^{2+}$. As shown in Table III, Mn$^{2+}$ or Cu$^{2+}$ can replace Mg$^{2+}$ to a limited extent, whereas other divalent cations tested are without effect.

Kinetic Constants—The standard reaction mixture contains high concentrations of inorganic phosphate, and Mg$^{2+}$ is present...
FIG. 4. Effect of pH on PRPP synthetase activity. The buffers were: O—O, 0.1 M potassium phosphate; —, 0.1 M potassium phosphate-Tris. The pH was measured with a Radiometer 26 pH meter on duplicate samples for each point on the activity curve.

**TABLE II**

Pyrophosphoryl donors

The hypoxanthine-guanine phosphoribosyltransferase assay is converted to a two-step procedure as described by Fox and Kelley (6). Pyrophosphoryl donors are substituted for ATP and are present at a final concentration of 0.1 or 0.5 mM. With 0.1 mM ATP, 2.7 nmoles of PRPP per min were formed and with 0.5 mM ATP, 9.6 nmoles of PRPP per min.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Activity (0.1 mM)</th>
<th>Activity (0.5 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>GTP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>XTP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ITP</td>
<td>11.1</td>
<td>6.9</td>
</tr>
<tr>
<td>UTP</td>
<td>9.0</td>
<td>10.3</td>
</tr>
<tr>
<td>CTP</td>
<td>14.0</td>
<td>9.5</td>
</tr>
<tr>
<td>dATP</td>
<td>113</td>
<td>110</td>
</tr>
<tr>
<td>dTTP</td>
<td>9.0</td>
<td>4.0</td>
</tr>
<tr>
<td>ADP</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>GDP</td>
<td>0</td>
<td>1.7</td>
</tr>
<tr>
<td>Sodium pyrophosphate</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

in excess of ATP. Under these conditions, a hyperbolic curve is obtained for initial velocity when plotted against MgATP as the variable substrate (Fig. 5). This relationship becomes sigmoidal when ATP is present in excess of MgATP, and an intermediate curve exists at equimolar concentrations of the two. Double reciprocal plots of these data are shown in the inset to Fig. 5. While it is difficult to estimate kinetic constants precisely because of nonlinear relationships when MgATP is not present in excess, the major effect of free MgATP appears to be a decrease in the apparent Michaelis constant for MgATP with a lesser change in apparent maximal velocity. With MgATP present in excess, the apparent $K_m$ for MgATP is 0.22 mM.

Fig. 6 shows the substrate saturation curve for ribose-5-P. The apparent $K_m$ for ribose-5-P is 0.29 mM, and substrate inhibition is seen at concentrations above 1.5 mM.

**Phosphate Requirement**—PRPP synthetase shows an unusual requirement for inorganic phosphate for catalytic activity. Optimal activity of the rat liver enzyme is found at phosphate concentrations of 100 mM (Fig. 7). Dialysis of the enzyme for 40 hours against Enzyme Buffer in which Tris-HCl (pH 7.6), replaces phosphate results in no appreciable loss of activity as long as phosphate is present in the subsequent assay. Lowering the phosphate concentration by a 20-fold dilution with Enzyme Buffer containing Tris-HCl instead of phosphate also fails to inactivate the enzyme. Phosphate has a slight effect in stabilizing enzyme which is diluted and incubated at 37°C, but this is insufficient to account for the phosphate requirement in the reaction.

The effect of phosphate on the reaction was examined by determining the substrate saturation curves at different concentrations of phosphate. Increasing the phosphate concentration from 10 to 100 mM raises the $V_{max}$ 1.4-fold and also decreases the apparent $K_m$ for MgATP 2.8-fold (Fig. 8). The apparent $K_m$ for ribose-5-P was the same at the two phosphate concentrations (data not shown).

**TABLE III**

Metal requirement

All metals are used as the chloride and are present in the $^32P$ transfer assay at 1 mM. The MgCl$_2$ sample gave 1.24 nmoles of PRPP per min. Values shown for other divalent cations represent per cent activity in the assay in which divalent cation has replaced MgCl$_2$, compared to the control assay with MgCl$_2$ alone.

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnesium</td>
<td>100</td>
</tr>
<tr>
<td>Manganese</td>
<td>14.7</td>
</tr>
<tr>
<td>Calcium</td>
<td>1.4</td>
</tr>
<tr>
<td>Cadmium</td>
<td>3.6</td>
</tr>
<tr>
<td>Cobalt(ous)</td>
<td>9.5</td>
</tr>
<tr>
<td>Barium</td>
<td>1.5</td>
</tr>
</tbody>
</table>
DISCUSSION

Studies of purified rat liver PRPP synthetase have shown similarities as well as differences compared with the enzyme from Salmonella and human erythrocytes. In common with the enzyme from all sources examined thus far, the rat liver enzyme has shown a requirement for phosphate in the catalytic reaction. In contrast to the findings with PRPP synthetase from Salmonella (1), phosphate is not required for stability, as shown by the failure of dialysis against a buffer containing no phosphate to inactivate the enzyme. The minor increase in stability seen when the rat liver enzyme is diluted in the presence of phosphate under conditions of the assay is too small to account for its requirement in the reaction. The apparent $K_m$ for MgATP changes when phosphate concentrations are altered, and thus changing intracellular phosphate levels may modify the activity of PRPP synthetase considerably when MgATP levels are limiting.

Rat liver PRPP synthetase is reproducibly inhibited by high concentrations of ribose-5-P. The enzyme from Salmonella shows this substrate inhibition only in the presence of $Ca^{2+}$ or ADP (16). Neither $Ca^{2+}$ nor ADP is required to show this effect with the enzyme from rat liver.

The relationship between MgATP and ATP in determining PRPP synthetase activity is complex. When MgATP is added to the assay at equimolar concentrations or in the presence of excess ATP, the relationship between activity and MgATP concentration is sigmoidal, with a sharp rise in activity over a small range of substrate concentrations. When Mg$^{2+}$ is present in excess, a hyperbolic relationship exists. The concentration of Mg$^{2+}$ as well as MgATP levels thus are important determinants of catalytic activity; this feature appears to be characteristic of this enzyme from several sources (1, 4).

The enzyme from rat liver exhibits a strict specificity for ribose-5-P. Deoxyribose-5-P and related sugars are not pyrophosphoryl acceptors and also fail to inhibit the reaction. Human erythrocyte PRPP synthetase has been reported to exhibit 34% activity with ribulose 5-phosphate as substrate compared to activity with ribose-5-P (8). Because of possible contamination of commercial ribulose 5-phosphate preparations with ribose-5-P, the activity may not reflect the formation of P-P-ribulose-P. The erythrocyte enzyme is, however, inhibited by deoxyribose-5-P (7), and true differences may exist in the specificity of the two enzymes with respect to this substrate.

Results from gel filtration studies as well as the appearance of the enzyme in electron micrographs show that PRPP synthetase from rat liver is purified as an aggregate of large molecular weight. Attempts to disaggregate the enzyme with removal of substrates or by removal of phosphate were not successful, as judged by sucrose gradient experiments. Exposure to high concentrations of sodium chloride does dissociate the enzyme, and the lower molecular weight species obtained after this treatment are catalytically active in subsequent assay. Reaggregation of the enzyme occurs when the salt is removed. An aggregated form of PRPP synthetase has also been shown for the enzymes from Salmonella (1) and human erythrocytes (6). Aggregation of the erythrocyte enzyme is modified by protein concentration, salt, and saturating levels of MgATP, and associated loss of activity is seen when the enzyme is disaggregated with 1 M NaCl or 2 M urea.

Several of the properties of rat liver PRPP synthetase presented here have potential importance for the regulation of this enzyme. In the accompanying paper, the results of inhibitor
studies and the significance of these findings in the over-all regulation of PRPP synthesis are presented.

Acknowledgment—It is a pleasure to acknowledge the technical assistance of Ms. Christine White.

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