Expression of Rat Phosphoribosylpyrophosphate Synthetase Subunits I and II in *Escherichia coli*

ISOLATION AND CHARACTERIZATION OF THE RECOMBINANT ISOFORMS*

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The 34-kDa subunit of rat liver phosphoribosylpyrophosphate synthetase is a mixture of the two highly homologous isoforms, PRS I and PRS II. Heretofore, it was not possible to separate the two. We now describe isolation and characterization of the recombinant isoforms, named rPRS I and rPRS II. The respective rat cDNAs were inserted into vectors constructed from pKK233-2 by replacing its replication origin with that of pGEM-1 and expressed in *Escherichia coli*. The rPRS I and rPRS II were purified to apparent homogeneity with specific activities of 33,400 and 46,200 millionits/mg, respectively; these values were at least 2.5-fold higher than the highest value for the mammalian enzyme so far reported. Both isoforms showed a similar dependency on Pᵢ, as an absolute activator. Sulfate partially substituted for Pᵢ. The maximal activities of rPRS I and rPRS II with sulfate were 43 and 7%, respectively, of those seen with Pᵢ. The two isoforms differed in sensitivity to inhibition by ADP and GDP. Inhibition of rPRS I and rPRS II by 0.3 mM ADP was 87 and 54%, respectively, and inhibition by 1 mM GDP was 93 and 24%, respectively. rPRS II was 180-fold more sensitive than rPRS I to heat inactivation at 49 °C.

Phosphoribosylpyrophosphate (PRPP) synthetase (ATP: D-ribose-5-phosphate pyrophosphotransferase, EC 2.7.6.1) catalyzes the formation of PRPP from ATP and ribose 5-phosphate. PRPP provides an important substrate for synthesis of almost all nucleotides (1, 2), and is a critical control factor for de novo synthesis of purines (3-5) and pyrimidines (6-8).

PRPP synthetase has been purified from *Salmonella typhimurium* (9, 10), *Escherichia coli* (11), *Bacillus subtilis* (12), human erythrocytes (13), and rat liver (14, 15). The enzyme is an oligomeric complex composed of about 34-kDa subunits. We reported that the rat liver enzyme exists as complex aggregates of 34-, 38-, and 40-kDa components, with the 34-kDa species being the catalytic subunit (15). Our cloning of rat cDNA (16) and amino acid sequencing of the enzyme purified from rat liver (15) revealed that the 34-kDa component is actually a mixture of two highly homologous isoforms, designated as PRS I and PRS II. The deduced amino acid sequence of both sets of 317 residues differs only by 13 residues (96% homology) (16). These two isoforms are encoded by different X-linked genes (PRPS I and PRPS II, respectively) (17). The amino acid sequences of both PRS I and PRS II are highly conserved. The deduced amino acid sequences of human (18, 19) and rat (16) PRS II differ only by 3 residues (99% homology), and those of human (19, 20) and rat (16) PRS I are completely conserved (100% homology). Rat mRNAs of PRPS I and PRPS II genes are expressed in almost all tissues, but the amounts differ with the tissue (21). All these observations suggest functional differences between catalytic and/or regulatory properties of PRS I and PRS II. The kinetic and physical characteristics of the individual isoforms have remained unknown due to difficulty in isolating the respective species.

We now report the expression of respective rat PRS I and PRS II in *E. coli*. The strategy we used produced the unfused proteins, possessing catalytic activity. We isolated and characterized the recombinant isoforms.

**EXPERIMENTAL PROCEDURES**

Materials—Synthetic oligonucleotides were prepared using an Applied Biosystems DNA synthesizer, model 390B. Restriction and DNA-modifying enzymes were obtained from Takara Shuzo, Toyobo, and New England Biolabs. Vectors pGEM-1 and pKK233-2 were from Stratagene and Pharmacia LKB Biotechnology Inc, respectively. TSK DEAE-5PW and G4000SW columns were purchased from Tosoh Manufacturing. Sources of all other reagents were as described (15).

**Construction of Expression Vectors**

The expression vectors were constructed by two modifications of pKK233-2. (a) The replication origin of pKK233-2, contained in the 3.4 kb of the EcoRI-PvuI fragment (22), was replaced with that of pGEM-1. (b) Multiple cloning sites, contained in the 45 bp of the HindIII-EcoRI fragment of pGEM-1, were inserted into the HindIII site located immediately downstream from the ATG initiation codon (22). The 31 bp of the EcoRI-HindIII fragment of pBR322 was used as an adaptor. The resulting plasmid is designated pG1KHB. Another vector, pG1KHM, was constructed from pG1KHB by inserting the MluI linker (GACGCCGTC) into the Smal site in the multiple cloning sites.

**Construction of Expression Plasmids for Rat PRS I and PRS II—**

For expression of PRS I, the rat PRS I cDNA fragment spanning bases 28-1193 (16) was cloned in pG1KHB between the NcoI and the Smal sites. The complementary synthetic oligonucleotides encoding the NH₂-terminal 9 amino acids were used as an adaptor. The constructed pKRI contained the complete coding region of 364 bp and
0.24 kb of the 3′-noncoding region to the Dral site of rat PRS I cDNA (23).

For expression of PRS II, the rat PRS II cDNA fragment spanning bases 14–2086 (16) was cloned in pG1KH between the NcoI and the BarnHI sites. The synthetic oligonucleotides encoding the terminal 5 amino acids were used as an adaptor. The resulting plasmid, pKrI, contained the complete coding region of 954 bp and 1.1 kb of the 3′-noncoding region to the BamHI site of rat PRS II cDNA (24).

The sequences of the resulting expression plasmids were confirmed by restriction mapping and sequence analysis. The plasmids were introduced into the E. coli strain MV1304, which contains a lacF represor. The transformed strains were designated MV1304/pKrI and MV1304/pKrII, respectively. As a negative control, pG1KH alone was also transformed into MV1304. The cloning steps described above were performed essentially as described by Maniatis et al. (25).

Cell Growth—The transformed MV1304 cells were grown at 30 °C for 15 h in 10 liters of M9 medium (25) containing 1 mM thiamin and ampicillin (20 μg/ml). After adding 1 mM IPTG, the cells were grown for an additional 3 h at 30 °C and then harvested by centrifugation.

Purification of Recombinant PRS I and PRS II—All procedures for enzyme purification were carried out at 4 °C, and all buffers used contained enzyme-stabilizing agents (13, 14), 0.3 mM ATP, 6 mM MgCl₂, 0.1 mM EDTA, and 2.5 mM 2-mercaptoethanol, unless otherwise stated. The pH values indicated were measured at 4 °C.

The E. coli cells were suspended in a minimum volume of a solution containing 50 mM potassium phosphate (pH 7.4) (adjusted at 37 °C), 6 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride. The protein concentration was adjusted to 3.3 mg/ml. The enzyme was then precipitated at pH 6.15.

The next two steps, polyethylene glycol precipitation and acid precipitation, were performed according to the protocol described previously (15), with the following modifications. (a) rPRS I was precipitated with 3% (w/v) polyethylene glycol 6000. The precipitate was dissolved in 50 mM potassium phosphate (pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride. The protein concentration was adjusted to 3.3 mg/ml and then precipitated at pH 5.85. (b) rPRS II was precipitated with 5% (w/v) polyethylene glycol 6000 and then precipitated at pH 7.4.

The acid precipitates of rPRS I and rPRS II were dissolved in 50 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride. The protein concentrations were adjusted to 5 mg/ml and the pH to 7.4. The purification up to this step was performed within 1 day, and the preparations were stored at −80 °C.

A portion of the above preparations (5 mg of protein for each) was applied to a DEAE-5PW HPLC column (0.75 × 30 cm) equilibrated with 30 mM potassium phosphate (pH 7.4) containing 0.1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. The protein concentration was adjusted to 0.3 mg/ml. The enzyme was then precipitated at pH 6.15.

The apparent increase in total activity after the first purification step of rPRS I and rPRS II is probably due to elimination of endogenous inhibitors. The bulk of the enzyme was purified at this step. The precipitated protein was removed at the step of polyethylene glycol precipitation. The enzyme activity increased within 2 h after addition of IPTG and reached a maximum in 3–4 h. The specific enzyme activities of the extracts of the cells containing pKrI, pKrII, and pG1KH as the negative control were 720, 400, and 20 milliunits/mg, respectively, at 4 h. A low level of enzyme activity detected in the control cells may represent the constitutive PRP synthetase of the E. coli itself (11).

Purification of rPRS I and rPRS II—Since homology of the deduced amino acid sequences of the E. coli and recombinant enzymes is high (47%), their separation may be difficult. Therefore, it is important to repress the synthesis of the bacterial PRP synthetase, and this synthesis is regulated (30, 31). We examined the enzyme levels in MV1304/pKrI and MV1304/pG1KH cells grown at 30 or 37 °C in M9 minimal or LB medium. As shown in Table I, the levels in the cells grown at 30 °C in M9 medium, the cells containing the vector alone showed the lowest level of the enzyme activity, and the level in the cells containing pKrI was higher. The cells containing pKrII or pKrII were grown at 30 °C in M9 medium, and the rPRS I and rPRS II were purified from the soluble fractions of the extracts as described under "Experimental Procedures." The results are summarized in Table II. The apparent increase in total activity after the first purification step of rPRS I and rPRS II is probably due to elimination of endogenous inhibitors. The bulk of the E. coli proteins was removed at the step of polyethylene glycol precipitation.

**RESULTS**

Construction of Expression Plasmids—The cDNA fragments of rat PRS I and PRS II were cloned into expression vectors derived from pKK233-2 (Fig. 1), as described under "Experimental Procedures." For a high level expression (29), the cDNA fragments that did not contain A-T tails were inserted into the expression plasmids. The constructed pKrI and pKrII consisted of a highly inducible trc promoter, a ribosome-binding site, the PRS I and PRS II cDNAs, respectively, and a strong rrnB transcription terminator.

Expression of Rat PRS I and PRS II in E. coli Cells—The induction of rPRS I and rPRS II by IPTG in the bacterial strains MV1304/pKrI and MV1304/pKrII was examined by the enzyme assay on soluble fractions of the crude cellular lysates. The enzyme activity increased within 2 h after addition of IPTG and reached a maximum in 3–4 h. The specific enzyme activities of the extracts of the cells containing pKrI, pKrII, and pG1KH as the negative control were 720, 400, and 20 milliunits/mg, respectively, at 4 h. A low level of enzyme activity detected in the control cells may represent the constitutive PRP synthetase of the E. coli itself (11).

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**FIG. 1.** Construction of plasmids pKrI and pKrII for expression of rat PRS I and PRS II, respectively. The solid regions indicate the coding regions of cDNAs, and the open regions indicate the 3′-noncoding regions. The black triangles, boxes, and arrows represent the trc promoter, transcription terminators-rrnBT₁₂, and the ampicillin resistance gene, respectively. RBS, ribosome-binding site.
PRPP synthetase activities in E. coli cells cultured under various conditions

The E. coli cells were cultured under the indicated conditions and harvested after a 3-h induction with 1 mM IPTG. The cells were disrupted by sonication and centrifuged at 10,000 x g for 15 min. The supernatant was assayed for PRPP synthetase activities and protein concentrations.

| Culture medium | Temperature | PRPP synthetase activities |
|----------------|-------------|----------------------------|
|                |             | MV1304/pG1KH | MV1304/pKrl |
|                | °C          | milliunits/mg | milliunits/mg |
| M9             | 30          | 1,900        | 0.39          |
| M9             | 37          | 2,000        | 4.7           |
| LB             | 30          | 720          | 9.5           |
| LB             | 37          | 1,040        | 41            |

* Containing 0.4% (w/v) glucose.
* Containing 0.1% (w/v) glucose.

rPRS I and rPRS II were purified from transformed E. coli MV1304 cells as described under “Experimental Procedures.” The cells were cultured in 10 liters of M9 medium at 30 °C and harvested after a 3-h induction with 1 mM IPTG. The purified rPRS I had a final specific activity of 33,400 milliunits/mg, and rPRS II had a slightly higher value of 46,200 milliunits/mg.

For assay of the activity of the purified enzymes, we used method 2 measuring [14C]AMP production from [14C]ATP, as described under “Experimental Procedures.” This approach can assay an exchange reaction between AMP and ATP, independent of PRPP synthesis (32). We examined the [14C]AMP-ATP exchange reaction under the assay conditions of method 2, except that 0.4 mM [14C]ATP was replaced with unlabeled ATP and 0.05 mM [14C]AMP was added. The purified rPRS I and rPRS II produced 0.055 and 0.013 nmol of [14C]ATP, respectively, for 5 min, and these values were 1.9 and 0.4%, respectively, of [14C]AMP production from [14C]ATP measured in parallel. Thus, the activities assayed here by method 2 were not a measure of the AMP-ATP exchange reaction.

NH2-terminal Sequence Analysis—The E. coli host cells possessed their own PRPP synthetase, as described above. The amount of the E. coli enzyme included in the rPRS I and rPRS II preparations was estimated by NH2-terminal sequence analysis. Analyses of the first 9 amino acids indicated that those preparations did not contain any detectable E. coli PRPP synthetase and that the initiator Met was not completely removed from the recombinant proteins. For example, at the third cycle of Edman degradation of rPRS I, we detected 114 pmol of Ile, 77 pmol of Asn, but no Met (less than 0.1 pmol), which should be derived from the E. coli enzyme (11). For rPRS I, the major sequence found was Met-Pro-Asn-Ile-Lys-Ile-Phe-Ser-Gly, in agreement with the sequence deduced from the cDNA sequence, with a secondary sequence beginning with Pro. The relative yields of the two sequences indicated that the initiator Met was removed from 40% of the rPRS I protein. Sequencing of the rPRS II showed that the initiator Met was removed from 65% of the rPRS II protein.

Physical and Kinetic Properties of rPRS I and rPRS II—Some physical and kinetic properties of rPRS I and rPRS II were compared (Table III).

The mammalian PRPP synthetases exist in multiple, highly aggregated states (33), and the purified rat liver enzyme has a molecular mass of over 1,000 kDa (15). The purified rPRS I and rPRS II were analyzed by HPLC gel filtration on TSK G4000SW. rPRS I was eluted as a broad single peak near the void volume, showing that the molecular mass was as high as that of rat liver PRPP synthetase. rPRS II was eluted as a sharp peak at 550 kDa.

The apparent Michaelis constants for ATP and ribose 5-phosphate were determined. There was no marked difference between the apparent Km values for rPRS I and rPRS II.

A remarkable difference was found between the heat stabilities of rPRS I and rPRS II. rPRS II was much more labile than rPRS I; at 49 °C, the half-life of inactivation of rPRS II was 0.5 min, whereas the half-life of rPRS I was 90 min, 180-fold greater.

Activation by P1—PRPP synthetase is activated by P1, which has been accepted to play a primary role in the control of intracellular PRPP synthesis (33). Activation by P1 of rPRS I and rPRS II were compared. The two isozymes had no activity when assayed at P1 concentrations lower than 0.4 mM; therefore the P1 concentration-activity relationship showed a slight sigmoidicity. At higher P1 concentrations, activation of the two isozymes followed a hyperbolic curve. Double-reciprocal plots were linear at P1 concentrations from 1 to 50 mM. K, values for P1 of rPRS I and rPRS II were 1.8 and 2.4 mM, respectively. Thus, the two isozymes have a similar P1 dependency.

**Table II**

| Purification step | Total protein | Total activity | Specific activity | Purification Yield |
|-------------------|---------------|----------------|-------------------|-------------------|
| Purification of rPRS I | 1,430 | 960 | 1 |
| Polyethylene glycol | 1,820 | 9,730 | 10 |
| Acid precipitation | 1,580 | 19,700 | 21 |
| DEAE-5PW | 1,310 | 25,700 | 27 |

**Table III**

| Purification step | Total protein | Total activity | Specific activity | Purification Yield |
|-------------------|---------------|----------------|-------------------|-------------------|
| Soluble cell extract | 942 | 565 | 1 |
| Polyethylene glycol | 662 | 9,450 | 22 |
| Acid precipitation | 567 | 21,000 | 50 |
| DEAE-5PW | 518 | 34,500 | 82 |

* Protein concentrations were determined by the method of Bradford (26).
* The specific activities based on the protein determination from dry weight measurements for rPRS I and rPRS II were 33,400 and 46,200 milliunits/mg, respectively.

**Fig. 2**—SDS-PAGE analysis of various steps in the purification of rPRS I and rPRS II. Each purification step is described under “Experimental Procedures.” Panel A, purification of rPRS I. Lane 1, 12 μg of protein of soluble fraction from E. coli cell lysate; lane 2, 3 μg of protein of polyethylene glycol precipitate; lane 3, 2 μg of protein of acid precipitate; lane 4, 2 μg of protein of pooled fraction from DEAE-5PW HPLC. Panel B, 2 μg of PRPP synthetase purified from rat liver. Panel C, purification of rPRS II. Lanes 1–4, the same as in panel A. Proteins were stained with Coomassie Brilliant Blue. The black triangle designates the mobility of the 34-kDa subunit of the rat liver PRPP synthetase.
Sulfate ion can partially substitute for P<sub>i</sub>, whereas at 50 and 100 mM, monovalent anions Cl<sup>-</sup>, HCO<sub>3</sub> -, and CH<sub>3</sub>COO<sup>-</sup> with K<sup+</sup> as the cation had no activating effects. The effects of sulfate ion on rPRS<sub>I</sub> and rPRS<sub>II</sub> are considerably different. rPRS<sub>I</sub> gave a maximum activity at 50 mM K<sub>2</sub>SO<sub>4</sub> as the activator (Fig. 3), and the value was 43% of the activity with 50 mM P<sub>i</sub>. In contrast, the rPRS<sub>II</sub> activity reached a maximum at 20–40 mM sulfate, and higher concentrations were inhibitory. The maximal activity was only 7% of that seen with P<sub>i</sub>. Sulfate inhibited P<sub>i</sub> activation of rPRS<sub>II</sub> in a competitive manner (with a K<sub>i</sub> value of 5.7 mM). On the other hand, P<sub>i</sub> activation of rPRS<sub>I</sub> was inhibited by sulfate but not in a simple competitive manner; increasing P<sub>i</sub> concentrations could not completely overcome the suppressive effects of sulfate (data not shown).

**Inhibition by Nucleotides**—PRPP synthetases from various sources are inhibited by nucleotides, of which ADP is the most potent (12, 34–36). When we compared the inhibition of rPRS<sub>I</sub> and rPRS<sub>II</sub> by nucleotides (Table IV), ADP, GDP, and the reaction product AMP were seen to be the most effective. Other nucleotides only weakly inhibited both isoforms (0–19%). Noteworthy is the different sensitivity of rPRS<sub>I</sub> and rPRS<sub>II</sub> to inhibition by ADP and GDP; the inhibition of rPRS<sub>I</sub> and rPRS<sub>II</sub> by 0.3 mM ADP was 87 and 54%, respectively, and inhibition by 1 mM GDP was 93 and 24%, respectively.

Thus, rPRS<sub>I</sub> and rPRS<sub>II</sub> possess differential properties. Since the 34-kDa component of the native rat liver PRPP synthetase is a mixture of rPRS<sub>I</sub> and rPRS<sub>II</sub>, and the enzyme contains other polypeptides (15), we are now examining the combination of rPRS<sub>I</sub>, rPRS<sub>II</sub>, and other components for catalytic and/or regulatory properties of the enzyme.

**DISCUSSION**

The catalytically active 34-kDa species of rat liver PRPP synthetase was partly isolated from 38- and 40-kDa components by gel filtration in the presence of 1 mM MgCl<sub>2</sub> (15). The 34-kDa component was a mixture of rPRS<sub>I</sub> and rPRS<sub>II</sub>, and no means were available to separate the two. We expressed the cDNAs of rat PRS<sub>I</sub> and PRS<sub>II</sub> in E. coli, isolated each of the 34-kDa catalytic subunits, and studied some properties of the respective isoforms.

The recombinant isoforms were purified to apparent homogeneity by a three-step procedure (Table II and Fig. 2), free from the endogenous E. coli PRPP synthetase. The yield of purified rPRS<sub>I</sub> from a 10-liter culture was about 50 mg.
and that of purified rPRS II was 15 mg, in contrast to a low yield of the enzyme from rat liver (about 2 mg from 600 g of liver) (15). The specific activities of the purified rPRS I and rPRS II were 2.5- and 3.3-fold higher, respectively, than that of the most purified rat liver enzyme, in terms of the 34-kDa component (15). The lower activity of the rat liver enzyme might be due to inhibitory effects of the 38- and/or 40-kDa components in the aggregate.

The NH₂-terminal sequence analysis showed a partial removal of the initiator Met from rPRS I and rPRS II, unlike the complete removal of the residue in the native enzymes of rat liver (15) and E. coli (11). Incompleteness of the removal of an extra Met has presented problems in case of other foreign proteins overexpressed in E. coli (37). Attempts to remove the extra Met using aminopeptidase M were unsuccessful, possibly due to obstruction by folded and aggregated forms of rPRS I and rPRS II. The manner in which the extra Met affects the enzyme activity remains to be elucidated.

PRPP synthetases from mammals and bacteria show an absolute requirement for P₄, for catalytic activity (33). The two isoforms also showed a similar P₄, dependency. The new findings are that sulfate also acts as an activator and that the effects on rPRS I and rPRS II are considerably different (Fig. 3); the extent of activation of rPRS I by sulfate is much greater than that of rPRS II. Furthermore, sulfate inhibited P₄, activation in a different manner with rPRS I than with rPRS II. Although the effects of sulfate may be of little physiological significance, the different responses of the two isoforms to the ion may be helpful in the approximate determination of the isoform composition of tissue PRPP synthetases. Differences between the two isoforms were also found in the sensitivity to inhibition by ADP and GDP. rPRS I was more sensitive to the two inhibitors than rPRS II.

The two isoforms differ only by 13 deduced amino acid residues, 7 out of which are conservative substitutions (16). Regarding the structure-function relationships, the substitutions of Lys for Val and Lys for Gln at positions 4 and 152 are notable. These two substitutions give net additional positive charges to PRS I. The different sensitivity to nucleotide inhibition and/or the different responses to sulfate may be due to either or both of the substitutions. Existence of two isoforms with different regulatory properties and their relative amounts that can vary with tissues in rats (21) suggest that PRPP synthetase has tissue-specific regulatory properties. Furthermore, attention should be given to the above possibility in studies on PRPP synthetase superactivity (38-40) as the molecular basis of human X-linked disease. Alterations in their relative amounts may produce various phenotypes of PRPP synthetase.

The important contribution of this work is the development of a simple and efficient system for overexpressing cDNAs. Since the expression vectors contain nine restriction cloning sites placed immediately downstream from the ATG initiation codon, a cDNA fragment can be conveniently inserted using an appropriate restriction site. Furthermore, an increased copy number of the vectors in E. coli cells was ensured by using the replication origin of pGEM-1 derived from pUC12 instead of pKK233-2 derived from pBR322 (41).

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