Class II Recombinant Phosphoribosyl Diphosphate Synthase from Spinach: Phosphate-Independence and Diphosphoryl Donor Specificity

Britta N. Krath and Bjarne Hove-Jensen

Department of Biological Chemistry, Institute of Molecular Biology, University of Copenhagen, 83H Sølvgade, DK-1307 Copenhagen K, Denmark

Running Title: Class II PRPP Synthase

§ To whom correspondence should be addressed. Tel.: +45 3532 2027; Fax: +45 3532 2040; E-mail: hove@mermaid.molbio.ku.dk.
SUMMARY

A recombinant form of spinach (*Spinacia oleracea*) phosphoribosyl diphosphate (PRPP) synthase isozyme 3 resembling the presumed mature enzyme has been synthesized in an *Escherichia coli* strain in which the endogenous PRPP synthase gene was deleted, and has been purified to near homogeneity. Contrary to other PRPP synthases the activity of spinach PRPP synthase isozyme 3 is independent of P\(_\text{i}\), and the enzyme is inhibited by ribonucleoside diphosphates in a purely competitive manner, which indicates a lack of allosteric inhibition by these compounds. In addition spinach PRPP synthase isozyme 3 shows an unusual low specificity towards diphosphoryl donors by accepting dATP, GTP, CTP and UTP in addition to ATP. The kinetic mechanism of the enzyme is an ordered steady state Bi Bi mechanism with \(K_{\text{ATP}}\) and \(K_{\text{Rib-5-P}}\) values of 170 and 110 \(\mu\text{M}\), respectively, and a \(V_{\text{max}}\) value of 13.1 µmol (min \(\times\) mg of protein\(^{-1}\)). The enzyme has an absolute requirement for magnesium ions, and maximal activity is obtained at 40 °C at pH 7.6.
The compound 5-phospho-D-ribosyl α-1-diphosphate (PRPP) is a precursor in several important metabolic pathways, including purine, pyrimidine and pyridine nucleotide de novo and salvage synthesis, and histidine and tryptophan synthesis (1, 2). Certain microorganisms also utilize PRPP for the synthesis of methanopterin or polyprenylphosphate pentoses (3, 4). The synthesis of PRPP is catalyzed by PRPP synthase: Rib-5-P + ATP → PRPP + AMP (5). PRPP synthase is encoded by the PRS gene(s). Eukaryotic organisms usually contain more than one PRS gene. Analysis of a cDNA library as well as genome sequencing have revealed the presence of at least five PRS genes in the flowering plant Arabidopsis thaliana (6, Data base accession no. AC004521). Spinach (Spinacia oleracea) appears to contain at least four PRS genes, coding for PRPP synthase isozyme 1 to 4, all of which have enzymatic activity. This was established by analysis of a cDNA library for complementation of an Escherichia coli Δprs allele (7). Spinach PRPP synthase isozyme 2 and 3 contain 76 and 87 additional amino acids at their N-terminal ends, respectively, compared to spinach PRPP synthase isozyme 4 and PRPP synthases from E. coli (8), Bacillus subtilis (9) or man (10). These transit peptides of the spinach PRPP synthases contain consensus motifs for maturation of the polypeptides during entry into organelles. Experimental evidence and amino acid sequence comparison revealed that spinach PRPP synthase isozyme 2 was found within the chloroplast, whereas isozyme 3 appeared to be located within the mitochondrion and isozyme 4 in the cytosol. The location of spinach PRPP synthase isozyme 1 is unknown. The deduced N-terminal sequence of the presumed mature isozyme 3 polypeptide is Asn-Ser-Val-Glu-Phe; the polypeptide contains 320 amino acids, and has a calculated molecular mass of 35,497 Da. This value is similar to that for PRPP synthases from most other organisms (7).

In the present work we report the properties of spinach PRPP synthase isozyme 3. The enzyme was synthesized in E. coli and was specified by a gene, which had been
manipulated to make the gene product resemble the presumed mature polypeptide, *i.e.* without a transit peptide. Spinach PRPP synthase isozyme 3 and the "classical" PRPP synthases from *E. coli, B. subtilis* and man differ greatly in their specificity for diphosphoryl donor, dependence of P_i for activity and in their allosteric properties. We show that spinach PRPP synthase isozyme 3 belongs to a novel class of PRPP synthases, Class II.

**EXPERIMENTAL PROCEDURES**

*Materials*—Oligonucleotides were provided by Hobolth DNA Syntese (Hillerød, Denmark). Restriction endonucleases were purchased from Amersham, PRPP and Rib-5-P from Sigma, and nucleotides from Boehringer. [α-^32P]dATP, [α-^32P]GTP, [α-^32P]CTP and [α-^32P]UTP were obtained from New England Nuclear. [γ-^32P]ATP was prepared as previously described (11). Other chemicals were from Sigma or Merck.

*Bacterial Strains and Growth Media*—The *E. coli* strains used were XL1-Blue (*araD Δ(ara-leu) galE galK Δ(lac)X74 rpsL hsdR (r_k^- m_k^-) mcrA mcrB/F lacI q zzf::Tn10*) (Stratagene), HO773 (*araC_an araD Δ(lac)U169 trp_an mal_an rpsL relA thi supF deoD gsk-3 udp Δprs-4*) (12) and HO1088, which is identical to HO773 except for the presence in the former strain of an F episome harboring an *lacI* allele as well as a Tn10 transposon. Episome transfer was performed by mixing exponentially growing cultures of HO773 and XL1-Blue in a 50:1 ratio at 37 °C. After incubation at 37 °C for 1 h leucine prototrophic-tetracycline resistant exconjugants were selected. Cells were grown at 37 °C in NZY broth (13). When necessary the culture medium was supplemented with ampicillin (100 mg L^-1_), tetracycline (10 mg L^-1_), isopropyl 1-thio-β-d-galactopyranoside (5 μM) or NAD (40 mg L^-1_). Cell growth was monitored in an Eppendorf PCP6121 photometer as optical density at
436 nm. An optical density at 436 nm of 1 (1-cm path length) corresponds to approximately $3 \times 10^{11}$ cells L$^{-1}$.

Manipulation of Spinach PRPP Synthase Isozyme 3-Specifying cDNA—To construct pBK862, DNA of pBK843 (7) was digested by restriction endonucleases BstBI and EcoRV. The resulting DNA fragments were subjected to electrophoresis in a 1% agarose gel and the 1048 bp DNA fragment containing PRS3 was cut out and purified (Qiagen). Ten picomoles of each of the oligodeoxyribonucleotides 5´-AATTCATCAAAGAGGAGAAATTAACTAATGAATTCCGTCGAGTTTT (MNS) and 5´-CGAAAACTCGACGGAATTCATAGTTAATTTCTCCTCTTTAATG (MNS-komp) were annealed by heating to 95 °C and slowly cooled to 0 °C. This annealing resulted in the formation of a double stranded DNA fragment with EcoRI and BstBI overhangs. The underlined nucleotides of the MNS oligodeoxyribonucleotide indicate the codons specifying the N-terminal end of the presumed mature spinach PRPP synthase isozyme 3 polypeptide. DNA of pUHE23-2 (H. Bujard, University of Heidelberg, personal communication) was digested by restriction endonucleases PvuII and EcoRI. The three DNA species were mixed and ligated by T4 DNA ligase (Promega), transformed (14) to E. coli strain HO1088 followed by a selection for prs+ (2). The nucleotide sequence of the resulting plasmid (pBK862) was confirmed by sequencing using an Abi Prism 310 DNA Sequencer as recommended by the supplier (Perking-Elmer).

Purification of PRPP Synthase and Protein Methods—Two liters of NZY broth supplemented with ampicillin, tetracycline and isopropyl 1-thio-β-d-galactopyranoside were inoculated with 100 mL overnight culture of strain HO1088/pBK862 and incubated with shaking for 20 h. The following procedures were carried out at 4 °C. Cells (6 g of wet weight) were harvested by centrifugation (Sorvall, GS3 rotor) at 6000 rpm for 12 min, washed in 0.9% NaCl, collected by centrifugation (Sorvall, SS34 rotor),
resuspended in 30 mL of 50 mM Tris/HCl, pH 7.6 and homogenized for 6 × 1 min in a Soniprep ultrasonic disintegrator (Measuring and Scientific Equipment, model 150). Debris was removed by centrifugation at 10,000 rpm for 15 min. Streptomycin sulfate (10% (w/v) in 50 mM Tris/HCl, pH 7.6) was added to a final concentration of 1% and the precipitate was removed by centrifugation at 10,000 rpm for 15 min. The supernatant fluid was 45% saturated with solid (NH₄)₂SO₄. The precipitate was removed by centrifugation and the resulting supernatant fluid was 65% saturated with solid (NH₄)₂SO₄. After centrifugation the precipitate was dissolved in 50 mM Tris/HCl, pH 7.6 and an equal volume of a solution of 10% (w/v) polyethyleneglycol 6000 was added. The precipitate was collected by centrifugation, dissolved in 50 mM Tris/HCl, pH 7.6, and applied to a column (1.0 × 25 cm) of Dyematrex Gel Blue B (Millipore). After washing the column with three volumes of 50 mM Tris/HCl, pH 7.6 protein was eluted at 1 mL min⁻¹ with a 50-mL linear 0.0 to 0.5 M KCl gradient in 50 mM Tris/HCl, pH 7.6. Fractions containing PRPP synthase activity were combined and applied to a DE52 anion exchange column (Whatman) (1.0 × 30 cm). Protein was eluted at 1 mL min⁻¹ with a 100-mL linear 0.0 to 0.5 M NaCl gradient in 50 mM Tris/HCl, pH 7.6. Fractions containing PRPP synthase activity were combined, concentrated by (NH₄)₂SO₄ precipitation, dialyzed against 50 mM Tris/HCl, pH 7.6 containing 50% glycerol, and stored at −20 °C. The purity was evaluated by SDS-PAGE and Coomassie Brilliant Blue staining (15). Protein content was determined by the bicinchoninic acid procedure with chemicals provided by Pierce (16). Bovine serum albumin was used as the standard. Amino acid sequencing by automated Edman degradation was performed by the Department of Biochemistry and Nutrition, Technical University of Denmark.

Assay of PRPP Synthase Activity—PRPP synthase activity was assayed at 37 °C as follows: Extract or enzyme (10 µL), appropriately diluted, was mixed with 40 µL of a
reaction cocktail (both prewarmed at 37 °C) containing radiolabeled (deoxy)ribonucleoside triphosphate (1 kBq per assay), Rib-5-P, MgCl₂ or MnCl₂, 20 mM NaF (omitted after chromatography in DE52), 50 mM Tris/HCl, pH 7.6. Unless otherwise indicated the concentrations of ATP, Rib-5-P and MgCl₂ (or MnCl₂) were 2.0, 3.0 and 5.0 mM, respectively. Samples (10 µL) were removed at intervals and mixed with 5 µL of 0.33 M HCOOH. This 15 µL was applied to polyethyleneimine-cellulose plates (Baker-flex, J. T. Baker) and dried. When [γ-3²P]ATP was used as diphosphoryl donor, PRPP and ATP were separated by thin layer chromatography in 0.85 M KH₂PO₄, which had been previously adjusted to pH 3.4 with 0.85 M H₃PO₄, as solvent. When [α-3²P]dATP, [α-3²P]GTP, [α-3²P]CTP or [α-3²P]UTP were used as diphosphoryl donors, chromatography was performed as follows: CH₃OH (2 cm), 1 M CH₃COOH (2 cm) and 0.9 M CH₃COOH-0.3 M LiCl (16 cm) (17, 18). By this procedure [³²P](deoxy)ribonucleoside triphosphate was separated from [³²P](deoxy)ribonucleoside monophosphate. Radioactivity was quantitated in an Instant Imager (Packard, model 2024).

**Kinetic Analysis**—Results of initial velocity determinations and of product inhibition studies, both of which were average of at least three determinations, were fitted to the following equations using the program UltraFit, (Biosoft, version 3.01). Equation 1 is the Michaelis-Menten equation for hyperbolic substrate saturation kinetics, whereas Equation 2 is the rate equation for a sequential Bi Bi mechanism. For competitive inhibition, noncompetitive inhibition and substrate inhibition the initial velocities were fitted to Equation 3, 4 and 5, respectively (19, 20).

\[
v = \frac{V_{\text{app}} S}{K_m + S}
\]  
\[\text{Eq. 1}\]

\[
v = \frac{V_{\text{max}} [\text{ATP}][\text{Rib-5-P}]}{(K_{\text{ATP}}[\text{Rib-5-P}] + K_{\text{Rib-5-P}}[\text{ATP}]) + K_{\text{iATP}} K_{\text{Rib-5-P}} + [\text{ATP}][\text{Rib-5-P}]})
\]  
\[\text{Eq. 2}\]

\[
v = \frac{V_{\text{app}} S}{K_m (1 + I/K_i) + S}
\]  
\[\text{Eq. 3}\]
\[ v = \frac{V_{\text{app}} S}{K_m (1 + I/K_{i_s}) + S(1 + I/K_{i_i})} \]  \hspace{1cm} (Eq. 4)

\[ v = \frac{V_{\text{app}} S}{K_m + S + (S/K_i)} \]  \hspace{1cm} (Eq. 5)

where \( v \) is the initial velocity, \( V_{\text{app}} \) is the apparent maximal velocity, \( K_m \) is the apparent Michaelis–Menten constant for the varied substrate \( S \), \( V_{\text{max}} \) is the maximal velocity, \( K_{i\text{ATP}} \) and \( K_{\text{Rib-5-P}} \) are the Michaelis–Menten constants for ATP and Rib-5-P, respectively. \( K_{i\text{ATP}} \) is the dissociation constant for ATP, \( K_{i_s} \) and \( K_{i_i} \) are inhibitor constants for the inhibitor I obtained from the effect on slopes and intercept, respectively, and \( K_i \) is the inhibitor constant for the substrate \( S \) (19, 20). Calculation of the free \( \text{Mg}^{2+} \) concentration was performed as previously described (21).

RESULTS

Synthesis and Purification of Spinach PRPP Synthase Isozyme 3—The DNA encoding the 87 codons specifying the presumed transit peptide of spinach PRPP synthase isozyme 3 were removed and replaced by an ATG triplet as described in "Experimental Procedures". Nucleotide sequencing of pBK862 as well as amino acid sequencing of the purified recombinant PRPP synthase demonstrated its N-terminal amino acid sequence to be Met-Asn-Ser-Val-Glu-Phe. This was identical to the sequence of the presumed mature polypeptide with an additional methionine, which served as the translation initiation residue. Induction of expression of the resulting \( \text{PRS}3 \) allele, harbored in pBK862, resulted in overproduction of the enzyme. PRPP synthase was purified by (NH\(_4\))\(_2\)SO\(_4\) and polyethylene glycol precipitations, followed by affinity chromatography and anion exchange chromatography. The yield was 7.2 mg of 95% pure enzyme with a specific activity of 13.1 µmol (min × mg of protein\(^{-1}\)).

Characteristics of Spinach PRPP Synthase Isozyme 3—The enzyme required the presence of \( \text{Mg}^{2+} \) for activity. When ATP and \( \text{Mg}^{2+} \) were added at equimolar
concentrations the saturation curve for ATP appeared sigmoid. When Mg$^{2+}$ was added at 2 mM excess of ATP an increase in enzyme activity was observed compared to the activity with equimolar concentrations of ATP and Mg$^{2+}$, and the saturation curve for ATP appeared hyperbolic with a decrease in the $K_m$ for ATP. These results indicate that Mg$^{2+}$ was required as a free cation to activate the enzyme and that the true substrate for the reaction was MgATP. The enzyme accepted Mn$^{2+}$ as an alternative cation with an activity of 15% of that obtained with Mg$^{2+}$. The enzyme was inactive with the following divalent cations: Ca$^{2+}$, Cd$^{2+}$ and Co$^{2+}$. The activity of the enzyme was independent of $P_i$, and in addition no activation by $P_i$ was observed as the activity of the enzyme remained at 11 µmol (min × mg of protein)$^{-1}$ at $P_i$ concentrations between 0 and 80 mM. The effect of pH on the activity was determined in the range 6.5 to 9.0 with Tris/HCl as buffer. The enzyme showed maximal activity at pH 7.6. The activity increased steeply up to pH 7.2 and decreased with a shallower slope above pH 7.6. The shape of the curve for the dependence of temperature on the activity was symmetrical with maximal activity at 40 °C. As a result of these analyses we chose to assay the activity of the enzyme at 37 °C, at pH 7.6 and at a free Mg$^{2+}$ concentration of at least 1.2 mM.

**Kinetic Mechanism**—Initial velocity versus the concentration of either ATP or Rib-5-P was found to follow typical Michaelis-Menten kinetics. To determine whether the reaction occurred by a sequential or a ping-pong mechanism, the effect of the ATP concentration on the initial velocity was measured at different concentrations of Rib-5-P and visa versa. In double reciprocal plots of the data, intersecting lines indicated that the reaction followed a sequential mechanism (Fig. 1). The data were fitted to Equation 2, and resulted in a $K_{ATP}$ value of 170 µM, a $K_{Rib-5-P}$ value of 110 µM, and a $V_{max}$ value of 13.1 µmol (min × mg of protein)$^{-1}$.

The binding order was analyzed by product inhibition studies. Rib-5-P or ATP was varied at different fixed concentrations of the products PRPP or AMP. Inhibition by PRPP
was competitive with respect to ATP (Fig. 2A) and noncompetitive with respect to Rib-5-P (Fig. 2B). Inhibition by AMP was noncompetitive with respect to both ATP and Rib-5-P (Fig. 2C, D). The calculated inhibition constants are given in Table I. The data agrees with a steady state ordered Bi Bi mechanism, in which ATP binds to the enzyme first, followed by Rib-5-P and a leaving order of products where AMP release is followed by release of PRPP.

*Alternate Diphosphoryl Donors*—Spinach PRPP synthase isozyme 3 readily accepted nucleoside triphosphates other than ATP as diphosphoryl donors. Saturation with dATP, CTP or UTP followed Michaelis-Menten kinetics and data were fitted to Equation 1. With GTP substrate inhibition was observed (Fig. 3). The calculated kinetic constants, \( K_m \), \( V_{\text{app}} \) and \( K_i \) for GTP, are shown in Table II. The \( K_m \) values for dATP, CTP and UTP varied little from \( K_{\text{ATP}} \), whereas the \( K_m \) value for GTP was 3 to 4-fold higher. The \( V_{\text{app}} \) values for CTP and UTP were 10% of \( V_{\text{max}} \) (Table II). As a result dATP and ATP were essentially equally effective as diphosphoryl donors, whereas GTP, CTP and UTP were less efficient compared to ATP.

In contrast to the diphosphoryl donor the enzyme was strictly specific for the ribose moiety. Thus, activity of the enzyme with deoxyribose 5-phosphate as substrate was undetectable.

*Inhibition by Ribonucleotides*—ADP and in some cases GDP, are potent inhibitors of PRPP synthases from a variety of organisms. Both of these ribonucleotides also inhibited spinach PRPP synthase isozyme 3. However, both ADP (Fig. 4A, Table I) and GDP (data not shown) were linear competitive inhibitors of ATP saturation. Furthermore inhibition by ADP was noncompetitive with respect to Rib-5-P (Fig. 4B, Table I). The inhibitory effect of GTP on PRPP synthase activity was analyzed further (Fig. 5). The results are interpreted as simple competitive inhibition of binding of ATP by GTP. The \( K_{\text{GTP}} \) value at a given ATP concentration can be estimated as \([\text{ATP}] \times K_{\text{GTP}} / K_{\text{ATP}}\). At 1 mM ATP this value is 3.8 mM, whereas at 3 mM ATP the value is 11.5 mM. Both values are
consistent with those extrapolated from the data of Fig. 5. Thus, ATP relieved the inhibition by GTP. This indicates that the cause of substrate inhibition by GTP most likely resulted from the formation of a catalytically noncompetent complex of two or more GTP molecules bound in the active site.

Neither tryptophan nor histidine (up to 8 mM, each), which are also products of the PRPP consuming pathways, inhibited the activity of spinach PRPP synthase isozyme 3.

DISCUSSION

We here show that spinach PRPP synthase isozyme 3 had properties very different from other well-characterized PRPP syntheses. These properties were independence of P_i for activity and stability, lack of allosteric inhibition and relaxed specificity for diphosphoryl donor. The “classical” PRPP syntheses share common properties, among others, of being dependent on P_i for maximal activity and being allosterically inhibited by ADP. In fact evidence has accumulated, which indicate that P_i and ADP both bind to the same allosteric site. This evidence comes from results of characterization of six point mutations of human PRPP synthase 1. These mutant variants are associated with gouty arthritis due to overproduction of purine compounds. The mutant forms are less sensitive or insensitive to ADP inhibition and simultaneously are activated by lower concentrations of P_i than the normal enzyme (22). In addition the crystal structure of B. subtilis PRPP synthase has been obtained either in complex with α,β-methylene ADP, an analog of ADP, or with SO_4^{2-}, an analog of P_i. In these two complexes the β-phosphorus of α,β-methylene ADP and SO_4^{2-} occupy the same position (23). Finally analysis of ADP inhibition of P_i activation of E. coli PRPP synthase suggests that the two compounds bind to the same site (24). The present work demonstrates that the activity of spinach PRPP synthase isozyme 3 was independent of P_i and that the enzyme was inhibited by ADP only in a manner competitive with ATP. This is in contrast to the complex ADP inhibition found
for the *Salmonella typhimurium* PRPP synthase (25). Together these observations suggest that spinach PRPP synthase isozyme 3 lacks a functional allosteric site for ribonucleoside diphosphates. GTP also inhibited spinach PRPP synthase isozyme 3. This effect appeared to be caused by binding of two molecules of GTP to the active site. Similar observations have been done with various nucleotides inhibiting PRPP synthase from *S. typhimurium* (25). From the data of Fig. 5 we conclude that there seems to be no alternative allosteric binding site to which binding of GTP will inhibit the enzyme activity. In addition, the “classical” PRPP synthases use ATP exclusively as diphosphoryl donor, except for the mammalian enzymes, which may also use dATP (26, 27). Contrary to this spinach PRPP synthase isozyme 3 had a much broader specificity towards diphosphoryl donor by using, in addition to ATP or dATP, GTP or the pyrimidine ribonucleoside triphosphates CTP or UTP.

The amino acid sequence identity of spinach PRPP synthase isozyme 3 with the “classical” PRPP synthases from *E. coli*, *S. typhimurium*, *B. subtilis*, and mammalian sources is low. Thus, the identity of spinach PRPP synthase isozyme 3 with *E. coli* and *B. subtilis* PRPP synthases and human PRPP synthase 1 is 23 to 25%, whereas the identity of *E. coli* PRPP synthase with *B. subtilis* PRPP synthase and human PRPP synthase 1 is 52 and 49%, respectively. Based on amino acid sequence comparison we previously suggested a distinction between Class I (*i.e.* “classical”) PRPP synthases and Class II PRPP synthases (6). This low sequence identity of spinach PRPP synthase isozyme 3 with the “classical” PRPP synthases is reflected in the dramatic differences in enzymatic properties between spinach PRPP synthase isozyme 3 and the “classical” PRPP synthases reported in the present work. Class II appears to be specific for plants, and contains a few other members: spinach PRPP synthase isozyme 4, *A. thaliana* PRPP synthase isozymes 3 and 4. These PRPP synthases have been shown to be independent of Pi for activity (6, 7). Six additional partial sequences with high homology to spinach PRPP synthase isozyme 3 have been identified by data base search. All of these sequences
originate from plant sources (Data base accession no. AI973393 (maize), AW620825 (soybean), AW696707 (barrel medic), BE041040 (rice), BE341210 (potato), and H75122 (loblolly pine)). Spinach and *A. thaliana* contain at least two and three additional PRPP synthases, respectively. Enzymatic analysis or amino acid sequence comparison showed that these enzymes belong to Class I (6, 7).

Spinach PRPP synthase isozyme 3 also shares common properties with the “classical” PRPP synthases. The kinetic mechanism of spinach PRPP synthase isozyme 3 was steady state ordered Bi Bi with MgATP binding first and PRPP leaving last similar to that shown previously for *S. typhimurium* and *E. coli* PRPP synthases (21, 28). In addition spinach PRPP synthase isozyme 3 required free Mg$_{2+}$ as an activator, similar to what has been shown for the bacterial and human PRPP synthases (8, 21, 26, 29).

Previous reports on plant PRPP synthases describe some properties of an enzyme purified 14-fold from spinach leaves (30) or an enzyme purified from rubber tree latex (31). The latter enzyme apparently is different from all other PRPP synthases in size and specific activity. The molecular mass of the subunit is estimated as 57,000 Da, compared to 35,497 Da of spinach PRPP synthase isozyme 3. The activity of the purified rubber tree latex enzyme is 0.7 µmol (min × mg of protein)$^{-1}$, which is much lower than the 13 and 170 µmol (min × mg of protein)$^{-1}$ determined for the spinach PRPP synthase isozyme 3 and *E. coli* PRPP synthase (28), respectively. It is very likely that these preparations of PRPP synthases from spinach leaves and from rubber tree latex are composed of more than one subunit species. In contrast, we used a highly purified enzyme specified by an allele of the spinach *PRS3* cDNA in the present work.

**Acknowledgments**—We thank Robert L. Switzer for careful reading of the manuscript, Martin Willemoës for invaluable discussions and for careful reading of the manuscript, and Tonny D. Hansen for excellent technical assistance.
REFERENCES

1. Hove-Jensen, B. (1988) *J. Bacteriol.* **170**, 1148-1152
2. Hove-Jensen, B. (1989) *Mol. Microbiol.* **3**, 1487-1492
3. White, R. H. (1996) *Biochemistry* **35**, 3447-3456
4. Sherman, M. S., Kalbe-Bournonville, L., Bush, D., Xin, Y., Deng, L., and McNeil, M. (1996) *J. Biol. Chem.* **271**, 29652-29658
5. Khorana, H. G., Fernandes, J. F., and Kornberg, A. (1958) *J. Biol. Chem.* **230**, 941-948
6. Krath, B. N., Eriksen, T. A., Poulsen, T. S., and Hove-Jensen, B. (1999) *Biochem. Biophys. Acta* **1430**, 403-408
7. Krath, B. N., and Hove-Jensen, B. (1999) *Plant Physiol.* **119**, 497-505
8. Hove-Jensen, B., Harlow, K. W., King, C. J., and Switzer, R. L. (1986) *J. Biol. Chem.* **261**, 6765-6771
9. Nilsson, D., Hove-Jensen, B., and Arning, K. (1989) *Mol. Gen. Genet.* **218**, 565-571
10. Roessler, B. J., Bell, G., Heidler, S., Seino, S., Becker, M., and Palella, T. D. (1990) *Nucleic Acids Res.* **18**, 193
11. Jensen, K. F., Houlberg, U., and Nygaard, P. (1979) *Anal. Biochem.* **98**, 254-263
12. Post, D., Switzer, R. L., and Hove-Jensen, B. (1996) *Microbiology* **142**, 359-365
13. Hove-Jensen, B., and Maigaard, M. (1993) *J. Bacteriol.* **174**, 6852-6865
14. Mandel, M., and Higa, A. (1970) *J. Mol. Biol.* **53**, 159-162
15. Laemmli, U. K. (1970) *Nature* **227**, 680-685
16. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76-85
17. Randerath, K., and Randerath, E. (1965) *Anal. Biochem.* **13**, 575-579
18. Hove-Jensen, B. (1992) *J. Bacteriol.* **174**, 6852-6856
19. Cleland, W. W. (1963) *Biochem. Biophys. Acta* **67**, 173-187

20. Cleland, W. W. (1979) *Methods Enzymol.* **63**, 103-138

21. Switzer, R. L. (1971) *J. Biol. Chem.* **246**, 2447-2458

22. Becker, M. A., Smith, P. R., Taylor, W., Mustafi, R., and Switzer, R. L. (1995) *J. Clin. Invest.* **96**, 2133-2141

23. Eriksen, T. A., Kadziola, A., Bentsen, A.-K., Harlow, K. W., and Larsen, S. (2000) *Nat. Struct. Biol.* **7**, 303-308

24. Willemoës, M., Hove-Jensen, B., and Larsen, S. (2000) *J. Biol. Chem.* **275**, 35408-35412

25. Switzer, R. L., and Sogin, D. C. (1973) *J. Biol. Chem.* **242**, 1063-1073

26. Fox, I. H., and Kelly, W. N. (1971) *J. Biol. Chem.* **246**, 5739-5748

27. Roth, D. G., Shelton, E., and Deuel, T. F. (1974) *J. Biol. Chem.* **249**, 291-296

28. Willemoës, M., and Hove-Jensen, B. (1997) *Biochemistry* **36**, 5078-5083

29. Arnvig, K., Hove-Jensen, B., and Switzer, R. L. (1990) *Eur. J. Biochem.* **192**, 195-200

30. Ashihara, H. (1977) *Z. Pflanzenphysiol.* **83**, 379-392

31. Gallois, R., Prévôt, J.-C., Clément, A., and Jacob, J.-L. (1997) *Plant Physiol.* **115**, 847-852
* This work was supported by the Center for Enzyme Research and the Danish Natural Science Research Council.

‡ Author to whom correspondence should be addressed. Tel: +45 3532 2027. Fax: +45 3532 2040. E-mail: hove@mermaid.molbio.ku.dk.

1 The abbreviations used are: PRPP, 5-phospho-D-riboyl α-1-diphosphate; Rib-5-P, ribose 5-phosphate.
**Figure legends**

**Fig. 1. Reaction mechanism of PRPP synthase.** Activity was determined as described in “Experimental Procedures”. The MgCl$_2$ concentration was 5.0 mM. Lines represent fitting of the data to Equation 2. A. Double reciprocal plot of velocity versus ATP at five concentrations of Rib-5-P. The concentration of ATP was varied from 0.05 to 1.0 mM in the presence of different concentrations of Rib-5-P: 1, 0.125 mM; 2, 0.25 mM; 3, 0.50 mM; 4, 1.00 mM and 5, 2.00 mM. B. Double reciprocal plot of initial velocity versus Rib-5-P at four concentrations of ATP. The concentration of Rib-5-P was varied from 0.125 to 2.00 mM in the presence of different concentrations of ATP: 1, 0.05 mM; 2, 0.10 mM; 3, 0.25 mM and 4, 1.00 mM.

**Fig. 2. Product inhibition of PRPP synthase.** Activity was determined as described in “Experimental Procedures”. A. Double reciprocal plot of initial velocity versus ATP at four concentrations of PRPP. The concentration of Rib-5-P and MgCl$_2$ were 3.0 and 5.0 mM, respectively. The concentration of ATP was varied from 0.1 to 2.0 mM in the presence of different concentrations of PRPP: 1, 0.0 mM; 2, 0.5 mM; 3, 1.0 mM and 4, 2.0 mM. The lines represent fitting of the data set to Equation 3. B. Double reciprocal plot of initial velocity versus Rib-5-P at four concentrations of PRPP. The concentration of ATP and MgCl$_2$ were 2.0 and 5.0 mM, respectively. The concentration of Rib-5-P was varied from 0.1 to 1.0 mM in the presence of different concentrations of PRPP: 1, 0.0 mM; 2, 0.5 mM; 3, 1.0 mM and 4, 2.0 mM. The lines represent fitting of the data set to Equation 4. C. Double reciprocal plot of initial velocity versus ATP at four concentrations of AMP. The concentration of Rib-5-P and MgCl$_2$ were 3.0 and 5.0 mM, respectively. The concentration of ATP was varied from 0.1 to 1.0 mM in the presence of different concentrations of AMP: 1, 0.0 mM; 2, 0.5 mM, 3, 1.0 mM and 4, 2.0 mM. The lines represent fitting of the data set to Equation 4. D. Double reciprocal plot of initial velocity versus Rib-5-P at four concentrations of AMP. The concentration of ATP and MgCl$_2$ were 2.0 and 5.0 mM,
respectively. The concentration of Rib-5-P was varied from 0.1 to 1.0 mM in the presence of different concentrations of AMP: 1, 0.0 mM; 2, 0.2 mM; 3, 0.4 mM and 4, 1.0 mM. The lines represent fitting of the data set to Equation 4.

**Fig. 3. Saturation of PRPP synthase with GTP.** Activity was determined as described in "Experimental Procedures”. The concentration of GTP was varied from 0.125 to 8.0 mM in the presence of 3.0 mM Rib-5-P and MgCl₂ in at least 2 mM in excess of diphosphoribosyl donor concentration. $v$ is expressed as µmol (min $\times$ mg of protein)$^{-1}$.

Data were fitted to Equation 5.

**Fig. 4. Inhibition of PRPP synthase activity by ADP.** Activity was determined as described in "Experimental Procedures”. The MgCl₂ concentration was 5.0 mM. A. Double reciprocal plot of initial velocity versus ATP at four concentrations of ADP. The concentration of ATP was varied from 0.2 to 2.0 mM in the presence of different concentrations of ADP: 1, 0.0 mM; 2, 0.5 mM; 3, 1.0 mM and 4, 2.0 mM. The lines represent fitting of the data set to Equation 3. B. Double reciprocal plot of initial velocity versus Rib-5-P at four concentrations of ADP. The concentration of Rib-5-P was varied from 0.1 to 1.0 mM in the presence of different concentrations of ADP as described in "A". The lines represent fitting of the data set to Equation 4.

**Fig. 5. Inhibition of PRPP synthase activity by GTP.** Activity was determined with [$γ$-$^{32}$P]ATP as described in “Experimental Procedures”. The concentration of Rib-5-P was 3 mM, whereas the concentration of MgCl₂ was 2 mM in excess of the total ribonucleoside triphosphate concentration. The concentration of unlabelled GTP was varied from 0.0 to 3.0 mM at an ATP concentration of 5.0 mM (●), 3.0 mM (■) or 1.0 mM (▲).
**Table I**

*Inhibition constants and mode of inhibition of PRPP synthase*

Inhibition constants were determined as described in "Experimental Procedures". Standard errors are those given by the computer program.

| Substrate | Inhibitor | Mode of inhibition   | $K_{is}$ (µM) | $K_{ii}$ (µM) |
|-----------|-----------|----------------------|----------------|----------------|
| ATP       | AMP$^a$   | non competitive       | 1620 ± 500     | 2170 ± 390     |
| ATP       | PRPP$^b$  | competitive           | 520 ± 50       |                |
| ATP       | ADP$^c$   | competitive           | 680 ± 50       |                |
| Rib-5-P   | AMP$^d$   | non competitive       | 450 ± 180      | 1130 ± 300     |
| Rib-5-P   | PRPP$^e$  | non competitive       | 820 ± 360      | 1700 ± 450     |
| Rib-5-P   | ADP$^f$   | non competitive       | 1220 ± 200     | 2290 ± 230     |

$^a$ The concentration of ATP was varied from 0.1 to 1.0 mM in the presence of 0.0 to 2.0 mM AMP, 3.0 mM Rib-5-P and 5.0 mM MgCl$_2$.

$^b$ The concentration of ATP was varied from 0.1 to 2.0 mM in the presence of 0.0 to 2.0 mM PRPP, 3.0 mM Rib-5-P and 5.0 mM MgCl$_2$.

$^c$ The concentration of ATP was varied from 0.2 to 2.0 mM in the presence of 0.0 to 2.0 mM ADP, 3.0 mM Rib-5-P and 5.0 mM MgCl$_2$.

$^d$ The concentration of Rib-5-P was varied from 0.1 to 1.0 mM in the presence of 0.0 to 1.0 mM AMP, 2.0 mM ATP and 5.0 mM MgCl$_2$.

$^e$ The concentration of Rib-5-P was varied from 0.1 to 1.0 mM in the presence of 0.0 to 2.0 mM PRPP, 1.0 mM ATP and 5.0 mM MgCl$_2$.

$^f$ The concentration of Rib-5-P was varied from 0.1 to 1.0 mM in the presence of 0.0 to 2.0 mM ADP, 1.0 mM ATP and 5.0 mM MgCl$_2$. 
TABLE II

*Kinetic constants of PRPP synthase for diphosphoryl donors other than ATP*

Kinetic constants were determined as described in "Experimental Procedures". Standard errors are those given by the computer program.

| Diphosphoryl donor | $K_m$ $^a$ (µM) | $V_{app}$ (µmol (min $\times$ mg)$^{-1}$) | $K_i$ (µM) |
|-------------------|-----------------|---------------------------------|-------------|
| dATP              | 233 ± 13        | 12.8 ± 0.1                      |             |
| GTP               | 650 ± 280       | 6.9 ± 1.7                       | 350 ± 160   |
| CTP               | 116 ± 20        | 1.5 ± 0.1                       |             |
| UTP               | 137 ± 20        | 1.2 ± 0.02                      |             |

$^a$ The concentration of dATP, GTP, CTP or UTP was varied from 0.125 to 8.0 mM at 3.0 mM Rib-5-P and MgCl$_2$ in at least 2 mM excess of diphosphoryl donor concentration.
Krath and Hove-Jensen, Fig. 1A
Krath and Hove-Jensen, Fig. 1B
Krath and Hove-Jensen, Fig. 2A
Krath and Hove-Jensen, Fig. 2B
Krath and Hove-Jensen, Fig. 2C
Krath and Hove-Jensen, Fig. 2D
Krath and Hove-Jensen, Fig. 3
Krath and Hove-Jensen, Fig. 4A
Krath and Hove-Jensen, Fig. 4B
Krath and Hove-Jensen, Fig. 5
Class II recombinant phosphoribosyl diphosphate synthase from spinach: phosphate-independence and diphosphoryl donor specificity
Britta N. Krath and Bjarne Hove-Jensen

J. Biol. Chem. published online February 27, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010172200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts