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D-Ribose-5-Phosphate Isomerase from Spinach: Heterologous Overexpression, Purification, Characterization, and Site-Directed Mutagenesis of the Recombinant Enzyme[1]

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A cDNA encoding spinach chloroplastic ribose-5-phosphate isomerase (RPI) was cloned and overexpressed in *Escherichia coli,* and a purification scheme for the recombinant enzyme was developed. The purified recombinant RPI is a homodimer of 25-kDa subunits and shows kinetic properties similar to those of the homodimeric enzyme isolated from spinach leaves (A. C. Rutner, 1970, *Biochemistry* 9, 178–184). Phosphate, used as a buffer in previous studies, is a competitive inhibitor of RPI with a K_i of 7.9 mM. D-Arabinose 5-phosphate is an effective inhibitor, while D-xylulose-5 phosphate is not, indicating that the configuration at carbon-3 contributes to substrate recognition. Although D-arabinose 5-phosphate binds to RPI, it is not isomerized, demonstrating that the configuration at carbon-2 is crucial for catalysis. Alignment of RPI sequences from diverse sources showed that only 11 charged amino acid residues of the 236-residue subunit are conserved. The possible function of four of these residues was examined by site-directed mutagenesis. D87A, K100A, and D90A mutants show greatly diminished k_{cat} values (0.0012, 0.074, and 0.38% of the wild type, respectively), while E91A retains substantial activity. Only insignificant or moderate changes in K_m of D-ribose 5-phosphate are observed for D87A, K100A, and D90A, indicating a direct or indirect catalytic role of the targeted residues. © 2000 Academic Press

Key Words: **ribose-5-phosphate isomerase; active site; mutagenesis.**

As the catalyst for the interconversion of D-ribose 5-phosphate and D-ribulose 5-phosphate, RPI[5] (EC 5.3.1.6), plays an essential role in the Calvin cycle of photosynthesis and in the oxidative pentose phosphate pathway of both photosynthetic and nonphotosynthetic organisms (1). RPI, in concert with ribulose-5-phosphate epimerase, facilitates partitioning of pentose phosphates between these two pathways in photosynthetic organisms, depending on metabolic needs and the redox status of cells. D-Ribose 5-phosphate itself is the substrate for the synthesis of phosphoribosyl pyrophosphate, which serves as a precursor for histidine, tryptophan, and nucleotides (2), and D-ribulose 5-phosphate in turn is a precursor for riboflavin (3).

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[5] Abbreviations used: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; ATP, adenosine 5′-triphosphate; Bicine, *N,N′*-bis-(2-hydroxyethyl)glycine; BSA, bovine serum albumin; DTT, dithiothreitol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PCR, polymerase chain reaction; PEP, phospho(enol)pyruvate; PMSF, phenylmethylsulfonyl fluoride; QAE, diethyl-(2-hydroxypropyl)aminoethyl; RPI, ribose-5-phosphate isomerase; R5P, D-ribose 5-phosphate; Ru5P, D-ribulose 5-phosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TIM, triose phosphate isomerase.

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RPI, which appears to be ubiquitous in all living cells (4, 5), has been isolated and partially characterized from many different prokaryotes and eukaryotes (6–11). The metabolic necessity of RPI is clearly shown by the fact that *Escherichia coli* strains defective in RPI are ribose auxotrophs (12). Despite the metabolic importance of RPI, however, the molecular properties and mechanism of RPI have not been well studied. The cloning of spinach and mouse cDNAs encoding RPI has been reported (13, 14), and the nucleotide sequences of RPI from 12 prokaryotes have been determined by partial or complete genome sequencing (15–21). However, to our knowledge, high-level expression and purification of enzymatically active recombinant RPI have not been achieved heretofore.

In this report, we describe overexpression of the mature form of spinach chloroplastic RPI, an efficient purification procedure for the recombinant enzyme, and some general properties of the highly purified recombinant RPI. Importantly, three active site residues, which facilitate catalysis substantially, are also identified by site-directed mutagenesis.

EXPERIMENTAL PROCEDURES

Materials. Materials and vendors were as follows: *Pfu* DNA polymerase and a library of *Spinacia oleracea* L. cv. Melody cDNAs in lambda ZAP II (Stratagene, Inc.); oligonucleotide primers for PCR, mutagenesis, and dye-terminator sequencing (GIBCO BRL); T4 DNA ligase and *Nco*I restriction endonuclease (New England Biolabs); pyruvate kinase, lactate dehydrogenase, NADH, PEP, R5P, Ru5P, D-arabinose 5-phosphate, D-xylulose 5-phosphate, leupeptin, and PMSF (Sigma); AEBSF (Calbiochem); and 3,3′-diaminobenzidine tetrahydrochloride dihydrate (Bio-Rad). Common laboratory reagents for enzyme purification and assays were procured at the highest level of purity readily available. Spinach phosphoribulokinase was prepared as described earlier (22, 23).

Rabbit serum containing polyclonal antibodies raised against purified recombinant spinach RPI was prepared by the Berkeley Antibody Company, CA.

Construction of RPI expression cassette. The mature form of spinach RPI was constructed by PCR amplification (30 cycles of 94°C/45 s, 50°C/45 s, 72°C/120 s) from the lambda ZAP II cDNA library, based on the published sequence (13). The PCR primers introduced one *Nco*I site 3′ to the termination codon and a second *Nco*I site coincident with the P54 codon of the transit peptide, thereby creating the new initiation site, P54M. The sequence of the forward primer was aca cca tgg tgt att ctc agg acg atc tca, and the reverse primer was caa cca tgg tca ctt ggt ttt cac act aac. The *tac* promoter vector pFL260 (24) was cleaved by *Nco*I, and the amplified product was ligated as an *Nco*I fragment adjacent to the *tac* promoter. The sequence of the expression cassette was confirmed by DNA sequencing, performed on an ABI 373A sequencer using either dye-primer or dye-terminator cycle sequencing chemistries.

Expression in E. coli. A culture of the expression cassette plasmid in host strain XL-1 or MV1190 was grown overnight at 37°C in 2× YT medium (25) containing ampicillin (50 μg/ml) and 1% (v/v) glycerol. It was then diluted 1:100 into the same medium and grown 4 h with vigorous shaking (250 rpm). β-D-Thiogalactopyranoside was added to 0.1 mM, and the incubation was continued an additional 3 h followed by harvesting of cells by centrifugation.

Protein and enzyme assays. Protein was determined by the Bradford method (26) using BSA as standard, according to the instructions of the manufacturer (Pierce Co.). RPI activity was measured routinely at 25°C as NADH oxidation through coupling to phosphoribulose kinase, pyruvate kinase, and lactate dehydrogenase (22, 27). The reaction mixture (1 ml) contained 40 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 3 mM PEP, 0.24 mM NADH, 2 mM R5P, 2 units of phosphoribulose kinase, 4 units of pyruvate kinase, 5 units of lactate dehydrogenase, and 0.01 to 0.1 unit of RPI in 50 mM Bicine buffer, pH 8.0. In order to measure the reverse reaction, RPI activity was coupled via transketolase, TIM, and glycerol phosphate dehydrogenase (28). The reaction mixture (0.2 ml) at 25°C contained 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.1 mM thiamine pyrophosphate, 0.25 mM NADH, 1 mM Ru5P, 5 mM xylulose 5-phosphate, 0.2 units of transketolase, 4 units of TIM, 0.4 units of glycerol phosphate dehydrogenase, and 0.002 to 0.02 unit of RPI in 50 mM Bicine buffer, pH 8.0. For each assay, NADH oxidation was monitored at 340 nm, and one unit of activity was defined as 1 μmol of NADH oxidized min^{-1}.

Isomerization of R5P to Ru5P was also determined directly from the absorbance change at 290 nm as described by Wood (29). An absorbance of 0.072 for 1 mM Ru5P was used to calculate Ru5P concentration (29).

Purification of recombinant RPI from E. coli. All steps were carried out at 4°C. Transformed *E. coli* paste (14 g), containing wild-type RPI, was suspended in 28 ml of 50 mM Bicine buffer, pH 8.0, containing 1 mM EDTA, 1 mM DTT, 10 μM leupeptin, 0.2 mM AEBSF, 1 mM PMSF, and 5% (v/v) glycerol. The cells were broken by two passes through a French press at 12,000 to 16,000 p.s.i. After centrifugation of the extract (100,000*g* for 45 min), the supernatant was diluted to 60 ml with cold water and applied to a 2.5 × 8-cm column of QAE (TosoHaas, QAE-550C, 100 μm) equilibrated with 20 mM Tris–Cl buffer, pH 7.0. RPI activity was eluted by the same buffer containing 250 mM NaCl, and the pooled active fractions were applied to a 2.5 × 6-cm column of hydroxyapatite (Bio-Rad, CHT Type I, 40 μm) equilibrated with 5 mM potassium phosphate buffer, pH 7.0. RPI was eluted with 400 ml of a linear gradient from 5 to 200 mM phosphate buffer, pH 7.0. Pooled active fractions were concentrated (Centricon 30, Amicon Co.) and applied to an FPLC MonoQ HR 10/10 (Pharmacia) column equilibrated with 25 mM potassium phosphate buffer, pH 7.0. Subsequent to elution with a 90-ml gradient of 25 to 200 mM phosphate buffer, pH 7.0, the active fractions were concentrated and applied to another Mono Q HR 10/10 column equilibrated with 20 mM Tris–Cl buffer, pH 7.0. The RPI activity was eluted with 90 ml of a 0 to 0.3 M NaCl linear gradient in the same buffer. Pooled fractions were concentrated to ~10 mg protein ml^{-1} and stored at −80°C in the presence of 20% (v/v) glycerol.

The same protocol was effective for the purification of each of the site-directed mutants of RPI examined in this study.

Molecular weight estimations. The subunit molecular weight of RPI was estimated by SDS–PAGE at 15°C on 8–20% PhastGels in conjunction with a PhastSystem (Pharmacia Biotech). Gels were stained with Coomassie blue R-250 according to the supplier's protocol. The molecular weight of native RPI was determined by gel filtration and by PAGE under nondenaturing conditions on gradient gels. A Superose-12 HR column (1 × 30 cm) from Pharmacia was equilibrated with 100 mM Tris–Cl buffer, pH 7.0. Blue dextran was used for determining the void volume (V_o) of the column, and BSA (67 kDa), hen egg albumin (45 kDa), and chymotrypsinogen (25 kDa) were used as molecular weight standards. Nondenaturing PAGE was achieved on 8–20% PhastGels (Pharmacia Biotech) and 8–16% Tris–Glycine gels (Novel Experimental Technology).

Isoelectric point of recombinant RPI. The isoelectric point of recombinant RPI was estimated on p*I* 4–6.5 range PhastGels according to the manufacturer's instructions. The p*I* of RPI was calculated using bovine carbonic anhydrase B (p*I* 5.85), β-lactoglobulin A (p*I* 5.2), and soybean trypsin inhibitor (p*I* 4.55) as standards.

Western blotting. Purified recombinant RPI was compared with that from spinach extract by Western blotting of SDS–PAGE gels (30). To prepare the latter sample, about 1 g of fresh spinach leaves was ground with a mortar and pestle in 1 ml of the extraction medium used for *E. coli.* The resulting slurry was centrifuged for 20 min at 4600*g* at 4°C, and the supernatant was used for Western blot analysis. Denaturing electrophoresis was carried out on 4–12% NuPAGE Tris gels with Mes–SDS running buffer (Novel Experimental Technology). Proteins were transferred to nitrocellulose membranes with a Novex XCell blot module. The membranes were fixed with 25% (v/v) isopropanol–10% (v/v) acetic acid. RPI was visualized by the rabbit serum containing RPI antibodies, peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad), 1.3 mM 3,3′-diaminobenzidine tetrahydrochloride, and 9 mM H_2O_2, according to the instructions of the supplier (Bio-Rad).

Site-directed mutagenesis. RPI mutants were constructed by use of linear PCR as described by Weiner *et al.* (31), using *Pfu* DNA polymerase. Following mutagenesis, plasmids were digested with *Dpn*I to destroy the template DNA and electroporated into *E. coli* XL-1 (32). Plasmid template was isolated from mutants and sequenced across the region of interest to confirm the desired constructs.

RESULTS

Cloning and expression of rpi spinach cDNA. A DNA fragment encoding the mature form of RPI was cloned from a commercial lambda ZAPII spinach cDNA library. The introduction of a *Nco*I site at the P54 codon of the transit peptide creates a new initiation site so that the N-terminal sequence of the recombinant protein is MVLT instead of PTPVLT as occurs in the authentic enzyme isolated directly from spinach leaves (13). Otherwise, our clone matches the published sequence (13).

Protein sequence alignment of RPI from various sources (Fig. 1) shows that only 25 residues of the 236-residue subunit are strictly conserved. E91[6] is also invariant with the exception of replacement by Q in *Archaeoglobus fulgidus.* Since only one nucleotide substitution (GAG to CAG) would result in this change, confirmation is warranted to exclude the possibility of a sequencing error.

Purification of recombinant wild-type and mutant RPIs. The purification method described under Experimental Procedures provides electrophoretically homogeneous recombinant wild-type RPI (Fig. 2) in good yield in 3 working days, as summarized in Table I. About 1.3% of total soluble protein in the *E. coli* extract is recombinant spinach RPI. Recombinant RPI has been expressed in two different hosts, XL-1 and MV1190, but there was no noticeable difference in expression level and purification profile between them. RPI indigenous to the *E. coli* host (about 10% of the total activity extracted) is separated from spinach RPI during the hydroxyapatite step, in which the former is eluted prior to initiation of the phosphate gradient. Discounting the activity of the host RPI in crude extracts, the recovery of the recombinant RPI after the final purification step is near 80%. RPI from the second Mono Q step is homogeneous based on SDS–PAGE (Fig. 2) and isoelectric focusing (data not shown).

The phosphate dependence of the elution position of spinach RPI, but not that of contaminating proteins, from Mono Q simplified the purification strategy. By successive chromatography on Mono Q columns with a phosphate gradient and a NaCl gradient, or vice versa, homogeneity of RPI is readily achieved. *E. coli* RPI, on the other hand, does not show this sensitivity to phosphate and is only partially purified by the same procedure. The recombinant RPI is eluted near 180 mM NaCl and 120 mM phosphate from Mono Q, while *E. coli* RPI is eluted near 150 mM NaCl and 95 mM phosphate. This would provide further removal of residual *E. coli* RPI activity and hence eliminate any possible interference by *E. coli* RPI in RPI mutant studies.

The same purification procedure developed for the wild-type enzyme was equally effective in achieving apparent electrophoretic homogeneity of the mutant RPIs (Fig. 2), thereby indicative of their conformational integrity. The only noted difference in chromatographic behavior was that K100A eluted somewhat later in the gradients applied to both of the Mono Q columns (230 rather than 180 mM NaCl and 200 rather than 120 mM phosphate).

Comparison of the recombinant RPI with that from a spinach leaf extract. When subjected to SDS–PAGE and detected by Western blotting, the mobility of the recombinant RPI was slightly greater than that of RPI in a crude spinach extract or purchased from Sigma. This barely discernible difference might be due to the altered N-terminal sequence encoded by our construct. *E. coli* RPI did not cross-react with the antibody raised against spinach RPI.

Kinetic constants. The spinach recombinant and partially purified *E. coli* RPIs exhibit typical Michaelis–Menten kinetics. Under our assay conditions, the K_m (R5P) for spinach RPI was 0.63 mM. This value is significantly lower than those reported by Kiely *et al.* (5.3 mM) (33), Knowles *et al.* (4.6 mM) (34), and Woodruff and Wolfenden (3.3 mM) (35), but in a good agreement with that by Rutner (0.46 mM) (11).

The k_{cat} of 3440 s^{-1} for recombinant RPI is higher than 2020 s^{-1} and 1880 s^{-1} as reported by Rutner (11) and Knowles *et al.* (34), respectively, for the spinach enzyme from the native source. This higher activity likely reflects the more rapid and efficient purification scheme developed in this study rather than a difference between native and recombinant RPI.

[6] Residue numbers refer to the mature recombinant enzyme, unless otherwise indicated. The N-terminal methionine corresponds to position 54 of the transit protein as described under Experimental Procedures.

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                        1                                                                         70
         M. jannaschii  ~~~~~~~~~~ ~~~~~~~~VS NEDLKLKVAK EAVKLVKDGM VIGLGTGSTA ALFIRELGNR IREEELT.VF
 Pyrococcus horikoshii  ~~~~~~~~~~ ~~~~~~~~MN VEEMKKIAAK EALKFIEDDM VIGLGTGSTT AYFIKLLGEK LKRGEISDIV
           A. fulgidus  ~~~~~~~~~~ ~~~~~~~~~M DSSGKYNAAK LALELVKDGM VLGIGSGSTV EVFLNLLGDK IREEGL.EIY
M. thermoautotrophicum  ~~~~~~~~~~ ~~~~~~~MEV FMNLKKMAAL RAVDEIDDGD VVGLGTGSTT HYFIEELGRR VREEGL.EVM
       E. coli (RPI A)  ~~~~~~MRVK FHTTGETIMT QDELKKAVGW AALQYVQPGT IVGVGTGSTA AHFIDALGTM ..KGQIEG..
 Edwardsiella ictaluri  ~~~~~~~~~~ ~~~~~~~~MT QDELKKAVGW AALKYVRPGT IVGVGTGSTA SHFIDALATM ..KGQIEG..
         H. influenzae  ~~~~~~~~~~ ~~~~~~~~MN QLEMKKLAAQ AALQYVKADT IVGVGSGSTV NCFIEALGTI ..KDKIQG..
  Borrelia burgdorferi  ~~~~~~~~~~ ~~~~MENQKI LVAKHAIDHY IK.....SNM NLGIGTGTTI YYAIKYLSEK IKSGSLKNLK
    Treponema pallidum  ~MHERNTTTN TPLDVTAQKL LVAQRSVDTL VQEGVLHAHM SIGLGTGSTA MPAVKRIADH LARGTLSDIA
      Synechocystis sp  ~~~~~~~~~~ ~~~~MAELDA ANLMKQAVGK AAADRVKSNT IVGLGTGSTT AYALEFIGDR LKKGELENVV
         S. cerevisiae  MAAGVPKIDA LESLGNPLED AKRAAAYRAV DENLKFDDHK IIGIGSGSTV VYVAERIGQY LHDPKFYEVA
Caenorhabditis elegans  ~~~~MVTSTG PEAELAPIEQ AKKRAAFACG EKYVQ..SGC RLGVGSGSTV KYLVEYLKQG FQNGSLKDI.
          Mus musculus  ~~~~~~~~~~ ~~~~MSKAEE AKKLASHTAV ENHVK..NNQ VLGIGSGSTI VHAVQRIAER VKQENL.DL.
          Homo sapiens  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
     Spinacia oleracea  ~~~~~~~~~~ ~~~~~~MVLT QDDLKKLAAE KAVDSVKSGM VLGLGTGSTA AFAVSRIGEL LSAGKLTNIV
                                                                      G G GST
                        71                                                                       140
         M. jannaschii  G....IPTSF EAKMLAMQYE IPLVTLDEYD .VDIAFDGAD EVEETTLFLI KGGGGCHTQE KIVDYNANEF
 Pyrococcus horikoshii  G....VPTSY QAKLLAIEHD IPIASLDQVD AIDVAVDGAD EV.DPNLNLI KGRGAALTME KIIEYRAGTF
           A. fulgidus  G....IPSSY QSYFAAIRNG VEIVDLVEFE P.DLCIDGAD QV.DAKLNCI KGGGGAMTRE KIVAKASRKV
M. thermoautotrophicum  G....VPTSY QSMFLAAESG IKVTSLAEHD .VDVAVDGAD EV.DPDLNLI KGGGAAHTLE KIVDSSAASF
       E. coli (RPI A)  A....VSSSD ASTEKLKSLG IHVFDLNEVD SLGIYVDGAD EI.NGHMQMI KGGGAALTRE KIIASVAEKF
 Edwardsiella ictaluri  A....VSSSD ASTARLKSLG IPVFDLNEVD SLDIYVDGAD EI.NGAMQMI KGGGAALTRE KIVAAVAKKF
         H. influenzae  A....VAASK ESEELLRKQG IEVFNANDVS SLDIYVDGAD EI.NPQKMMI KGGGAALTRE KIVAALAKKF
  Borrelia burgdorferi  FYTTSSDTKY LLSKEQIPY. ESNFSK.LNK NLDIAIDGAD EILLEKKSLI KGMGGAHLME KVIAYNSETL
    Treponema pallidum  AVPTSFQTAL ICERYNIPL. FSLSSKRIGG KLDVTIDGAD EIDTQ.NFVI KGGGAALLQE KIAAYNSAHF
      Synechocystis sp  G....IPTSF QAEVLARKYG IPLTTLDVAD RIDIAIDGAD EV.DPQKNLI KGGGAAHTRE KIVDALAETF
         S. cerevisiae  SKFICIPTGF QSRNLILDNK LQLGSIEQYP RIDIAFDGAD EV.DENLQLI KGGGACLFQE KLVSTSAKTF
Caenorhabditis elegans  ...ICVPTSF LTKQWLIESG LPVSDLDSHP ELDVCIDGAD EV.DGQFTCI KGGGGCLAQE KIVQTAAKNF
          Mus musculus  ...ICIPTSF QARQLILQYG LTLSDLDQHP EIDLAIDGAD EV.DAELNLI KGGGGCLTQE KIVAGYASRF
          Homo sapiens  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~AGYASRF
     Spinacia oleracea  G....IPTSK RTAEQAASLG IPLSVLDDHP RIDLAIDGAD EV.DPDLNLV KGRGGALLRE KMVEAASDKF
                                                       DGAD            KG G     E K
                        141                                                                      210
         M. jannaschii  VVLVDESKLV KKL.GEKF.. PIPVEVIPSA YRVVIRALS. ...EMGGEAV IRLGDR.KRG PVITDNGNMI
 Pyrococcus horikoshii  IVLVDERKLV DYL.CQKM.. PVPIEVIPQA WKAIIEELS. ...IFNAKAE LRMGVN.KDG PVITDNGNFI
           A. fulgidus  VIIVDESKLV EKL...SM.. PVPVEVLPFA YGWVLREIE. ...KMGCKAR LREGKG.KIG PVITDNGNFV
M. thermoautotrophicum  IVIVDESKLV ERL.G.AF.. PLPVEVIPAA CRPVKLKLE. ...SMGASVN IRSSEG.KDG PVVTDNGNFV
       E. coli (RPI A)  ICIADASKQV DIL.GK.F.. PLPVEVIPMA RSAVARQL.. ..VKLGGRPE YRQG...... .VVTDNGNVI
 Edwardsiella ictaluri  VCIVDASKQV DIL.GS.F.. PLPVEVIPMA RAYVAREL.. ..VKLGGQPV YRQG...... .VLTDNGNVI
         H. influenzae  ICIVDSSKQV DVL.GSTF.. PLPVEVIPMA RSQVGRKL.. ..AALGGSPE YREG...... .VVTDNGNVI
  Borrelia burgdorferi  LIIADETKIV KKLG...TKM PIPIEVAQNA VGFIMTRLE. ...EMNLEAT LRICKEKK.G PTITDNNNYI
    Treponema pallidum  VIIVDETKVV ETLG...TRA ALPIEVVPEA RMSVMRTLQ. ...DWGLSVH IREAVRKK.G PVVTDHGNFI
      Synechocystis sp  LVVVDSGKLV DKL.GSTF.. LLPVEVIPMA LTPVMRALA. ...KLGGKPE LRMGVK.KAG PVVTDQGNLV
         S. cerevisiae  IVVADSRKKS PKHLGKNWRQ GVPIEIVPSS YVRVKN..DL LEQLHAEKVD IRQGGSAKAG PVVTDNNNFI
Caenorhabditis elegans  YVIADYLKDS .KHLGDR.YP NVPIEVLPLA AQPLLR...S IPRAEGGSCQ LRQAVK.KCG PIVTDNGNFI
          Mus musculus  IVIADFRKDS .KNLGDRWHK GIPIEVIPMA YVPVSR...A VAQKFGGEVE LRMAVN.KAG PVVTDNGNFI
          Homo sapiens  IVIADFRKDS .KNLGVQWHK GIPIEVIPMA YVPVSR...A VSQKFGGVVE VRMAVN.KAG PVVTDNGNFI
     Spinacia oleracea  IVVVDDTKLV DGLGGSRL.. AMPVEVVQFC WKYNLKRLQE IFKELGCEAK LRM.EG.DSS PYVTDNSNYI
                            D  K                  E                                R             TD  N
                        211                                                        269
         M. jannaschii  IDV.FMNIDD A.I..ELEKE INNIPGVVEN GIFTK.V.DK VLVG.TKKGV KTLKK~~~~
 Pyrococcus horikoshii  IDAKFPRIDD P.L..DMEIE LNTIPGVIEN GIFAD.IADI VIVG.TREGV KKLER~~~~
           A. fulgidus  VDCDFGVIEE D.RVEGLEGE IKLISGVVEN GIFSKELIDA VIAGSSRSAR FL~~~~~~~
M. thermoautotrophicum  LDAAFGVIDD P.G..AMESR LNNIPGVVEN GIFAG.IADM VIAG.TSEGL KILR~~~~~
       E. coli (RPI A)  LDVHGMEILD P.I..AMENA INAIPGVVTV GLFANRGADV ALIG.TPDGV KTIVK~~~~
 Edwardsiella ictaluri  LDVHNLQIME P.C..KLENA INAIAGVVTV GLFANRGADV ALVG.CADGV KTLTL~~~~
         H. influenzae  LDVHNFSILN P.V..EIEKE LNNVAGVVTN GIFALRGADV VIVG.TPEGA KVID~~~~~
  Borrelia burgdorferi  LDVK...M.H VENPEGTEKY FKLFPGILEI GIFNHKNTRI .VYYQDKQIK EA~~~~~~~
    Treponema pallidum  LDARWQSL.P TRTPQDMERA LNALPGVIEN GLFTERTVRV FVAHADGSVE ERSASF~~~
      Synechocystis sp  IDVKFDAITN P.A..ELEKT INNLPGVLEN GLFVG.VADV ILVGEIIDGQ PTVREF~~~
         S. cerevisiae  IDADFGEISD PRK...LHRE IKLLVGVVET GLFIDN.ASK AYFGNSDGSV EVTEK~~~~
Caenorhabditis elegans  IDWQFEKNVS GRDWFAIQQR LANTPGIVET GLFIGC.VDA VFFAYSDGSV KEIVNSKKH
          Mus musculus  LDWKFDR... VHKWSEVNTA IKMTPGVVDT GLFINM.AER VYFGMQDGSV NVREKPF~~
          Homo sapiens  LDWKFDR... VHKWSEVNTA IKMIPGVVDT GLFINM.AER VYFGMQDGSV NMREKPFC~
     Spinacia oleracea  VDLYFPTSIK D.A.EAAGRE ISALEGVVEH GLFLGMASEV IIAGKTGVSV KTK*~~~~~
                         D                          G    G F
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FIG. 1. Comparison of RPI sequences. The RPI sequences from *Methanococcus jannaschii* (U67600), *Pyrococcus horikoshii* (AB005215), *Archaeoglobus fulgidus* (AE001039), *Methanobacterium thermoautotrophicum* (AE000842), *E. coli* (X73026), *Edwardsiella ictaluri* (AF037440), *Haemophilus influenza* (U32729), *Borrelia burgdorferi* (AE001167), *Treponema pallidum* (ORF00728), *Synechocystis* sp. (D64002), *Saccharomyces cerevisiae* (Z75003), *Caenorhabditis elegans* (P41994), *Mus musculus* (L35034), *Homo sapiens* (L35035), and *Spinacia oleracea* (this study and Ref. 13) are compared. The strictly conserved residues are denoted in the last line of each grouping of comparative sequences.

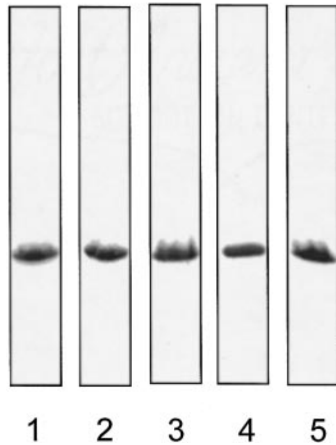

FIG. 2. SDS–PAGE of purified wild-type and mutant RPIs. Approximately 0.5 μg of protein was applied in each case. Lane 1, wild-type RPI; lane 2, D87A mutant; lane 3, D90A; lane 4, E91A; lane 5, K100A.

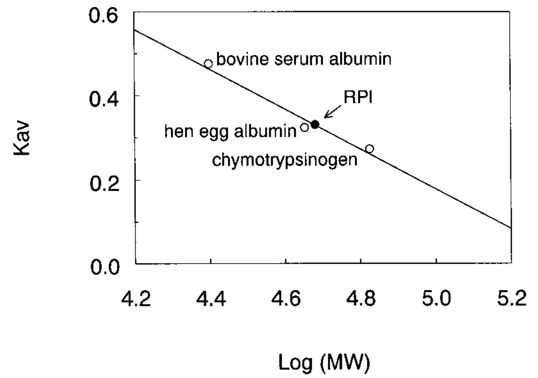

FIG. 3. Molecular weight of recombinant RPI as determined by gel filtration on Superose 12. Blue dextran 2000 and NaCl were used to determine the void volume (8.8 ml) and the total liquid phase (21.4 ml). Experimental details are provided in the text.

E. coli contains two RPIs, constitutive RPI A and inducible RPI B (36). We determined the K_m (R5P) of partially purified *E. coli* RPI to be 0.89 mM, virtually identical to that of RPI B (0.83 mM), but in stark contrast to that of RPI A (4.4 mM) as reported by Essenberg and Cooper (36). Since the subunit molecular weight of the partially purified *E. coli* RPI as determined by SDS–PAGE (25 kDa) suggests that the partially purified RPI is equivalent to RPI A (23 kDa) rather than RPI B (16 kDa) and since RPI A accounts for at least 99% of the total RPI activity of strains grown in nutrient broth (12), these seemingly discrepant results deserve further study.

Molecular weight and pI. The subunit molecular weight of RPI was calculated as 25,066 from the deduced amino acid sequence (Fig. 1) and estimated to be 25 kDa by SDS–PAGE (Fig. 2). When assessed by gel filtration in comparison with BSA, chymotrypsinogen, and hen egg albumin, the molecular weight of recombinant RPI was 49 kDa (Fig. 3), which indicated that the recombinant enzyme is a homodimer.

According to isoelectric focusing analysis, the p*I* for RPI was 5.1, which is quite similar to the value of 4.86 calculated from the deduced amino acid sequence and the values for the corresponding enzymes from pea (4.95) and tobacco leaves (5.13) (37, 38).

Phosphate and substrate analogs. Phosphate is a competitive inhibitor for the recombinant spinach and *E. coli* RPIs, exhibiting K_i values of 7.9 and 10.2 mM, respectively. In order to evaluate structural determinants for substrate recognition, the stereoisomers of R5P, D-arabinose 5-phosphate, and D-xylulose 5-phosphate, were examined as RPI inhibitors. Arabinose 5-phosphate is a strong inhibitor with a K_i of 0.70 mM, quite similar to the K_m of 0.63 mM for R5P. Xylulose 5-phosphate, on the other hand, is a weaker inhibitor with a K_i estimated to be 4.0 mM, only slightly lower than that of Pi. Thus, the C3 hydroxyl of R5P may be directly involved in binding.

The possibility of isomerization of arabinose 5-phosphate to Ru5P by RPI was examined directly by monitoring the absorbance at 290 nm. Even with a 400-fold greater concentration of RPI, relative to that used in the standard assay, no increase in absorbance could be detected (data not shown). Thus, although bound by RPI

TABLE I

Purification of Recombinant Spinach RPI from *Escherichia Coli*

Purification step	Protein (mg)	Activity (units)	Specific activity (units mg^{-1})	Recovery (%)	Purification fold
Crude extract	1260	118,000	93.7	100	1
QAE	377	112,000	297	95	3.2
Hydroxyapatite	46.9	99,800	2,130	85	22.7
Mono Q (Pi gradient)	16.7	92,100	5,510	78	58.8
Mono Q (NaCl gradient)	13.4	84,400	6,280	72	67.0

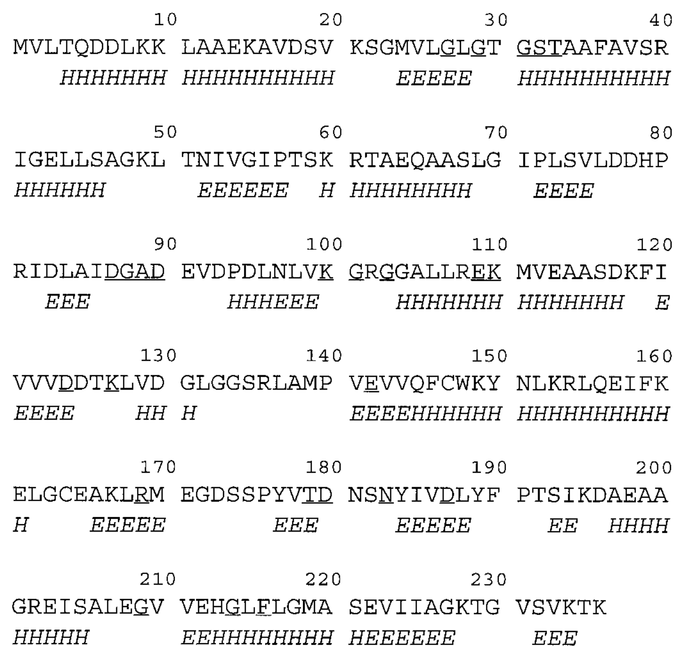

FIG. 4. Tentative secondary structure of spinach RPI. The predicted secondary structure of RPI is based on algorithms of Rost and Sander (39). The putative helical regions are marked as *H* and the β-strands as *E*. The absolutely conserved residues are underlined.

almost as well as R5P, arabinose 5-phosphate is not isomerized. This demonstrates that the orientation of the hydrogen at the C-2 position is crucial for catalysis, as predicted by a single-base mechanism. Xylulose 5-phosphate, which binds to RPI only slightly better than phosphate itself, is also not isomerized, as examined by the absorbance at 290 nm (data not shown).

Site-directed mutagenesis. Although the three-dimensional structure of RPI has not been determined, sequence homology analysis identifies only 11 invariant acid–base side chains as potential candidates for catalytic roles (Fig. 1): D87, D90, K100, E109, K110, D124, K127, E142, R169, D180, and D187. Furthermore, only three segments encompass contiguous, conserved sequences: G27–T33, D87–D90, and K100–G103. Although not definitive, the secondary structure prediction (Fig. 4), based on algorithms of Rost and Sander (39), is consistent with a β/α-barrel folding motif for RPI as frequently observed for isomerases, epimerases, and racemases (40–42). If this is indeed the case, D87, D90, and K100 appear to be located at the carboxyl end of β-strands, as typifies active-site residues in TIM and other β/α-barrel proteins (40, 41, 43). Based on these considerations, we probed the potential functionality of D87, D90, K100, and E91 by site-directed mutagenesis. E91 was included because of its adjacency to a segment containing invariant DGAD residues, its presence in all but one species (*A. fulgidus*) sequenced to date (Fig. 1), and the utilization of glutamyl residues as proton-abstracting bases by TIM and phosphoglucose isomerase (44–46).

The D87A mutant shows greatly diminished activity with a k_{cat} of 0.042 s^{-1} (0.0012% of wild-type) (Table II). No significant change in K_m is observed, indicating a direct or indirect role of the targeted carboxyl group in catalysis. D87N and D87E mutants were also constructed, but the respective *E. coli* transformants grew poorly, and the expression levels were only 1/10th of those for wild-type and D87A RPI, as assessed by Western blotting analysis. However, based on analysis of *E. coli* crude extracts, D87N and D87E appeared as inactive as D87A, although precise comparisons were precluded. Likewise, the activity of D90A is greatly impaired (0.38% of wild-type) without significant change in K_m. Thus, D90 facilitates catalysis but to a considerably lesser extent than D87. In contrast to D87A and D90A, E91A retains substantial activity, thereby excluding any catalytic role for E91.

The K100A mutant also shows a greatly diminished k_{cat} of 2.54 s^{-1} (0.074% of wild-type) but a sevenfold increase in the K_m for R5P. Ethylamine slightly enhanced the activity of the K100A mutant with an apparent K_d (ethylamine) of 70 mM and a maximal k_{cat} in the presence of saturating ethylamine of 10.7 s^{-1}. Numerous amines such as ammonia, butylamine, propylamine, trifluoroethylamine, methylamine, and *t*-methylamine were also examined but a thorough study was not undertaken, because these amines strongly interfere with the coupling enzymes. Except for *t*-methylamine, all of these amines slightly enhanced K100A activity (data not shown).

When assayed in the reverse direction, i.e., Ru5P to R5P, D87A, E91A, and K100A showed about the same degree of impairment in k_{cat} as was determined in the forward direction. However, the k_{cat} of D90A in the reverse direction was 3% of wild-type compared to only 0.4% in the forward direction (data not shown).

DISCUSSION

Because an efficient heterologous overexpression of the RPI gene from any source has not been reported heretofore, an immediate imperative was to authenti-

TABLE II

Kinetic Constants of Wild-Type and Mutant RPIs[a]

	K_m (R5P) (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Wild Type	0.63 ± 0.048	3440 ± 60	5.46×10^6
D87A	0.56 ± 0.011	0.0421 ± 0.0002	7.58×10^1
D90A	0.52 ± 0.025	13.2 ± 0.2	2.52×10^4
E91A	1.17 ± 0.06	946 ± 17	8.10×10^5
K100A	4.41 ± 0.52	2.54 ± 0.52	5.75×10^2

[a] Averages of three determinations.

cate the purified recombinant spinach enzyme. Although the design of our expression vector introduced a slight change at the N-terminus of the encoded RPI subunit (MVLT vs PTPVLT for the authentic spinach enzyme (13)), the purified recombinant enzyme is nevertheless a homodimer akin to the enzyme isolated from spinach (11). Furthermore, the k_{cat} of the recombinant RPI actually exceeds that of preparations from spinach by 70–80% (11, 34). The K_m for R5P of 0.63 mM that we observe agrees well with an earlier reported value of 0.46 mM (11). Other literature values that are five- to eightfold higher are likely due to differences in assay conditions (33–35). In one of these prior cases (35), the assay included 40 mM Pi, which we show competitively inhibits RPI with a K_i of 7.9 mM. Although an apparent K_i of 50 mM was approximated in a previous study (11), the single fixed concentration of 2.5 mM R5P (five times K_m) that was used would have masked inhibition by Pi. Thus, based on subunit structure and catalytic parameters, we conclude that the recombinant spinach RPI is a valid surrogate for the authentic enzyme for mechanistic and structural studies.

The 1,2-proton transfer catalyzed by RPI presumably proceeds via a single-base mechanism and entails a *cis*-enediol(ate) intermediate (47, 48). Such a mechanism is supported by the relative stereochemistry of the α-proton of substrate and product subject to abstraction, by the observed exchange of these α-protons with solvent protons during enzyme turnover, and by analogy with rigorously characterized aldose–ketose isomerases such as TIM. Apart from the acid–base group that actually shuttles the proton, another active-site feature common to isomerases, epimerases, and racemases is the presence of a general acid to polarize the substrate carbonyl and thereby facilitate α-proton abstraction. Because of the absence of crystallographic or chemical modification studies of RPI, the identities of this enzyme's general base and general acid have not been established. In fact, any information about the residues that constitute the active site of RPI is glaringly lacking. Consequently, we were prompted to take advantage of our expression system and pursue site-directed mutagenesis as an avenue for active-site characterization of RPI.

In consideration of the prevalence of enzymes that abstract and transfer α-protons of carbon acids, we anticipated that searches of sequence data bases would uncover regions of sequence similarities between RPI and other mechanistically related enzymes, thereby providing candidate residues for mutagenesis. These expectations, however, were not realized, so we turned our attention to sequence comparisons among RPIs from diverse sources. Based on residue characteristics of species invariance, location within a segment of polypeptide chain with a preponderance of conserved residues, and presence of an ionizable side chain, we targeted D87, D90, E91, and K100 for substitution.

The retention of substantial catalytic activity by E91A (~30% of the wild-type level), in conjunction with only a slightly elevated K_m for R5P, excludes the possibility of E91 serving any significant role in catalysis or substrate binding. In vivid contrast, substitution of D87, D90, or K100 by an alanyl residue severely impairs the wild-type k_{cat} with reductions of approximately 10^5-, 10^2-, and 10^3-fold, respectively. The slight activity of D87A (0.0012% of wild-type) could be falsely high due to contamination by wild-type *E. coli* RPI or due to a translational error giving rise to wild-type spinach RPI. Contamination by the wild-type bacterial enzyme does not seem very likely, given the wide separation of recombinant spinach and *E. coli* RPIs on the hydroxyapatite (the former is bound while the latter emerges unretarded) and both mono Q columns used for purification. However, the apparent activity of D87A is only severalfold greater than would result from the typical frequency of translational errors in *E. coli* (49).

The K_m of D87A and D90A for R5P is virtually identical relative to that of wild-type enzyme and increased a moderate sevenfold in the case of K100A. Thus, the catalytic debilitation of the three mutants does not appear to be a consequence of major conformational perturbations but rather the absence of a side chain critical to catalysis. We conclude that the segment of the spinach RPI polypeptide that encompasses D87, D90, and K100 constitutes a portion of the active site.

Based on analogies to mechanistically similar enzymes, the 10^5-fold rate enhancement provided by D87 of RPI renders this residue a credible candidate for the general base or electrophile required for substrate enolization and subsequent proton transfer. For example, the general bases of TIM (Glu165) and 3-ketosteroid isomerase (Asp38) accelerate catalytic rates by $\sim 10^6$-fold and $>10^5$-fold, respectively (50, 51). Phosphoglucose isomerase also relies on a carboxylate as the general base (46, 52), but its quantitative contribution to catalysis has not been assessed. The electrophiles of TIM and 3-ketosteroid isomerase have also been identified and probed by site-directed mutagenesis. In TIM, His95 serves to polarize the substrate carbonyl and accelerates catalysis by $\sim 10^4$-fold (50). The role of electrophile in 3-ketosteroid isomerase is shared by Tyr14 and Asp99; the phenolic hydroxyl contributes $>10^4$-fold to overall rate enhancement (51), whereas the β-carboxylate (which may hydrogen bond with Tyr14 rather than directly with substrate carbonyl) (53) contributes $>10^3$-fold (54). Given these considerations, the comparatively lesser impacts of alanyl substitution of D90 and K100 in RPI argue against either of these residues serving directly as the general base or electrophile.

As a potential avenue for probing the role of K100, we screened numerous amines for their ability to chemically rescue the activity of K100A. Although this novel approach proved instructive when applied to the active-site mutant K258A of aspartate aminotransferase in pioneering studies of Toney and Kirsch (55), the extent of rescue of the isomerase mutant was so meager that in-depth structure–activity correlations were precluded.

The free carbonyl forms of R5P and Ru5P must be the true substrates for enolization and interconversion by RPI. The solution structure of Ru5P does not impose a constraint in this regard, because the equilibrium concentration of the ketone exceeds that of the hydrate by >9:1 (56). In contrast, R5P exists predominantly as cyclic furanoses (64% β and 34% α), with the acyclic hydrated and free aldehyde forms represented at only 0.5 and 0.1% of the total concentration, respectively (57). Thus, in the direction of Ru5P formation, questions arise as to whether the enzyme binds the furanose forms and catalyzes ring opening. Although some aldose–ketose isomerases (e.g., phosphoglucose isomerase, D-xylose isomerase, and L-arabinose isomerase) have been shown to catalyze ring opening and to display anomeric preference (58, 59), these issues have not been broached with RPI. If only the free carbonyl form of R5P were recognized by the enzyme, the true K_m would become 0.63 μM rather than 0.63 mM as based on the total chemical concentration of R5P. Such a low K_m for the carbonyl form of R5P seems untenable in view of the much higher K_m of ~0.2 (our determination) to 0.8 mM (33) for the structurally similar acyclic Ru5P. Furthermore, a K_m of 0.63 μM would increase the k_{cat}/K_m to 5.5×10^9 M^{-1} s^{-1}, which exceeds the diffusion-controlled limit by about 10-fold (60). The conclusion that RPI binds at least one of the furanose forms thus appears inescapable.

The rapidity of spontaneous ring opening may obviate active intervention by the enzyme in this step. At pH 8.0 and 24°C, the first-order rate constants for spontaneous ring opening are 27 s^{-1} for the α-form and 15 s^{-1} for the β-form (57). As the velocity of ring opening can be expressed as $v = k_\alpha[R5P_\alpha] + k_\beta[R5P_\beta]$, $v = 38.4 \times 10^{-3}$ M s^{-1} at 2 mM R5P. For the overall enzyme-catalyzed reaction at 2 mM R5P, $v = 0.76\ V_{max}$ or $0.76\ k_{cat}[E_T]$, which equals only 1.04×10^{-6} M^{-1} s^{-1} at 10 ng/ml (0.4 nM subunit) of RPI as typically used in assays or 3.7×10^4-fold slower than the velocity of spontaneous ring opening. Even at the much higher concentrations of RPI estimated to be present in chloroplasts ~0.5 mg/ml (20 μM subunit),[7] spontaneous ring opening would still not become cleanly rate limiting in overall isomerization.

In-depth mechanistic insights regarding wild-type RPI must be gleaned in order to ascertain whether the enzyme actually catalyzes ring opening of furanose R5P and, if so, whether any of the active-site residues uncovered in this study play a role in this process.

[7] This estimate is based on the known chloroplastic concentration of D-ribulose-1,5-bisphosphate carboxylase/oxygenase of 4 mM (61) and the relative amounts of the carboxylase/oxygenase (62) and the isomerase (11) proteins extracted from spinach leaves.

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