Phosphoprotein with Phosphoglycerate Mutase Activity from the Archaeon Sulfolobus solfataricus

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When soluble extracts of the extreme acidothermophilic archaeon Sulfolobus solfataricus were incubated with [$\gamma$-32P]ATP, several proteins were radiolabeled. One of the more prominent of these, which migrated with a mass of ~46 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), was purified by column chromatography and SDS-PAGE and subjected to amino acid sequence analysis via both the Edman technique and mass spectroscopy. The best match to the partial sequence obtained was the potential polypeptide product of open reading frame sso0417, whose DNA-derived amino acid sequence displayed many features reminiscent of the 2,3-diphosphoglycerate-independent phosphoglycerate (PGA) mutases [iPGMs]. Open reading frame sso0417 was therefore cloned, and its protein product was expressed in Escherichia coli. Assays of its catalytic capabilities revealed that the protein was a moderately effective PGA mutase that also exhibited low levels of phosphohydrolase activity. PGA mutase activity was dependent upon the presence of divalent metal ions such as Co2+ or Mn2+. The recombinant protein underwent autophosphorylation when incubated with either [$\gamma$-32P]ATP or [$\gamma$-32P]GTP. The site of phosphorylation was identified as Ser59, which corresponds to the catalytically essential serine residue in bacterial and eucaryal iPGMs. The phosphoenzyme intermediate behaved in a chemically and kinetically competent manner. Incubation of the 32P-labeled phosphoenzyme with 3-PGA resulted in the disappearance of radioactive phosphate and the concomitant appearance of 32P-labeled PGA at rates comparable to those measured in steady-state assays of PGA mutase activity.

MATERIALS AND METHODS

Materials. [$\gamma$-32P]ATP, [$\alpha$-32P]ATP, and [$\gamma$-32P]GTP were purchased from NEN Research Products (Boston, Mass.). Matrix Gel Blue A and Matrix Gel Blue B were purchased from Amicon Corp. (Danvers, Mass.). Hydroxyapatite was from Bio-Rad (Richmond, Calif.). Enolase was obtained from Sigma (St. Louis, Mo.). Oligonucleotide primers were from Life Technologies, Inc. (Gaithersburg, Md.). Genomic DNA from S. solfataricus was obtained from the American Type Culture Collection. The Gene Editor in vitro site-directed mutagenesis system and sequencing grade trypsin were from Promega (Madison, Wis.). EK Max enterokinase was from Invitrogen (San Diego, Calif.). The Jupiter C18 (10 μm/300 A) reversed-phase high-pressure liquid chromatography (HPLC) resin was from Phenomenex, Inc. (Torrance, Calif.). 3-PGA was purchased as the tricyclohexylammonium salt from Roche Diagnostics GmbH (Mannheim, Germany). General laboratory reagents, molecular biology supplies, all other sugars, and microbial culture media were obtained from Fisher (Pittsburgh, Pa.) or Sigma.

Standard procedures. S. solfataricus P1 (ATCC 35091) was grown in continuous culture with vigorous aeration at 70°C in de Rosa’s standard medium (11) with the level of yeast extract increased to 2 g liter−1. Protein concentrations were determined as described by Bradford (3), using a preeminent reagent and a standardized solution of bovine serum albumin from Pierce (Rockford, Ill.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (29), and gels were stained with Coomassie brilliant blue as described by Fairbanks et al. (15). 32P-labeled phosphoproteins were visualized in SDS-polyacrylamide gels by electronic autoradiography using a Packard (Meriden, Conn.) InstantImager.

Partial purification of the ~46-kDa phosphoprotein. Soluble extract was prepared from a 20-g (wet weight) sample of S. solfataricus and incubated with [$\gamma$-32P]ATP for 30 min as described by Solow et al. (41). The radiolabeled extract was then applied to a 3-by-50-cm column of DE-52 cellulose that had been equilibrated in 20 mM (N-morpholino)-ethanesulfonic acid (MES; pH 6.5) containing 50 mM NaCl and 0.5 mM EDTA. The column was washed with this same buffer and then developed with a 400-mM linear gradient of 50 to 500 mM NaCl.

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in 20 mM MES (pH 6.5) containing 0.5 mM EDTA. Fractions, 2 ml each, were collected and analyzed by SDS-PAGE, followed by autoradiography. Those containing the ~46-kDa phosphoprotein were pooled, dialyzed versus 4 liters of 20 mM MES (pH 6.5) containing 0.5 mM EDTA, and applied to a 2 by-25-cm column of Mono Qulfic acid (Pharmacia) in 10 mM Tris-HCl, pH 7.5. The column was washed in equilibration buffer (5 volumes) and then developed with a 60-mL linear gradient of 0 to 500 mM NaCl in 20 mM MES (pH 6.5) containing 0.5 mM EDTA. Fractions (2 ml) were collected and analyzed by SDS-PAGE, followed by autoradiography.

Fractions containing the ~46-kDa phosphoprotein were pooled, dialyzed versus 40 mM sodium phosphate (pH 6.8), and applied to a 1 by-9-cm column of hydroxyapatite that had been equilibrated in the same buffer. The column was washed with equilibration buffer and developed with a 60-mL linear gradient of 10 to 200 mM sodium phosphate (pH 6.8). Fractions were collected and analyzed as described above. Those containing the ~46-kDa phosphoprotein were pooled and dialyzed versus 20 mM MES (pH 6.5) containing 0.5 mM EDTA. The dialyzed material was applied to a 1 by-8-cm column of Matrix Gel Blue B that had been equilibrated in dialysis buffer. The column was washed with 20 mM MES (pH 6.5) containing 0.5 mM EDTA. Bound proteins were eluted with 20 mM MES (pH 6.5) containing 0.5 mM EDTA and 500 mM NaCl.

Partial sequence analysis of the ~46-kDa phosphoprotein. For Edman sequencing, a sample of the ~46-kDa phosphoprotein was treated with 8 M urea and V8 protease, and the resulting peptides were resolved on a high-resolution SDS-polyacrylamide gel according to established procedures (31). The peptides were electroblotted onto an Immobilon-P membrane, and the most visually prominent peptide subjected to 20 cycles of Edman sequencing at the Protein Chemistry Facility of the W. Alton Jones Cell Science Center in Lake Placid, N.Y., for sequence analysis by mass spectrometry, a 4.2-μg portion of partially purified ~46-kDa phosphoprotein was applied to a 10% (wt/vol) SDS-polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie blue, and the section of the gel containing the ~46-kDa phosphoprotein was excised and sent to the W. M. Keck Biomedical Mass Spectrometry Laboratory at the University of Virginia for analysis.

Cloning of sso0417 and expression of its recombinant protein product, rSso0417. ORF sso0417 was amplified from genomic DNA from S. solfataricus and cloned into expression vector pRSET-C, which had been cut with EcoRI and BglII, by standard procedures (39). The primers used for PCR amplification were 5′-GAAGATCTCCTGGGTTAGTTGGAAGC-3′ (forward primer) and 5′-GAGAGTATTTCTCTG-3′. To alter Ser59 to Thr, we used following primer 5′-TAATTCCTGGGGCTGATACTTCAC-3′. To alter Ser59 to Ala, the following primer was used: 5′-ATC-3′. The peptides were electroblotted onto an Immobilon-P membrane, and the most phosphorylated as described above were incubated for 15 min at 65°C with [32P]ATP used was not radiolabeled. A 10-μl portion of the autophosphorylated protein was applied to a SDS-polyacrylamide gel, and the gel stained with Coomassie blue after electrophoresis. The gel was destained by incubating with several changes of 25 mM ammonium bicarbonate containing 5 mM (vol/vol) acetonitrile. The section of the gel containing the protein was excised, incubated in 50 mM ammonium bicarbonate (pH 8.0) containing 10 mM DTT, and then soaked in a solution of 55 mM iodoacetamide in 50 mM ammonium bicarbonate (pH 8.0). The gel slice was rinsed several times with ammonium bicarbonate (pH 8.0) and then soaked in a solution of 10 mM HEPES (pH 7.5) containing 5 mM MnCl2. A portion of the incubation mixture (20 μl) was then spotted, 5 μl at a time, 2 cm from the bottom of a 20 by 24 cm (width by height) sheet of Whatman no. 1 filter paper. A second 20-μl portion was spotted coincident with 1 μl of 100 mM 2,3-diphosphoglycerate (2,3-dPG) that was dissolved in 10 mM HEPES (pH 7.5). Other lanes contained PGA and the 2,3-dPG standards alone. The filter paper was developed until the solvent front was 20 cm from the origin with solvent system I, and the positions of the PGA and diPGA standards were determined as described by Jacobs and Grissolo (25). Radiolabeled species were detected by electronic autoradiography.

Identification of [32P]PGA by paper chromatography. Autophosphorylated rSso0417 (~0.4 μg that contained 3.6 pmol of [32P]PGA was incubated with 15 mM 3-PGA for 5 min at 25°C in 50 μl of 25 mM HEPES (pH 7.5) containing 5 mM MnCl2. A portion of the incubation mixture (20 μl) was then spotted, 5 μl at a time, 2 cm from the bottom of a 20 by 24 cm (width by height) sheet of Whatman no. 1 filter paper. A second 20-μl portion was spotted coincident with 1 μl of 100 mM 2,3-diphosphoglycerate (2,3-diPGA) that was dissolved in 10 mM HEPES (pH 7.5). Other lanes contained PGA and the 2,3-diPGA standards alone. The filter paper was developed until the solvent front was 20 cm from the origin with solvent system I, and the positions of the PGA and diPGA standards were determined as described by Jacobs and Grissolo (25).

Identification of the phosphorylation site on rSso0417 by mass spectrometry. rSso0417 (20 μg) was autophosphorylated as described above except that the ATP used was not radiolabeled. A 10-μg portion of the autophosphorylated protein was applied to an SDS-polyacrylamide gel, and the gel stained with Coomassie blue after electrophoresis. The gel was destained by incubating with several changes of 25 mM ammonium bicarbonate containing 50% (vol/vol) acetonitrile. The section of the gel containing the protein was excised, incubated in 50 mM ammonium bicarbonate (pH 8.0) containing 10 mM DTT, and then soaked in a solution of 55 mM iodoacetamide in 50 mM ammonium bicarbonate (pH 8.0). The gel slice was rinsed several times with ammonium bicarbonate (pH 8.0) and then soaked in a solution of 10 mM HEPES (pH 7.5). The gel slice was rehydrated for 15 min in a solution of 0.02 mg of TPCK (tosylphenylalanyl chloride)-treated trypsin/ml and then transferred to a tube containing 25 μl of 25 mM ammonium bicarbonate (pH 8.0). The mixture was incubated for 8 h at 37°C. Trifluoroacetic acid (5 μl of a 5% (vol/vol) solution) was added, and the mixture was immediately agitated with a Vortex mixer to terminate proteolysis. The supernatant liquid was removed and stored at −20°C until needed.

For liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS-MS), 1-μl portions of the peptide mixture were applied to a 75-μm capillary column packed with Jupiter C18 (10 μm/300 A) resin to a bed length of 10 cm. Prior to loading, the column was equilibrated in 0.5% (vol/vol) acetic acid (HAc) at a flow rate of 50 μl/min using HPLC pump Ultimate 3000. The column was sequentially pulled by using a microtorch to eliminate the need for a frit. The column was developed with 0.5% (vol/vol) HOAc containing 5% (vol/vol) acetonitrile for 5 min, fol-
lowed by a linear gradient of 5 to 80% (vol/vol) acetonitrile in 0.5% (vol/vol) HOAc for 40 min. A low dead volume stainless steel tee was used to split the eluant and divert a 250-nl/min portion to the mass spectrometer. Mass spectra were acquired on a ThermoFinnigan LCQ DecaXP quadrupole ion trap mass spectrometer. The electrospray voltage was 2.3 kV, the capillary temperature was 150°C, and the capillary voltage was 10 V. Three microscans (~1.5 s each) were acquired for each spectrum recorded. The threshold for tandem mass spectrum acquisition was set at 5E6, and precursor ions were dynamically excluded for 5 min. Peptides were identified by using the SEQUEST algorithm searching an in-house FASTA-formated database for the S. solfataricus P2 genome, using a dynamic modification (80.0 atomic mass units [amu] on S, T, and Y residues and a static modification (carbamidomethylation, 58.03 amu) on cysteine residues.

RESULTS

Partial amino acid sequence of the ~46-kDa phosphoprotein of S. solfataricus. When a soluble extract of the archaeon S. solfataricus was incubated with [γ-32P]ATP, numerous polypeptides were observed to incorporate radiolabeled phosphate. Among the more visually prominent of these was a polypeptide with an apparent mass of ~46-kDa that contained [32P]phosphoserine (41). The large number of polypeptides present in the extract rendered the unambiguous identification of the Coomassie stained band corresponding to the ~46-kDa phosphoprotein problematic. Therefore, the phosphoprotein was partially purified by column chromatography on DE-52 cellulose, Matrix Gel Blue A, hydroxyapatite, and Matrix Gel Blue B as described in Materials and Methods. The resulting protein mixture was sufficiently simplified in its polypeptide composition to permit the ~46-kDa phosphoprotein to be identified on an SDS-polyacrylamide gel.

Two methods were employed in an effort to obtain amino acid sequence information from the ~46-kDa phosphoprotein. First, a sample was subjected to partial proteolysis with S. aureus V8 protease. The resulting peptides were resolved from one another by SDS-PAGE on a high-osmolarity gel and transferred to a polyvinyliden fluoride membrane. After being stained, the most visually prominent polypeptide was sent to the Protein Chemistry Facility of the W. Alton Jones Cell Science Center in Lake Placid, N.Y., for Edman sequence analysis. Although the low yields of phenylthiohydantoin (PTH) amino acids obtained rendered their identification difficult, a partial sequence was obtained (Table 1). In order to obtain further sequence information, a sample of the ~46-kDa phosphoprotein was sent to the W. M. Keck Biomedical Mass Spectrometry Laboratory at the University of Virginia for analysis by MS. Here, the protein was reduced, alkylated, and proteolyzed with trypsin, and the resulting peptides were separated by reversed-phase HPLC and sequenced by MS-MS using collisionally induced dissociation (CID) to induce peptide fragmentation. This analysis yielded complete or partial sequence information from 21 peptides (Table 1).

The ~46-kDa phosphoprotein is encoded by ORF sso0417. When the partial sequence obtained via Edman analysis was searched against the genome of S. solfataricus, the best discernible match was to the N-terminal sequence of the predicted polypeptide product of ORF sso0417 (Table 1). The calculated molecular mass of the deduced protein, 45,179 Da, closely matched the mass empirically determined for the phosphoprotein by SDS-PAGE. Additional homology searches utilizing the 21 peptide sequences obtained by LC-MS-MS indicated that one-third, i.e., 7, displayed discernible similarity to portions of the predicted protein product of ORF sso0417 as well. However, none of the empirically determined sequences matched the potential product of ORF sso0417 in an exact, residue-for-residue basis, a potential consequence of errors and ambiguities in residue assignments arising from the low levels of sample available for these analyses.

In the face of the large number of peptides left unaccounted for, as well as the many discrepancies between the predicted protein sequence and those of the putative “matching” peptides, the proposition that the ~46-kDa phosphoprotein was the product of ORF sso0417 was tested further. ORF sso0417 was cloned, and its protein product was expressed as a recombinant fusion protein in E. coli. rSso0417, which contained an N-terminal hexahistidine sequence, was purified to apparent homogeneity by using metal chelate chromatography. When incubated with [γ-32P]ATP, the protein became phosphorylated on serine to a stoichiometry of roughly 0.5 mol/mol, strongly suggesting that sso0417 did indeed encode the ~46-kDa phosphoprotein observed in soluble extracts of S. solfataricus. Radiolabeling also was observed when [γ-32P]GTP was substituted for ATP. After SDS-PAGE, rSso0417 could be renatured and phosphorylated with [γ-32P]ATP as phosphoryl donor while still immobilized within the gel matrix, indicating that phosphate incorporation resulted from an autophosphorylation event (data not shown).

Autophosphorylation of recombinant Sso0417 takes place on Ser59 in vitro. rSso0417 was incubated with ATP, and the phosphorylated protein was isolated by SDS-PAGE. After incubation with trypsin, the resulting peptide mixture was analyzed by LC-MS-MS. Thanks in part to the large quantity of recombinant protein available for analysis, tryptic peptides encompassing 94% of the DNA-derived amino acid sequence encoded by ORF sso0417, including every predicted serine residue, were identified and sequenced. Computer analysis of the data obtained, by using parameters that accounted for the possible phosphorylation of all serine residues and neutral fragment (phosphate) loss, coupled with manual spectral inspection, indicated that only a single serine residue was phosphorylated. This serine residue was contained in a peptide spanning Asn42 through Arg76 (Fig. 2). Although the singly charged molecular ion [M + H+] + (3,881.4 amu) for this peptide was not detected, strong signals were obtained for three multiply charged ions: [M + 2H+] 2+ (1,941.6 amu), [M + 3H+] 3+ (1,294.8 amu), and [M + 4H+] 4+ (971.4 amu). CID of the multiply charged ions was used to determine which of the five serines within the 35-residue peptide was phosphorylated. Figure 1 shows one of the patterns obtained when the doubly charged peptide ion [M + 2H+] 2+ was fragmented by CID. The most abundant peak corresponds to a peptide that has undergone neutral loss of phosphate [M + 2H+] 2+ − H3PO4] 2+. Singly charged ions corresponding to fragments Asn42 to Ile65 [b15] and Thr61 to Arg76 [y16] are both visible, but not their potential phosphorylation products. On the other hand, doubly charged ions corresponding to the phosphorylated form of peptide fragment Pro46 to Arg76 [y27] as well as the corresponding peptide fragment that had undergone neutral loss of phosphate (y27 − H3PO4), were readily apparent. The only serine residue whose phosphorylation would be predicted to produce this fragmentation pattern, as well as
the patterns that were obtained from the triply and quadruply charged ions (data not shown), was Ser59.

The assignment deduced from mass spectroscopic analysis was verified by using site-directed mutagenesis. Substitution of Ser59 with Ala produced a protein that failed to undergo autophosphorylation when incubated with \([\gamma^32P]ATP\), whereas substitution of Ser 59 with Thr produced a protein that underwent autophosphorylation exclusively on threonine residues (data not shown).

rsso0417 exhibits PGA mutase activity. Although the predicted protein product of ORF rsso0417 was originally annotated as a potential phosphonopyruvate decarboxylase (38), homology searches indicated the existence of significant sequence homology with 2,3-diPGA-independent PGA mutases [iPGM] from Eucarya and Bacteria (Fig. 2). PGA mutases catalyze the net transfer of phosphate between the 3- and 2-hydroxyl groups of PGA (16, 27, 35). In addition to the iPGMs, a second, structurally distinct family of PGA mutases—the 2,3-diPGA-dependent PGA mutases [dPGMs]—are present in many members of the Bacteria and Eucarya.

The regions of sequence similarity between Sso0417 and prototypic iPGMs from Bacteria and Eucarya were not symmetrically distributed along the polypeptide chain. Rather, they were confined almost exclusively to domain A of the latter, which includes residues drawn from both the N- and C-terminal portions of the polypeptide. X-ray crystallographic analysis indicates that domain A contains the residues responsible for binding the two divalent metal ions that serve as catalytic cofactors for iPGMs, whereas domain B includes many of the residues responsible for binding the substrate or product, PGA (26). Domain A also contains a conserved serine residue that is essential for activity (26). It is generally believed

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**TABLE 1. Partial sequence analysis of ~46-kDa phosphoprotein**

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<th>Peptide no.</th>
<th>Mass (Da)</th>
<th>Sequence</th>
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<td>QFEAXGQR</td>
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<tr>
<td>2</td>
<td>1,650.4</td>
<td>AFEALGAGAq</td>
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<td>6</td>
<td>1,267.8</td>
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<tr>
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</tr>
<tr>
<td>8</td>
<td>865.0</td>
<td>??QY ?IL(L/p)FI(a/p) (f/d) G?K??p</td>
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</tbody>
</table>

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a The partially purified ~46-kDa phosphoprotein was extracted from an SDS-polyacrylamide gel and digested with S. aureus V8 protease. The resulting peptides were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane, and one of the more visually prominent bands was analyzed by the Edman method. Shown is the sequence obtained, and below it is the region of the protein product of ORF ss00417 with which it shares sequence similarity. Lowercase letters indicate tentative identifications, question marks indicate amino acid residues of undetermined identity, and dashes designate the presence of unknown numbers of unidentified amino acid residues. Parentheses enclose possible alternative assignments.

b The partially purified ~46-kDa phosphoprotein was extracted from an SDS-polyacrylamide gel, digested with trypsin, and analyzed by LC-MS and LC-MS-MS by standard procedures at the W. M. Keck Mass Spectrometry Laboratory at the University of Virginia. Shown are the masses of the peptides obtained, as well as the amino acid sequences deduced from their patterns of fragmentation. Below peptides 1 to 7 are shown the regions of the protein product of ORF ss00417 with which they share sequence similarity. “X” indicates the presence of I or L, which cannot be distinguished on the basis of mass.
that the catalytic mechanism of the iPGMs involves the formation of a phosphoenzyme intermediate (2, 4, 26, 27), with this serine residue constituting the most likely site of modification (9, 26). Intriguingly, not only does Sso0417 contain a plausible candidate for this conserved serine residue, it was the same residue that was autophosphorylated when rSso0417 was incubated with ATP in vitro, i.e., Ser59.

The presence in the DNA-derived amino acid sequence of Sso0417 of plausible candidates for each of the metal ion binding residues conserved among the iPGMs, as well as the catalytically essential serine residue, suggested that it might also be a phosphomutase. Therefore, rSso0417 was assayed for PGA mutase activity by using a coupled enzyme assay system that utilizes enolase to convert the reaction product, 2-PGA, to phosphoenolpyruvate, which absorbs UV light at 240 nm. As can be seen in Fig. 3, rSso0417 exhibited PGA mutase activity when incubated with the components of this coupled assay system. As has been observed for prototypic iPGMs (7, 9, 28), the PGA mutase activity of rSso0417 was dependent upon the presence of exogenous metal ions such as Co$^{2+}$ or Mn$^{2+}$. The rate of phosphoenolpyruvate production remained constant when the concentration of enolase was doubled, indicating that, under these conditions, the conversion of 3-PGA to 2-PGA by rSso0417 was rate limiting. Activity was maximal at around pH 7.5.

The measured rate of reaction in the presence of Co$^{2+}$, i.e., 0.515 μmol/min/mg, was approximately three times that observed with Mn$^{2+}$ (0.182 μmol/min/mg), which in turn was a more effective activator than Cd$^{2+}$ (Table 2). Removal of the N-terminal fusion domain via proteolytic cleavage with enterokinase had no discernible effect on catalytic activity (data not shown). Substitution of the presumed catalytic Ser, Ser$_{59}$, with Ala eliminated the PGA mutase activity of rSso0417, while its replacement by Thr restored catalytic function (Fig. 3). The addition of exogenous 2,3-diPGA had no discernible effect on enzyme activity.

What is the fate of the enzyme-bound phosphoryl group?
The catalytic mechanism of the iPGMs remains a subject of controversy. The most widely accepted reaction sequence involves the transfer of a phosphoryl group from 3-PGA (or 2-PGA) to the enzyme’s catalytically essential serine residue, forming glycerate and a phosphoenzyme intermediate, followed by the subsequent rephosphorylation of the tightly bound glycerate to form 2-PGA (or 3-PGA) (reviewed in reference 27). This double-displacement mechanism readily accounts for three observations concerning prototypic iPGMs. First, catalysis does not require an exogenous cofactor such as 2,3-diPGA. Second, phosphoryl transfer is intramolecular, i.e., the phosphate group bound to the carbon backbone of the product is the same one that was bound to the backbone of the corresponding substrate molecule (4, 19). Third, phospho-transfer occurred with net retention of configuration by the chiral phosphoryl group of 2-[($R$)$_{2}$]$^{18}$O$_{2}$PGA in vitro (2). On the other hand, repeated attempts to detect the presumed intermediates in the iPGM reaction sequence, glycerate and the phosphoenzyme, have met with little success (4, 5, 9, 22, 24). Moreover, even when minute quantities (<0.05 mol/mol) of the presumed phosphoenzyme intermediate of an iPGM from *Trypanosoma brucei* were detected, the rate at which the phosphoryl group turned over was much too low (t$_{1/2}$ ~ 40 s) to be considered kinetically competent (9).

If autophosphorylated rSso0417 corresponds to the postulated phosphoenzyme intermediate of prototypic iPGMs from the *Bacteria* and *Eucarya*, then incubation with its cognate
reaction intermediate, glycerate, should result in completion of the catalytic cycle with the concomitant displacement of $[^{32}P]$phosphate from the enzyme. However, even when rSso0417 that had been autophosphorylated with $[^32P]ATP$ was incubated for a prolonged period (15 min) with excess glycerate, a significant portion of the $[^{32}P]$phosphate remained bound to the enzyme (Table 3). In contrast, incubation under similar conditions with equal or lower concentrations of 3-PGA resulted in the near-complete disappearance of protein-bound $[^{32}P]$phosphate. Several other phosphorylated sugars, including 2-PGA, $[^32P]$glycerol phosphate, erythrose-4-phosphate, and ribulose-5-phosphate, also proved noticeably more effective than glycerate in their ability to chase protein-bound radioactivity from autophosphorylated rSso0417 (Table 3). However, none was as effective as 3-PGA. These observations suggested either that rSso0417 is not a PGA mutase or that the FIG. 2. Comparison of DNA-derived amino acid sequence of Sso0417 with archaeal, bacterial, and eukaryotic iPGMs. Shown is the DNA-derived amino acid sequence of the protein product of ORF sso0417 from S. solfataricus (Sso0417 [38], GenBank accession no. Q980A0) and, immediately below it, the sequence of iPGMs from the archaean P. furiosus (Pfs iPGM [42]; GenBank accession no. AAL82083), the eukaryote T. brucei (Tbr iPGM [8]; GenBank accession no. CAB85498), and the bacterium B. stearothermophilus (Bst iPGM [6]; GenBank accession no. AF120091). The sequences were aligned by using the CLUSTALW program available from the European Bioinformatics Institute, with the C-terminal ca. 60 residues adjusted by eye. Amino acid identities between each of the established iPGMs and Sso0417 are indicated by colons, whereas nonidentical amino acids of very similar character are indicated by periods. Amino acids comprising subdomain A of iPGM from B. stearothermohpilus are underlined. Amino acids involved in metal ion binding in iPGM are marked by asterisks. Other amino acid residues that are conserved among the subdomain B regions of bacterial and eucaryal iPGMs are indicated by plus signs. The catalytically essential serine residue of the iPGM from B. steaothermophilus is marked by ‘$@$’.
Materials and Methods.

All metal ions were present at a concentration of 5 mM. For further details, see more readily detectable levels. Shown are the results of triplicate determinations.

Phosphatase assays was raised to 65 °C to increase reaction rates to phosphohydrolase assays was raised to 65 °C by the stability of the coupling enzyme, enolase. The temperature of the phosphohydrolase activity toward both 3-PGA and ATP. However, the rate at which it catalyzed their hydrolysis fell well below that at which it converted 3-PGA to 2-PGA. In addition, when autophosphorylated rSs0417 was incubated with 3-PGA, <20% of the 32P radioactivity that was displaced from the protein was recovered as inorganic phosphate, indicating that the majority was transferred to 3-PGA or some other solute and not water.

We next sought to determine whether Ss0417 was a kinase. Certainly, the protein’s autophosphorylation in the presence of ATP constitutes strong circumstantial evidence for such a function. However, when rSs0417 was incubated with [γ-32P]ATP and either 3-PGA or any one of a variety of other sugars, including glycerate, 2-PGA, glycerol, β-glycerol phosphate, erythrose-4-phosphate, or ribulose-5-phosphate, neither [α-32P]ADP nor [α-32P]AMP was produced in significant quantities. Specifically, under conditions in which rSs0417 catalyzed the production of ≈10 nmol of 2-PGA, <0.1 nmol of [α-32P]AMP was detected (data not shown).

The second predicted product of a potential PGA kinase reaction is 2,3-diPGA. However, analysis by paper chromatography—which separates monophosphorylated glycerates from 2-PGA (data not shown). Thus, under these circumstances autophosphorylated rSs0417 behaved more like a dPGM, with ATP serving as cofactor instead of 2,3-diPGA.

As with prototypic iPGMs, dPGMs utilize a double-displacement mechanism involving a phosophoenzyme intermediate (reviewed in reference 16). However, they must first be primed for catalysis via prior autophosphorylation of an active-site histidine residue by using the cofactor 2,3-diPGA as phosphohydrolase. Upon binding 3-PGA (or 2-PGA), the now catalytically

### TABLE 2. Catalytic activity of rSs0417

<table>
<thead>
<tr>
<th>Activity type (enzyme)</th>
<th>Substrate</th>
<th>Conc (mM)</th>
<th>Cofactor</th>
<th>Temp (°C)</th>
<th>Mean reaction rate (μmol/min/mg) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatase</td>
<td>3-PGA</td>
<td>15</td>
<td>Mn2⁺</td>
<td>25</td>
<td>0.182 ± 0.0045</td>
</tr>
<tr>
<td></td>
<td>3-PGA</td>
<td>15</td>
<td>Co2⁺</td>
<td>25</td>
<td>0.515 ± 0.032</td>
</tr>
<tr>
<td></td>
<td>3-PGA</td>
<td>15</td>
<td>Cd2⁺</td>
<td>25</td>
<td>0.113 ± 0.018</td>
</tr>
<tr>
<td></td>
<td>3-PGA</td>
<td>15</td>
<td>Mn2⁺ + Mg2⁺</td>
<td>25</td>
<td>0.165 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>2.5</td>
<td>Mn2⁺</td>
<td>65</td>
<td>0.017 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>2.5</td>
<td>Co2⁺</td>
<td>65</td>
<td>0.050 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>2.5</td>
<td>Mg2⁺</td>
<td>65</td>
<td>0.018 ± 0.009</td>
</tr>
</tbody>
</table>

*Shown are the results of steady-state analyses of the rates at which rSs0417, 40 μg per assay, catalyzed conversion of 3- to 2-PGA or the hydrolysis of 3-PGA and ATP. The temperatures of the assays of PGA mutase activity were dictated by the stability of the coupling enzyme, enolase. The temperature of the phosphohydrolase assays was raised to 65°C in order to increase reaction rates to more readily detectable levels. Shown are the results of triplicate determinations. All metal ions were present at a concentration of 5 mM. For further details, see Materials and Methods.

### TABLE 3. Substrate specificity of rSs0417 PGA mutase

<table>
<thead>
<tr>
<th>Potential substrate</th>
<th>0.01 mM</th>
<th>0.02 mM</th>
<th>0.04 mM</th>
<th>0.1 mM</th>
<th>1 mM</th>
<th>10 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-PGA</td>
<td>17</td>
<td>17</td>
<td>15</td>
<td>11</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>2-PGA</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Glyceraldehyde</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>69</td>
<td>34</td>
<td>24</td>
</tr>
<tr>
<td>β-Glycerolphosphate</td>
<td>51</td>
<td>–</td>
<td>–</td>
<td>37</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Erthyrose-4-phosphate</td>
<td>91</td>
<td>78</td>
<td>59</td>
<td>23</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ribulose-5-phosphate</td>
<td>57</td>
<td>40</td>
<td>29</td>
<td>17</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Potential substrates were assayed for their ability to cause the disappearance of bound [32P]phosphate from the putative PGA mutase. Recombinant Ss0417 PGA mutase was incubated with [γ-32P]ATP, and the [32P]phosphorylated protein was isolated by gel filtration chromatography. Portions (5 μg, each containing ~0.5 mol of P, mol) were incubated for 15 min at 65°C in 50 μl of 25 mM MOPS (pH 7.0) containing 5 mM MgCl2, 5 mM MnCl2, and one of the potential substrates listed at the indicated concentrations. Incubation was terminated by the addition of SDS sample buffer lacking DTT, followed by heating at 100°C for 5 min, followed by SDS-PAGE. Shown is the proportion of 32P radioactivity remaining bound to the protein relative to controls to which no sugar was added. A total of 70% or more of the radioactivity remained after incubation with the following compounds, each at a concentration of 0.1 mM: ribose-1-phosphate, ribose-5-phosphate, xylene-1-phosphate, glucose, glucose-1-phosphate, glucose-6-phosphate, fructose, fructose-1-phosphate, fructose-6-phosphate, mannose-1-phosphate, mannose-6-phosphate, ATP, or ADP. –. Not determined.

Phosphatases and ATP. The temperatures of the assays of PGA mutase activity were dictated by the stability of the coupling enzyme, enolase. The temperature of the phosphohydrolase assays was raised to 65°C in order to increase reaction rates to more readily detectable levels. Shown are the results of triplicate determinations. All metal ions were present at a concentration of 5 mM. For further details, see Materials and Methods.

![FIG. 3. rSs0417 possesses PGA mutase activity. The PGA mutase activity of both rSs0417 (●) and a mutationally altered form in which Ser202 was altered to Thr (○) was measured spectrophotometrically by using a coupled assay system that measures the increase in absorbance at 240 nm that takes place when 2-PGA is converted to phosphoenolpyruvate by enolase. Also shown are the changes in absorbance observed in control assays in which either the potential PGM (□), enolase (△), or 3-PGA (■) was omitted. All assays were performed in triplicate, with error bars indicating the magnitude of the standard error. For further details, see Materials and Methods.](https://jb.asm.org/content/jb/92/12/2118/F1.large.jpg)
TABLE 4. 3-PGA induces the rapid disappearance of radioactivity from autophosphorylated rSso0417.

<table>
<thead>
<tr>
<th>Incubation period</th>
<th>Addition</th>
<th>5 mM MnCl₂</th>
<th>15 mM 3-PGA</th>
<th>Amt of [³²P]phosphate remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 s</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>3.6 ± 0.1 109</td>
</tr>
<tr>
<td>10 s</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>3.7 ± 0.2 112</td>
</tr>
<tr>
<td>10 s</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>3.3 ± 0.2 100</td>
</tr>
<tr>
<td>10 s</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.8 ± 0.1 24</td>
</tr>
<tr>
<td>5 min</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.4 ± 0.1 12</td>
</tr>
</tbody>
</table>

* Portions, −0.4 μg each (7.5 pmol), of rSso0417 that had been autophosphorylated by using [γ⁻³²P]ATP were incubated at 25°C in a volume of 50 μl of 25 mM HEPES (pH 7.5) containing the compounds listed at the indicated final concentrations. The incubation was terminated by the addition of 500 μl of ice-cold acetone, and the denatured protein was collected by centrifugation at 10,000 × g for 3 min. The superantiput was decanted, and the pellet was resuspended in 25 mM Tris (pH 7.5) containing 0.5 mM EDTA. Then, 30 μl of SDS-PAGE sample buffer was immediately added and the mixture was heated for 5 min at 100°C. The mixture was applied to an SDS-gel. After, electrophoresis, the protein was then isolated by SDS-PAGE, and the amount of [³²P]phosphate remaining bound to the protein was determined by electronic autoradiography. Shown are the results of triplicate analyses.

The incubation was terminated by the addition of 500 μl of ice-cold acetone, and the denatured protein was collected by centrifugation at 10,000 × g for 3 min. The superantiput was decanted, and the pellet was resuspended in 25 mM Tris (pH 7.5) containing 0.5 mM EDTA. Then, 30 μl of SDS-PAGE sample buffer was immediately added and the mixture was heated for 5 min at 100°C. The mixture was applied to an SDS-gel. After, electrophoresis, the amount of [³²P]phosphate remaining bound to the protein was determined by electronic autoradiography. Shown are the results of triplicate analyses.

Competent phosphoenzyme transfers its phosphoryl group to the free hydroxyl group of the substrate to form 2,3-diPGA. This intermediate subsequently donates a phosphoryl group back to the active site histidine to produce 2-PGA (or 3-PGA) and regenerate the catalytically competent phosphoenzyme. Phosphoryl transfer by dPGMs is therefore intermolecular. The phosphoenzyme that is present on the 2-PGA (or 3-PGA) produced by a dPGM originates from the free phosphoenzyme, which acquired it either during the preceding catalytic cycle or from the priming cofactor 2,3-diPGA.

We next investigated whether transfer of the enzyme-bound phosphoryl group to PGA took place with sufficient rapidity to account for at which rSso0417 catalyzed the conversion of 3-PGA to 2-PGA in the steady state. Using Mn²⁺ as the cofactor, the ³²P radiolabel should disappear from rSso0417 with a half-life of ≤6 s under the conditions described in Table 2 if phosphotransfer was kinetically competent. In other words, ≤32% of the initial protein-bound [³²P]phosphate should remain after a 10-s incubation in the presence of Mn²⁺ and 3-PGA. As can be seen from Table 4, the experimentally determined figure was ≤24%.

As was observed in steady-state assay of PGA mutase activity, Mn²⁺ and Cd²⁺ proved to be effective cofactors for the 3-PGA-induced disappearance of [³²P]phosphate from autophosphorylated rSso0417, whereas Mg²⁺ proved relatively ineffective (Fig. 4). Assessing the role of Co²⁺ proved problematic, since this metal effectively supported the transfer of the enzyme-bound phosphoryl group to water when 3-PGA was not present. The same proved true for Ni²⁺ and Cu²⁺. Zn²⁺, on the other hand, facilitated substrate-dependent transfer much more readily than hydrolytic transfer to water, whereas Mg²⁺ had little discernible effect on phosphoenzyme hydrolysis.

**DISCUSSION**

Several lines of evidence indicate that the ~46-kDa phosphoprotein previously detected in soluble extracts of S. solfataricus was the protein product of ORF sso0417. First, whereas the deduced protein product of ORF sso0417 represented an imperfect and incomplete match to the amino acid sequence data generated from the ~46-kDa phosphoprotein, it plausibly accounted for a higher proportion of the sequence information obtained than any other component of the S. solfataricus genome. Second, the calculated mass of Sso0417, 45,179 Da, closely matched the estimated mass of the ~46-kDa phosphoprotein from S. solfataricus extracts. Third, both proteins became phosphorylated when incubated with [γ⁻³²P]ATP and did so on serine residues. The appearance of unaccounted-for peptides in the mass spectral analyses most likely reflected the presence of polypeptide contaminants in the sample. We suggest that the discrepancies noted between the empirically and genomically derived sequences for those peptides that displayed similarity to Sso0417 were the consequence of inaccuracies in amino acid assignments arising from the low levels of sample available for these analyses.

Although Sso0417 was originally annotated as a phosphopyruvate decarboxylase (38), our own homology searches indicated that it contained plausible candidates for virtually all of the mechanistically critical residues found in bacterial and eucaryal iPGMs. The iPGMs, in turn, are part of a larger family of metal-requiring phosphotransferases and phosphohydrolases that includes the alkaline phosphatases (reviewed in...
references 18 and 27). Enzyme assays indicated that rSso0417 displayed measurable levels of both activities but was a markedly more efficient PGA mutase than phosphohydrolase. As has proven to be the case for established iPGMs, the PGA mutase activity of rSso0417 was dependent upon the presence of divalent metal ions such as Mn$^{2+}$, Co$^{2+}$, or Cd$^{2+}$.

Portions of the amino acid sequence of this archaeal enzyme dramatically diverged from that of prototypical iPGMs from the Bacteria and Eucarya, specifically in the substrate-binding region, subdomain B. The specific activity of rSso0417, i.e., ~0.52 $\mu$mol/min/mg when Co$^{2+}$ was utilized as a cofactor, was also low relative to values reported for bacterial iPGMs. Even if one allows for the roughly 2$^\alpha$-fold increase in activity that would take place if the assays were conducted at the growth temperature of *S. solfataricus* (~75°C), the estimated specific activity of ~16 $\mu$mol/min/mg still falls an order of magnitude below that determined for prototypic iPGMs from *Bacillus megaterium* (568 $\mu$mol/min/mg [40]) and *Bacillus subtilis* (621 $\mu$mol/min/mg [43]). On the other hand, the estimated specific activity of rSso0417 falls near that reported for the iPGM from *T. brucei* (26 $\mu$mol/min/mg [9]).

As the present study neared completion, two proteins with PGA mutase activity were characterized from the archaeaen *Pyrococcus furiosus* and *Methanococcus jannaschii* (20, 42). Like Sso0417, the areas of similarity between these archaeal proteins and prototypical iPGMs from the Eucarya and Bacteria were restricted to domain A (Fig. 2). These similarities extended to the molecular masses of their component polypeptides (ca. 45 to 46 kDa) and their specific activities, an estimated 20 $\mu$mol/min/mg at 90°C for the iPGM from *P. furiosus* and an estimated 80 $\mu$mol/min/mg at 100°C for the iPGM from *M. jannaschii* (42). Although all three archaeal enzymes could utilize Mn$^{2+}$, Co$^{2+}$, or Zn$^{2+}$ as cofactors in vitro, only the iPGMs from *P. furiosus* and *M. jannaschii* utilized Mg$^{2+}$ as well.

The significance of Sso0417's most unusual property, the ability to autophosphorylate its catalytically essential serine residue using ATP as phosphorl donor, remains cryptic. The greater efficiency with which the phosphoenzyme transferred its phosphorl group to 3-PGA as opposed to glycera is exactly the reciprocal of what would be predicted based on the presumed mechanism of catalysis for eucaryal and bacterial iPGMs. Such behavior mirrors, at least superficially, that of dPGMs in which 3- and 2-PGA are interconverted by a previously phosphorylated enzyme via the transient formation of 2,3-diPGA. However, this sequence of events may represent an adventitious and physiologically irrelevant side reaction peculiar to the phosphoenzyme generated in the laboratory. It must be remembered that the autophosphorylated enzyme utilized in these studies was produced by incubation with ATP, not the presumed substrate 3-PGA. Nor did catalysis by rSso0417 in the coupled assay system require the addition of ATP, as would be expected if this nucleotide triphosphate functioned as a priming cofactor in vivo.

On the other hand, archaeal iPGMs differ significantly in sequence from their better-characterized bacterial and eucaryal counterparts. It is not unreasonable to suggest that these differences extend to the level of catalytic mechanism as well. Autophosphorylated rSso0417 behaved in an apparently chemically and kinetically competent manner as a PGA mutase. Moreover, the differences between the mechanisms of dPGMs and prototypic iPGMs are relatively small, as it is the order rather than the chemical nature of the individual events that vary. Further studies are needed to definitively determine the sequence of steps that take place within the active sites of Sso0417 and other archaeal iPGMs and whether these differ from those of bacterial and eucaryal iPGMs. However, the ability to produce reagent quantities of the phosphoencezyme in vitro using ATP will provide a potent tool for examining the catalytic mechanism of this metabolically critical enzyme family.

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REFERENCES


