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Characterization of a Thermostable Enzyme with Phosphomannomutase/Phosphoglucomutase Activities from the Hyperthermophilic Archaeon *Pyrococcus horikoshii* OT3

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The phosphomannomutase/phosphoglucomutase (PMM/PGM) enzyme catalyzes reversibly the intra-molecular phosphoryl interconverting reaction of mannose-6-phosphate and mannose-1-phosphate or glucose-6-phosphate and glucose-1-phosphate. Glucose-6-phosphate and glucose-1-phosphate are known to be utilized for energy metabolism and cell surface construction, respectively. PMM/PGM has been isolated from many microorganisms. By performing similarity searches using existing PMM/PGM sequences, the homologous ORFs PH0923 and PH1210 were identified from the genomic data of *Pyrococcus horikoshii* OT3. Since PH0923 appears to be part of an operon consisting of four carbohydrate metabolic enzymes, PH0923 was selected as the first target for the investigation of PMM/PGM activity in *P. horikoshii* OT3. The coding region of PH0923 was cloned and the purified recombinant protein was utilized for an examination of its biochemical properties. The enzyme retained half its initial activity after treatment at 95°C for 90 min. Detailed analyses of activities showed that this protein is capable of utilizing a variety of metal ions that are not utilized by previously characterized PMM/PGM proteins. A mutated protein with an alanine residue replacing the active site serine residue indicated that this residue plays an important but non-essential role in PMM/PGM activity.

Key words: carbohydrate metabolism, hyperthermophilic archaea, over expression, phosphoryl transfer, thermostable enzyme.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ORF, open reading frame; MOPS, (*N*-morpholino)propanesulfonic acid.

Most organisms, including thermophilic archaea, utilize glucose as an energy source, and as an important starting material for the biosynthesis of the cell surface structure. The glucose brought into the cytoplasm from the surrounding environment is converted to glucose-6-phosphate by glucokinase. In most microorganisms, glucose-6-phosphate is converted into two pyruvate molecules via the Embden-Meyerhof (EM) pathway accompanied by the synthesis of two molecules of ATP. Glucose-1-phosphate, created from glucose-6-phosphate, is utilized for cell surface construction by modification into other types of sugars. The reaction converting glucose-6-phosphate to glucose-1-phosphate is catalyzed by phosphoglucomutase. Consequently, phosphoglucomutase is a key enzyme that controls an important branch point in glucose metabolism for energy synthesis and cell surface construction.

Phosphoglucomutase has been isolated from many organisms (*1–9*) and includes two types of similar but distinguishable enzymes, an independent phosphoglucomutase and a bi-functional protein, phosphomannomutase/phosphoglucomutase (PMM/PGM), that catalyzes the phosphoryl group-converting reaction for both phosphoryl glucose and phosphoryl mannose. The bi-functional enzyme is a part of a family of phosphohexomutases that catalyzes reversibly the intra-molecular phosphoryl interconverting reaction of hexose-6-phosphate and hexose-1-phosphate, and vice versa. In the proposed mechanism for the PMM/PGM reaction (*10–14*), the first step is the transfer of the phosphoryl group, in the active site of the enzyme, from the enzyme to the substrate, thereby generating the bisphosphorylated intermediate. In the second step, the intermediate is reoriented, then another phosphoryl group is transferred back to the enzyme from the intermediate. Finally, the intra-molecular phosphoryl-transferred products are generated and the enzyme is regenerated into the active form, which is ready for another round of catalysis. In this proposed mechanism, the serine residue located at the active site is thought to be necessary for catalysis.

ORFs PH0923 and PH1210 were identified as PMM/PGM homologues in the genomic data of *Pyrococcus horikoshii* OT3 by similarity search using known PMM/PGM amino acid sequences (*15*). In the analysis of genomic sequence data from hyperthermophilic archaea,

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some archaea were found to contain multiple PMM/PGM orthologues (*9*), while other archaea were found to contain only one PMM/PGM homologue. In the genomic data of *P. horikoshii*, the PH0923 gene was identified in a gene cluster or operon consisting of four genes, PH0923, PH0925, PH0926 and PH0927 (*16*). These four genes encode the enzymes that catalyze the reaction in the pathway that converts fructose-6-phosphate to mannosyl glycerate, PMM/PGM (PH0923), phosphomannose isomerase/GDP-D-mannose pyrophosphorylase (PH0925), mannosyl-3-phosphoglycerate phosphatase (PH0926) and mannosyl-3-phosphoglycerate synthase (PH0927). In contrast, it was found that the PH1210 gene was not part of any gene cluster or operon. Since it was predicted that PH0923 would play a more important role in carbohydrate metabolism than PH1210, PH0923 was selected as the target for our investigation of archaeal PMM/PGM.

In *P. horikoshii*, an ORF showing similarity to the characterized glucokinase was also detected, and, consequently, it was expected that the PMM/PGM homologue plays an important role in glucose metabolism by converting glucose-6-phosphate to glucose-1-phosphate.

In this study, an ORF, PH0923, encoding a PMM/PGM homologous protein, was cloned into an expression vector, and the recombinant protein was produced in *E. coli*. The recombinant protein exhibited thermostable PMM and PGM activities, and was able to function with a variety of different metal ions.

Comparison between the native and mutant forms indicated that the conserved serine residue in the proposed active site is important, but not essential, for catalytic activity.

MATERIALS AND METHODS

Bacterial Strains and General Reagents—An anaerobic hyperthermophilic archaeon, *P. horikoshii* OT3 (JCM9974), was obtained from the Japan Collection of Microorganisms (JCM). The culture of this archaeon and the preparation of genomic DNA were carried out as previously described (*15*, *17*). The KOD-plus DNA polymerase used for PCR amplification was purchased from Toyobo Co., Ltd. (Osaka, Japan). The restriction enzymes and T4 DNA ligase used for gene manipulation were purchased from New England Bio Labs, Inc. (Beverly, MO, USA). The plasmid vector pET28a was purchased from Novagen (Merck, Darnstadt, Germany). *Escherichia coli* strain DH5α was obtained from Takara Bio Inc. (Ohtsu, Shiga, Japan) for plasmid cloning, and the strain BL21-CodonPlus(DE3)-RIL was obtained from Stratagene (La Jolla, CA, USA) for the expression of the recombinant protein. Glucose-1-phosphate, glucose-1,6-bisphosphate, mannose-1-phosphate, nicotinamide adenine dinucleotide phosphate oxidized form (NADP), and phosphomannose isomerase from *E. coli* were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). Glucose-6-phosphate dehydrogenase from *Geobacillus stearothermophilus* was obtained from Seikagaku Corporation (Tokyo, Japan). Phosphoglucose isomerase from *Saccharomyces cerevisiae* was purchased from Nacalai Tesque Co., Ltd. (Kyoto, Japan). Lysozyme from chicken egg white was obtained from Wako Pure Chemical Industries, Co., Ltd. (Osaka, Japan). Deoxyribonuclease I was purchased from Takara Bio Inc. (Ohtsu, Shiga, Japan).

Construction of Expression Vector—ORF PH0923 from *P. horikoshii* OT3 was amplified and cloned to obtain a recombinant protein with a histidine tag at the N-terminus. The primer, dPH0923-5-1: GGGAATTCCATATGGAAAGCTCTTTGGTA, was designed from the 5′ sequence of PH0923. The primer, dPH0923-3-1: CCGCTCGAGTCATGAAAGTGCTTTCTCAAG, was designed from the 3′ sequence of PH0923. The primer dPH0923-5-1 contained an *Nde*I site (shown by the single underline), and the primer dPH0923-3-1 contained an *Xho*I site (shown by the double underline) for directional cloning into the pET28a plasmid vector. Ten nanograms of *P. horikoshii* OT3 genomic DNA and 100 pmol each of the appropriate primer sets were added to 50 μl of standard PCR reaction mixture. Thirty cycles with a temperature profile of one min at 95°C, one min at 55°C, and two min at 72°C were performed with 2.5 units of KOD-plus DNA polymerase. The resulting PCR products were digested with *Nde*I and *Xho*I, and ligated with the pET28a vector that had been digested with the same restriction enzymes. After confirmation of the nucleotide sequence, the plasmid possessing the PH0923 coding region with the histidine tag at the N-terminus was designated as pPH0923.

Construction of a Site Directed Mutant—To convert serine-101 in PH0923 to alanine, the following primers were designed. The overlapping primers dPH0923-4-1: CGGTGGGTTGTGCGCTGCGGTTATTACCGC and dPH0923-4-2: GTAATAACCGCAGCGCACAACCCACCGGAG were designed from the nucleotide sequence around the 290 bp position of PH0923. The codon CAA, which encodes serine, was changed to CAG, which encodes alanine, (shown underlined in the primer sequences). The primers dPH0923-5-1 and dPH0923-4-1 were used for PCR amplification of the N-terminal region and dPH0923-4-2 and dPH0923-3-1 were used for amplification of the C-terminal region. The PCR products corresponding to the N- and C-termini were digested with *Nde*I and *Hha*I, and *Xho*I and *Hha*I, respectively. The digested products were then ligated with the *Nde*I/*Xho*I-digested pET28a vector. After confirmation of the nucleotide sequence, the plasmid possessing the mutant form of the PH0923 coding region with a histidine tag at the N-terminus was designated pPH0923S101A.

Expression and Purification of the Recombinant Proteins—*E. coli* strain BL21-CodonPlus(DE3)-RIL cells transformed by the expression vectors pPH0923 or pPH0923S101A were grown in 2 liters of LB medium containing 75 μg/ml kanamycin at 25°C until the absorbance at 600 nm reached 0.6. Isopropyl-1-thio-β-D-galactoside (IPTG) was added to a final concentration of 1 mM, and shaking was continued for 15 h at 25°C to induce the expression of recombinant proteins. After cultivation, *E. coli* cells were collected by centrifugation at 5,000 × *g* for 15 min, and suspended in 180 ml of 50 mM MOPS (pH 7.6) after washing with 300 ml of the same solution. The cells were treated with lysozyme and deoxyribonuclease I to a final concentration of 0.2 mg/g of the wet cell pellet and 10 μg/ml, respectively. The suspension was stored for 30 min on ice. The treated cells were ruptured by sonication with a Bioruptor (Cosmo Bio Co. Ltd., Tokyo, Japan), using 10 cycles of 30 s pulses followed by a 30 s

rest on ice. The lysate was centrifuged at 21,130 × *g* for 30 min at 4°C, and the supernatant was collected as the soluble fraction. The soluble fraction was treated at 65°C for 30 min and then centrifuged at 21,130 × *g* for 30 min at 4°C to remove the denatured protein. The recombinant proteins were purified on a nickel-loaded HiTrap Chelating HP column (Amersham Biosciences Corp., Piscataway, NJ, USA) according to the manufacturer's protocol. The purified proteins were concentrated by ultrafiltration using Amicon® Ultra (Millipore, Billerica, MA, USA) and dissolved in 4 ml of 50 mM MOPS (pH 7.6). The purified proteins were stored at 4°C. The protein concentration of the purified fraction was determined with a BCA protein assay reagent kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Proteins contained in 10 μl of supernatant were separated by 0.1% SDS–10% polyacrylamide gel electrophoresis and detected by staining with Coomassie Brilliant Blue R-250.

Enzymatic Assays—The enzymatic activities of the recombinant proteins were primarily analyzed by the method of Ye *et al.* (7) with some modification. The assay is based on the fact that the amount of reduced nicotinamide adenine dinucleotide phosphate (NADPH) that is produced from glucose-6-phosphate by the glucose-6-phosphate dehydrogenase can be monitored by the increase in the absorbance at 340 nm. The assay for the reverse reaction of phosphoglucomutase (PGM), which produces glucose-6-phosphate from glucose-1-phosphate, was performed in a 2 ml standard reaction mixture containing 50 mM MOPS (pH 7.6), 2 mM $MgSO_4$, 10 μM glucose-1,6-bisphosphate, 1 mM $NADP^+$, 1 U glucose-6-phosphate dehydrogenase from *G. stearothermophilus* and 2.0 μg of purified recombinant PH0923 protein. After pre-incubation of the reaction mixture at 65°C for 3 min, the reaction was started by the addition of glucose-1-phosphate to a final concentration of 0.4 mM. The reaction was allowed to proceed at 65°C for 10 min. Increases in the absorbance of the solution were monitored at 340 nm using a spectrophotometer (V-550, JASCO). The activities of the recombinant proteins were calculated from the initial velocity.

For measurement of phosphomannomutase (PMM) activity, phosphoglucose isomerase and phosphomannose isomerase, which are involved in the conversion of mannose-6-phosphate to glucose-6-phosphate, were added to the standard reaction mixture for PGM activity. The assay of the reverse reaction of PMM, which produces mannose-6-phosphate from mannose-1-phosphate, was carried out in the 2 ml standard reaction mixture for PGM with the addition of 1 U phosphomannose isomerase from *E. coli*, 1 U phosphoglucose isomerase from *S. cerevisiae*, 1 U glucose-6-phosphate dehydrogenase from *G. stearothermophilus* and 20 μg of the purified recombinant PH0923 protein. PMM activity assays were performed at 37°C including pre-incubation.

The kinetic constants for PGM and PMM activities were determined from the Michaelis-Menten equation.

To determine the effect of metal ions on PGM activity, reactions were performed in 2 ml of the standard reaction mixture for PGM containing 0.1 mM EDTA. After monitoring the absorbance at 340 nm for 100 s from the start of reaction initiated by the addition of glucose-1-phosphate, the appropriate metal ion was added to a final concentration of 2 mM, and further increases in the absorbance at 340 nm were monitored.

To analyze the thermostability of the recombinant protein, the purified recombinant protein was incubated at 95 or 100°C for different periods. The remaining PGM activity was measured in the standard reaction mixture under the standard conditions described above.

To investigate the effect of pH on PGM activity, 2 ml of the standard reaction mixture for PGM using 50 mM citrate buffer for pH 6.0, 50 mM MOPS for pH 6.5 to 7.6, 50 mM Tris-HCl for pH 8.0, 50 mM glycine-NaOH buffer for pH 9.0 to 10.0. The relative activities of glucose-6-phosphate dehydrogenase depending on pH were obtained in 2 ml of the reaction solution described above containing 0.2 mM glucose-6-phosphate. The relative PGM activities of the recombinant proteins were calculated from these values.

RESULTS AND DISCUSSION

Construction of an Expression Vector for PH0923—A 1,368 bp long ORF, PH0923, was predicted as a homologue of PMM/PGM on the basis of sequence similarity. A putative 455 amino acid residue gene product of PH0923 showed 28–31% identity with PMM/PGM enzymes previously characterized from *Pseudomonas aeruginosa* (gi: 12230879), *Pseudomonas putida* (gi: 33300965), *Vibrio furnissii* (gi: 37497224) and *Xanthomonas campestris* (gi: 155395). Amino acid sequences for a motif in the reaction center and a motif in the metal binding site of PMM/PGM were identified in PH0923 (Fig. 1). These findings suggest that PH0923 encodes a protein with PMM/PGM activity. To investigate the activity and function of the protein encoded by PH0923, the gene was cloned into an expression vector according to the procedure described in "MATERIALS AND METHODS."

Expression and Purification of the Recombinant PH0923 Protein—The recombinant protein containing a histidine tag at the N-terminus was detected in the soluble fraction, and remained soluble after 30 min at 80°C (data not shown). After heating and centrifugation, the recombinant protein was purified by nickel-affinity column chromatography. The purity of the recombinant protein was monitored by SDS-PAGE, which showed that the resulting purified protein migrated as a single band with a molecular mass of approximately 50 kDa, which is consistent with the molecular mass predicted from the nucleotide sequence (lane 2 in Fig. 2). The purified recombinant protein was stored at 4°C and used for subsequent enzymatic analyses.

Detection of PGM and PMM Activities—The PGM and PMM activities of the recombinant PH0923 protein for their reverse reactions producing sugar-6-phosphate from sugar-1-phosphate were detected by increases in absorbance at 340 nm over various time periods (data not shown). These results demonstrated that this archaeal recombinant PH0923 protein produced in *E. coli* exhibits thermostable PGM and PMM activities. The results indicate that this protein is actually a PMM/PGM enzyme from the hyperthermophilic archaeon, *P. horikoshii* OT3.

Optimum Conditions for Activity of the Recombinant PH0923 Protein—Previous work on the characterization of PGM and PMM activities in *P. aeruginosa* indicated

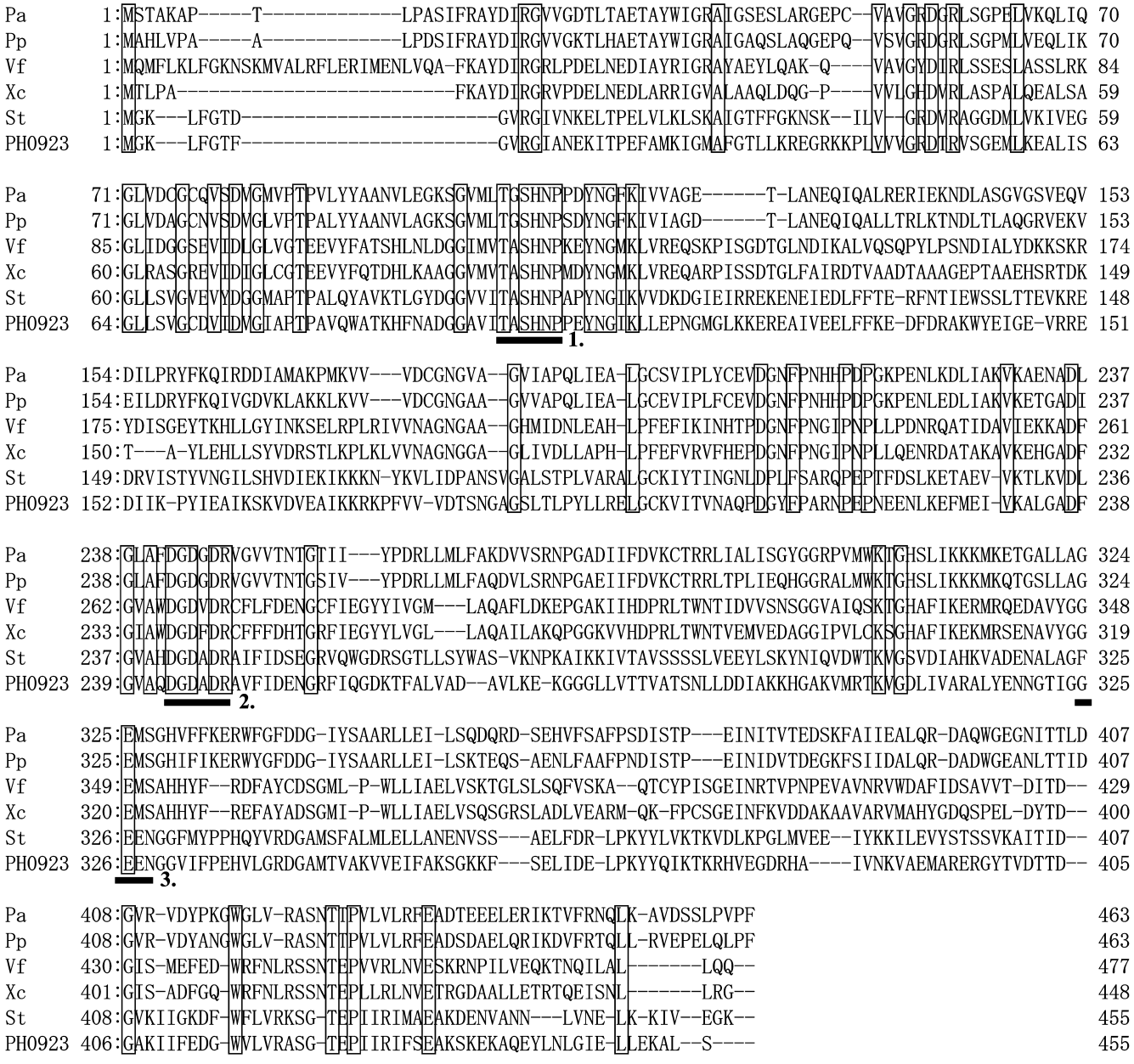

Fig. 1. **Comparison of amino acid sequences among PMM/PGMs.** Pa, Pp, Vf, Xc, and St indicate the PMM/PGM proteins from *P. aeruginosa* (gi: 12260879), *P. putida* (gi: 33300965), *V. furnissii* (gi: 37497224), *X. campestris* (gi: 155395) and *S. tokodaii* (gi: 15621207), respectively. PH0923 indicates the PMM/PGM homologous gene used in this work. Underlined residues indicate the core motif previously predicted (*12*). The letters within black boxes indicate residues conserved across these six sequences. The underlined residues marked 1, 2 and 3 indicate the highly conserved motifs for the recognition of substrate, metal ions and substrate in the PMM/PGM enzyme, respectively.

that the metal ions Mg^{2+} or Mn^{2+} are necessary as cofactors, but the metal ions Ca^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} and Li^{2+} are not (*7*). To investigate the recombinant PH0923 protein for the utilization of metal ions, the effects of different metal ions on the reverse reaction of PGM activity were analyzed according to the protocol described in the "MATERIALS AND METHODS."

PGM activity was not detectable when divalent metal ions were removed from the reaction buffer by the addition of 0.1 mM EDTA, indicating that the PGM activity of the recombinant PH0923 protein has an absolute requirement for divalent metal ions (Table 1). Since Co^{2+}, Ni^{2+}, and Ca^{2+} did not function as cofactors in PMM/PGM from *P. aeruginosa*, this phenotype is specific to the recombinant PH0923 protein.

Analysis of the concentration effect of the most common metal ion, Mg^{2+}, revealed no substantial changes in the enzymatic activity between 0.2 and 2.0 mM (data not shown). The previous report by Ye *et al.* indicated no activity in the presence of Ca^{2+}, Co^{2+} or Ni^{2+}, and that these divalent metal ions inhibited both the PGM and PMM activities, even when Mg^{2+} was present (*7*). Although

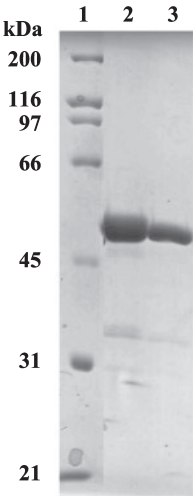

Fig. 2. **SDS-PAGE analysis of the purified wild type and mutant recombinant PH0923 proteins.** The recombinant protein expressed in *E. coli* harboring the plasmid indicated below was analyzed in a 10% polyacrylamide gel containing 0.1% SDS. Lane 1: standard molecular weight proteins, Lane 2: pPH0923, Lane 3: pPH0923S101A. All recombinant proteins were purified by nickel-affinity column chromatography on a HiTrap Chelating HP column after heat treatment at 65°C for 30 min. Proteins were visualized by Coomassie Brilliant Blue R-250 staining.

there was a difference in the relative effectiveness of divalent metal ions on the activities of the recombinant PH0923 protein and PMM/PGM enzyme from *P. aeruginosa*, the amino acid sequence in the metal binding sites of the PH0923 protein is highly conserved; six out of eight residues are identical in the metal binding site motif sequence (Fig. 1). In the previous analysis, the three aspartic acids located within the metal binding site were found to play an important role in Mg^{2+} binding (*18*). Although the utilization of metal ions by the recombinant PH0923 protein was distinctly different from that of PMM/PGM proteins isolated from *E. coli* or *P. aeruginosa*, the three aspartic acids predicted to be important for the recognition of metal ions are conserved in the PH0923 protein. This observation suggests that other

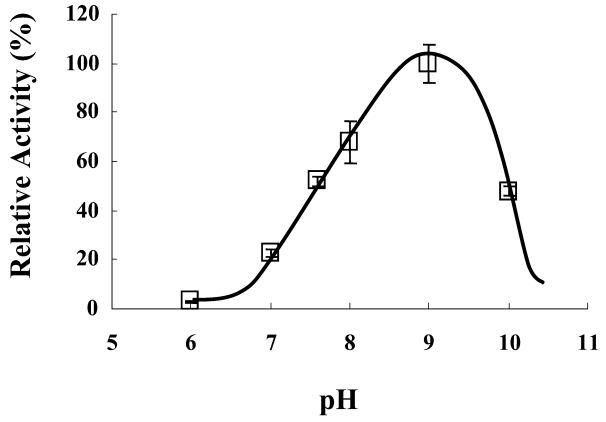

Fig. 3. **Relative activities of the recombinant PH0923 protein at varying pH.** The enzymatic activities were measured between pH 6.0 and 10.0. Relative activity is expressed as a percentage of the maximum activity at pH 9.0.

Table 1. **PGM activities of the recombinant PH0923 protein in the presence of different metal ions.**

Metal	Relative activity (%)
Mg^{2+}	100 ± 5
Co^{2+}	44 ± 1
Mn^{2+}	20 ± 3
Ni^{2+}	15 ± 2
Ca^{2+}	1 ± 0
none	ND

The metal ion concentration used was 2 mM. The assay conditions were as described in "MATERIALS AND METHODS." The relative activity is shown as % of the activity detected with Mg^{2+}. ND indicates non-detectable.

amino acid residues may play important roles in the recognition of metal ions. It has been previously shown that some enzymes isolated from thermophilic archaea are capable of recognizing substrates or cofactors that are not accepted by bacterial enzymes (*19–21*). If it can be concluded that this feature is unique among and common to archaeal enzymes, then an understanding of the mechanism would be useful for enzyme engineering.

The effect of pH on the reverse reaction of PGM activity was analyzed using four different solutions as described in "MATERIALS AND METHODS." The recombinant PH0923 protein exhibited relatively high activity at around pH 7.5 and 10.0, with maximum activity at pH 9.0 (Fig. 3).

Thermostability of the Recombinant PH0923 Protein—PH0923 was cloned from the hyperthermophilic archaeon *P. horikoshii* OT3, which grows optimally at 95°C. The thermostability of the recombinant PH0923 protein was examined. However, the optimal temperature for the PGM and PMM activities of the recombinant protein was not determined, since the thermostable temperatures of coupling enzymes used in the standard reaction mixture were lower than that of the recombinant PH0923 protein. Purified recombinant PH0923 protein at a concentration of 3.6 mg/ml in 50 mM MOPS (pH 7.6) was treated at 95°C or 100°C for the periods indicated in Fig. 4A, and then the residual activities were measured in 2 ml of the standard PGM reaction mixture. Figure 4A shows the half-life of the PH0923 enzymatic activity under treatment at 100°C to be approximately 5 min, but longer than 90 min at 95°C. These results indicate that the protein encoded by PH0923 is extremely thermostable.

The reduction in activity during heat treatment can be explained by two different hypotheses: (i) that although the activity of each active protein is not reduced, the number of active proteins is reduced; (ii) that the activity of all proteins is reduced depending on the period of heat treatment. To investigate by which mechanism the majority of the activity of the recombinant PH0923 protein is lost after treatment at 100°C, the amount and activity of the soluble protein recovered were measured after treatment at 100°C for the periods indicated in Fig. 4.

First, the solubility of the recombinant PH0923 protein was analyzed by SDS-PAGE. After treatment at 100°C, the denatured PH0923 protein was removed by centrifugation at 21,130 × *g* for 30 min at 4°C. The amount of protein remaining in the soluble fraction was reduced

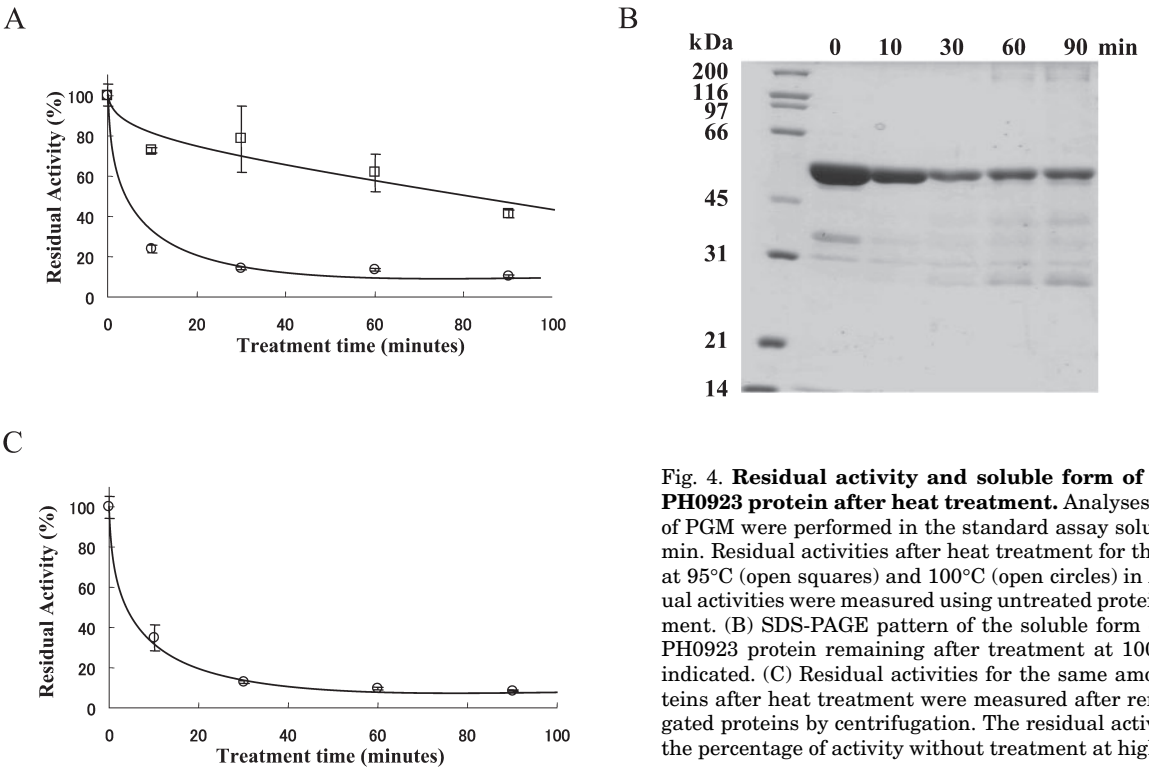

Fig. 4. **Residual activity and soluble form of the recombinant PH0923 protein after heat treatment.** Analyses of residual activity of PGM were performed in the standard assay solution at 65°C for 10 min. Residual activities after heat treatment for the periods indicated at 95°C (open squares) and 100°C (open circles) in A and C. (A) Residual activities were measured using untreated proteins after heat treatment. (B) SDS-PAGE pattern of the soluble form of the recombinant PH0923 protein remaining after treatment at 100°C for the periods indicated. (C) Residual activities for the same amount of soluble proteins after heat treatment were measured after removal of the aggregated proteins by centrifugation. The residual activity is expressed as the percentage of activity without treatment at high temperature.

according to the period of treatment at 100°C as shown in Fig. 4B, with treatment at 100°C for 10 min reducing the amount of soluble protein to approximately half. Consequently, we conclude that the soluble form of the recombinant PH0923 protein becomes insoluble according to the period of treatment at 100°C.

Next, after treatment at 100°C, the residual activity of the remaining soluble recombinant PH0923 protein was measured. After removing the insoluble protein by centrifugation, the concentration of the soluble protein in the standard reaction mixture was adjusted to that before heat treatment, and the PGM activity was measured. As indicated in Fig. 4C, the soluble form of the recombinant PH0923 protein remaining after treatment at high temperature showed a reduction in activity depending on the duration of treatment. This observation suggests that the recombinant PH0923 protein is present in three different forms, a soluble and active form, a soluble and inactive form and an insoluble form. This indicates that the recombinant PH0923 protein that retains its soluble structure but loses its activity after heat treatment (described as soluble and inactive form above) may be damaged only in the reaction center required for enzymatic activity.

The properties of the recombinant PH0923 protein described here may be useful for future investigations into the correlation between protein structure and activity.

Kinetic Constants of the PMM and PGM Activities—To determine the effect of substrate concentration on the enzymatic activity, plots of initial velocity were made. In this measurement, standard reaction mixtures in which the concentrations of other enzymes for the coupling reaction were 5–10 times higher than their sufficient concentration were used. In assays of PGM and PMM reverse reactions, the apparent K_m values of the recombinant PH0923 protein for glucose-1-phosphate or mannose-1-phosphate were 0.0952 and 0.0915 mM, respectively (Table 2). The V_{max} values for the reverse PGM and PMM reactions of the recombinant PH0923 protein were 24.2 and 0.7 μmol/min/mg, respectively. These results indicate that the K_m of this recombinant protein for glucose-1-phosphate is similar to that of *P. aeruginosa* (7). However, the K_m of the recombinant PH0923 protein for mannose-1-phosphate was five times higher than that of *P. aeruginosa*. This indicates that archaeal PMM/PGM has a higher affinity for glucose-phosphate than mannose-phosphate. Also the V_{max} of the recombinant PH0923 protein for mannose-1-phosphate was 35 times lower than that for glucose-1-phosphate, indicating that the recombinant PH0923 protein has an activity specific for glucose-1-phosphate.

Table 2. **Kinetic properties of the reverse reaction of PGM and PMM activities of the wild type and mutant recombinant proteins.**

	PMM		PGM	
	V_{max} (μmol/min/mg)	K_m (μM)	V_{max} (μmol/min/mg)	K_m (μM)
PH0923	0.7 ± 0.0	91.5 ± 10.8	24.2 ± 3.0	95.2 ± 24.2
PH0923S101A	ND	ND	0.4 ± 0.0	127.0 ± 29.7

ND indicates non-detectable.

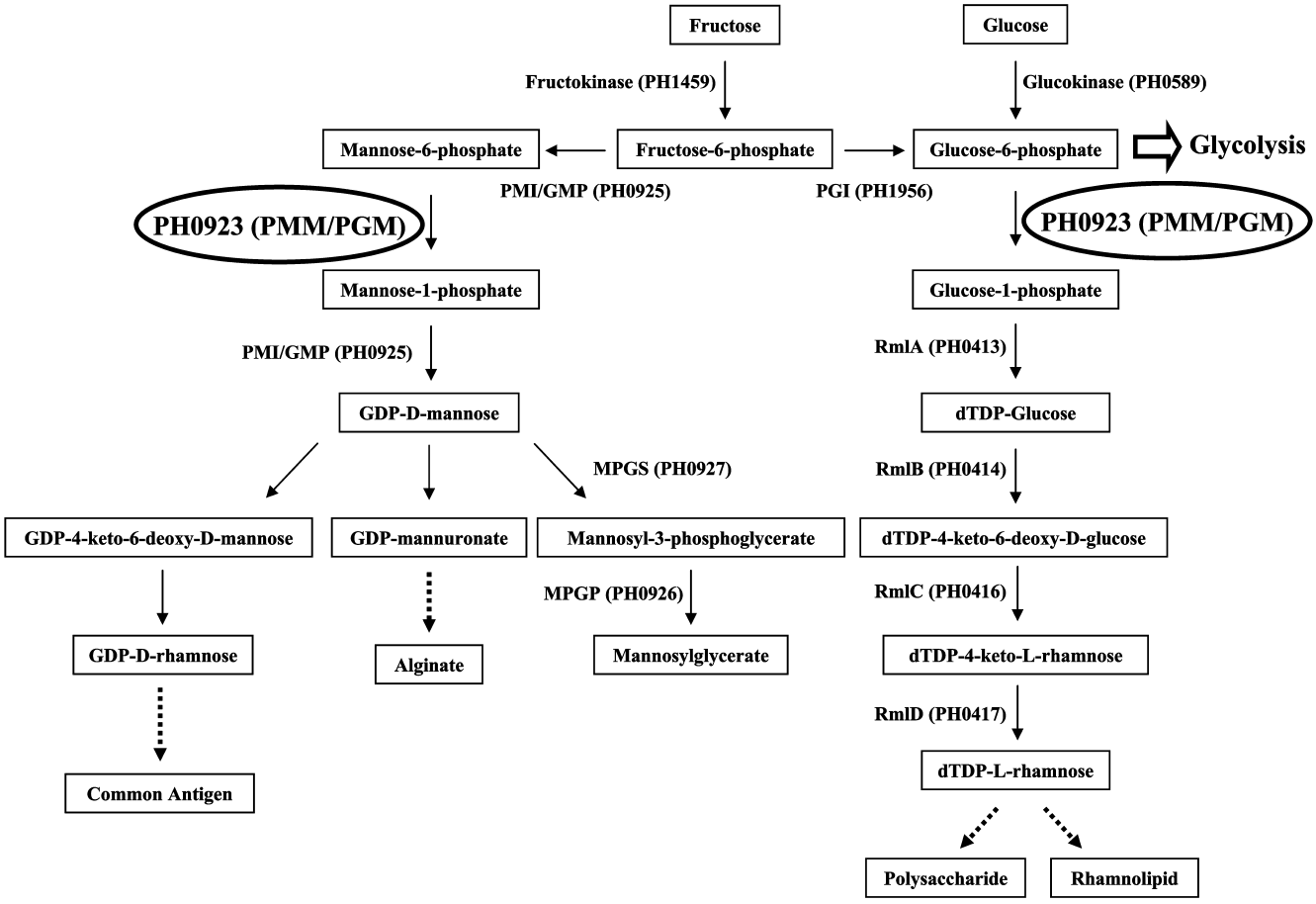

Fig. 5. **Proposed carbohydrate metabolic pathway in *P. horikoshii* OT3 predicted from the genomic data.**

Analysis of Mutant Protein—In previous research to elucidate the mechanism of the PMM/PGM reaction, it was predicted that the serine residue located at the reaction center is necessary for this reaction because the phosphoryl molecule binding to this serine residue is used for the kination of substrate. Thus, a mutant protein in which the serine residue at position 101 is replaced by alanine was constructed to investigate the role of the serine residue.

Analyses of the PGM activities of the recombinant PH0923S101A protein indicated that the K_m of the mutant protein was similar to that of the wild type enzyme (Table 2). However, the V_{max} value of the recombinant mutant protein was 60 times lower than that of the wild type protein. This suggests that the serine residue play an important role in the turnover rate, although the PMM/PGM protein of *P. aeruginosa* with the same mutation exhibited the same activity, which resulted from a 10-fold reduction in both V_{max} and K_m (*14*). We conclude that the serine residue plays an important role in the turnover rate in the recombinant PH0923 protein, but is not essential for activity.

Role of PMM/PGM in Carbohydrate Metabolism—In this study, we demonstrate that the actual PGM and PMM activities are present in the recombinant PH0923 protein isolated from *P. horikoshii*. It has been reported that members of the genus *Pyrococcus* generally use the Embden-Meyerhof (EM) pathway to convert glucose to pyruvate to obtain energy (*22*, *23*). As shown in Fig. 4, all enzymes catalyzing reactions in central carbohydrate metabolism are identified from the genomic data of *P. horikoshii* OT3. In this pathway, the PH0923 protein stands at a branch point between the central metabolic pathway and the polysaccharide synthesis pathway. The PH0923 protein plays an important role in supplying substrate for the synthesis of polysaccharides and other types of saccharides from glucose-6-phosphate or mannose-6-phosphate.

This work provides the first detailed analysis of a thermostable enzyme with both PMM and PGM activities from a hyperthermophilic archaeon, *P. horikoshii* OT3. Finally, the highly stable PH0923 protein may be useful for the industrial production of sugar-1-phosphate, and it would be worthwhile to explore this potential application in a future study.

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