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The Phosphoglucose Isomerase from the Hyperthermophilic Archaeon *Pyrococcus furiosus* Is a Unique Glycolytic Enzyme That Belongs to the Cupin Superfamily*

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Pyrococcus furiosus **uses a variant of the Embden-Meyerhof pathway during growth on sugars. All but one of the genes that encode the glycolytic enzymes of *P. furiosus* have previously been identified, either by homology searching of its genome or by reversed genetics. We here report the isolation of the missing link of the pyrococcal glycolysis, the phosphoglucose isomerase (PGI), which was purified to homogeneity from *P. furiosus* and biochemically characterized. The *P. furiosus* PGI, a dimer of identical 23.5-kDa subunits, catalyzes the reversible isomerization of glucose 6-phosphate to fructose 6-phosphate, with K_m values of 1.99 and 0.63 mM, respectively. An optimum pH of 7.0 has been determined in both directions, and at its optimum temperature of 90 °C the enzyme has a half-life of 2.4 h. The N-terminal sequence was used for the identification of the *pgiA* gene in the *P. furiosus* genome. The *pgiA* transcription start site has been determined, and a monocistronic messenger was detected in *P. furiosus* during growth on maltose and pyruvate. The *pgiA* gene was functionally expressed in *Escherichia coli* BL21(DE3). The deduced amino acid sequence of this first archaeal PGI revealed that it is not related to its bacterial and eukaryal counterparts. In contrast, this archaeal PGI shares similarity with the cupin superfamily that consists of a variety of proteins that are generally involved in sugar metabolism in both prokaryotes and eukaryotes. As for the *P. furiosus* PGI, distinct phylogenetic origins have previously been reported for other enzymes from the pyrococcal glycolytic pathway. Apparently, convergent evolution by recruitment of several unique enzymes has resulted in the unique *Pyrococcus* glycolysis.**

The hyperthermophilic archaeon *Pyrococcus furiosus* is capable of metabolizing sugars via a modified Embden-Meyerhof pathway (1). Novel enzymes and unique control points in this pathway have been elucidated and involve two phosphorylation and an oxidoreduction reaction (2–5).

A first variation of the pyrococcal glycolysis concerns the unique ADP-dependent sugar kinases, *i.e.* ADP-dependent glucokinase (ADP-GLK)[1] and ADP-dependent phosphofructokinase (ADP-PFK) have been characterized biochemically, and the paralogous genes were identified on the *P. furiosus* genome (2, 3). The recently determined crystal structure of the ADP-GLK from the related archaeon *Thermococcus litoralis* revealed that the ADP-dependent sugar kinase family (ADP-GLK and most likely ADP-PFK) belong to the ribokinase family (6), whereas their bacterial and eukaryal counterparts belong to the hexokinase and PFK family, respectively (7, 8).

A second variation concerns the glycolytic conversion of glyceraldehyde 3-phosphate to 3-phosphoglycerate in *P. furiosus* that was found to be catalyzed by the unique glyceraldehyde-3-phosphate ferredoxin oxidoreductase enzyme (4, 5). This ferredoxin-dependent, single-step conversion of glyceraldehyde 3-phosphate was shown to represent a novel site of glycolytic regulation in *P. furiosus* (5).

With the increasing number of available sequence data from different species, including bacteria, eucarya, and archaea, and functional characterization of the gene products, most of the genes encoding the other *P. furiosus* glycolytic enzymes (fructose-1,6-bisphosphate aldolase, triose-phosphate isomerase, phosphoglycerate mutase, enolase, and pyruvate kinase) encoding genes could readily be identified in its genome (9). Attempts to identify the gene encoding phosphoglucose isomerase (PGI) by a bioinformatics approach have hitherto been unsuccessful. Although significant PGI activity has previously been detected (0.2 units/mg) in a *P. furiosus* cell-free extract (1, 2, 10, 11), no ortholog of a bacterial/eukaryal PGI could be identified in the *P. furiosus* genome. This suggested that *P. furiosus* might possess a distinct type of PGI. To complete the *P. furiosus* glycolytic pathway and to obtain insight in the anticipated novel type of PGI, we here report on the purification of the PGI enzyme from *P. furiosus*, its characterization, and the isolation of the corresponding *pgiA* gene. This is the first molecular and biochemical characterization of an archaeal PGI, that indeed represents a novel type of this enzyme.

EXPERIMENTAL PROCEDURES

Materials—All chemicals and enzymes were purchased from Sigma, Merck, or Roche Molecular Diagnostics in analytical grade. *Aspergillus nidulans* mannitol-1-phosphate dehydrogenase was purified from an overproducing *A. nidulans* strain as described previously (12).

Organisms and Growth Conditions—*P. furiosus* was cultivated in artificial seawater medium as described before (3). *Escherichia coli* XL1 Blue was used as a host for the construction of pET24d derivatives. *E.*

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[1] The abbreviations used are: GLK, glucokinase; PFK, phosphofructokinase; PGI, phosphoglucose isomerase; PAGE, polyacrylamide gel electrophoresis; PCR, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid.

coli BL21(DE3) was used as an expression host. Both strains were grown in Luria Bertani medium with kanamycin (50 μg/ml) in a rotary shaker at 37 °C.

Preparation of Cell-free Extract from P. furiosus—*P. furiosus* cells from a 200-liter culture were harvested by continuous centrifugation (Sharples, Rueil, France) and stored at −20 °C until use. Cell-free extract was prepared by suspending a cell paste in 2 volumes (w/v) of 50 mM Tris/HCl buffer, pH 7.5, and treatment in a French press at 100 megapascals. Cell debris was removed by centrifugation for 1 h at 100,000 × *g* at 10 °C.

Purification of the PGI from P. furiosus Cell-free Extract—To prevent microbial contamination, all buffers contained 0.02% sodium azide. Cell-free extract (27 ml) was filtered (0.45 μm), brought to 1.7 M ammonium sulfate saturation and loaded onto a Phenyl-Sepharose fast flow column (69 ml, Amersham Pharmacia Biotech), equilibrated in 50 mM Tris/HCl buffer, pH 7.8, containing 1.7 M ammonium sulfate. During a 350-ml linear gradient (1.7–0.0 M ammonium sulfate) PGI activity eluted at 1.0 M ammonium sulfate. Active fractions were pooled and desalted by filtration (Macrosep, 10-kDa cutoff), using a 50 mM Tris/HCl buffer, pH 8.5. The desalted PGI pool was applied to a Q-Sepharose fast flow column (25 ml, Amersham Pharmacia Biotech) that was equilibrated in the same buffer. The PGI eluted in a 125-ml linear gradient (0.0–0.7 M NaCl) at 0.27 M NaCl. Active fractions were pooled and dialyzed against 20 mM potassium phosphate buffer, pH 7.0. The desalted PGI pool was applied to a hydroxyapatite column (20 ml, Bio-Rad) that was equilibrated in the same buffer. PGI activity eluted in a 200-ml linear gradient (20–500 mM potassium phosphate) at 140 mM potassium phosphate. Active fractions were pooled, the buffer was changed for a 50 mM Tris/HCl buffer, pH 7.6, by dialysis and the pool was loaded onto a Mono-Q HR 5/5 column (1 ml, Amersham Pharmacia Biotech) that was equilibrated in the same buffer. PGI activity eluted in a 30-ml linear gradient (0.0–0.7 M NaCl) at 0.18 M NaCl. Fractions showing PGI activity were pooled and concentrated 10-fold to a final volume of 100 μl. This concentrated pool was applied to a Superdex 200 HR 10/30 gel filtration column (24 ml, Amersham Pharmacia Biotech) that was equilibrated with a 50 mM Tris/HCl buffer, pH 7.8, containing 100 mM NaCl, from which the protein eluted after 14.5 ml. The purified PGI was desalted in 50 mM Tris/HCl, pH 7.8, using a Microsep filter with a 10-kDa cutoff.

Cloning of the PGI Gene—The N-terminal sequence of the purified PGI was determined by the Edman degradation method. The sample was subjected to SDS-PAGE and electroblotted on a polyvinylidene difluoride membrane prior to analysis. The N-terminal amino acid sequence was used for BLAST search of the *P. furiosus* data base (www.genome.utah.edu), and identification of the PGI gene (*pgiA*, accession number AF381250, NCBI GenBank™). The following primer set was designed to amplify this open reading frame by PCR: BG902 (5′-GCGCG**TCATGA**TGTATAAGGAACCTTTTGGAGTG, sense) and BG903 (5′-GCGCG**AAGCTT**CTACTTTTTCCACCTGGGATTAT, antisense), with *Bsp*HI and *Hin*dIII restriction sites in bold.

The 100-μl PCR mixture contained 100 ng of *P. furiosus* DNA, isolated as described before (13), 100 ng each of primer BG902 and BG903, 0.2 mM dNTPs, *Pfu* polymerase buffer, and 5 units of *Pfu* DNA polymerase and was subjected to 35 cycles of amplification (1 min at 94 °C, 1 min at 56 °C, and 1 min at 72 °C) on a DNA Thermal Cycler (PerkinElmer Life Sciences). The PCR product was digested (*Bsp*HI/*Hin*dIII) and cloned into an *Nco*I/*Hin*dIII-digested pET24d vector, resulting in pLUW557, which was transformed into *E. coli* XL1 Blue and BL21(DE3). Sequence analysis on pLUW557 was done by the dideoxynucleotide chain termination method with a Li-Cor automatic sequencing system (model 4000L). Sequencing data were analyzed using the computer program DNASTAR.

Overexpression of the PGI Gene in E. coli—An overnight culture of *E. coli* BL21(DE3) containing pLUW557 was used as a 1% inoculum in 1 liter of Luria Bertani medium with 50 μg/ml kanamycin. Gene expression was induced by adding 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at the A_{600} of 0.5. Growth was continued for 10 h at 37 °C, and cells were harvested by centrifugation (2,200 × *g* for 20 min) and resuspended in 10 ml of 50 mM Tris/HCl buffer, pH 7.6. The suspension was passed twice through a French press (100 megapascals), and cell debris was removed by centrifugation (10,000 × *g* for 20 min). The resulting supernatant was used for purification of the recombinant PGI.

Purification of Recombinant PGI—The *E. coli* cell-free extract containing pLUW557 was heat-treated for 30 min at 80 °C, and precipitated proteins were removed by centrifugation. The heat-treated cell-free extract was filtered through a 0.45-μm filter and applied to a Mono-Q HR 5/5 column (Amersham Pharmacia Biotech), equilibrated with 50 mM Tris/HCl, pH 7.6. The PGI activity eluted at 0.18 M NaCl during a linear gradient of 0.0–1.0 M NaCl. Active fractions were pooled and concentrated 10-fold to a final volume of 100 μl using a Microsep filter with a 10-kDa cutoff. The concentrated pool was loaded onto a Superdex 200 HR 10/30 gel filtration column (Amersham Pharmacia Biotech), equilibrated with 50 mM Tris/HCl, pH 7.8, containing 100 mM NaCl. The recombinant PGI eluted at 14.5 ml. The purified enzyme was desalted in 50 mM Tris/HCl, pH 7.8, using a Microsep filter with a 10-kDa cutoff.

Protein Concentration and Purity—Protein concentrations were determined with Coomassie Brilliant Blue G-250 as described before (14) using bovine serum albumin as a standard. The purity of the enzyme was checked by SDS-PAGE as described (15). Protein samples for SDS-PAGE were heated for 5 min at 100 °C in an equal volume of sample buffer (0.1 M citrate-phosphate buffer, 5% SDS, 0.9% 2-mercaptoethanol, 20% glycerol, pH 6.8).

Determination of Enzyme Activity—PGI activity was determined in 100 mM MOPS buffer, pH 7.0 (50 °C). Enzyme preparations were added in 5–50 μl. Enzyme activity on fructose 6-phosphate was determined by measuring the formation of NADPH in a coupled assay with yeast glucose-6-phosphate dehydrogenase. The assay mixture contained 0.5 mM NADP, 5 mM fructose 6-phosphate, and 0.35 units of D-glucose-6-phosphate dehydrogenase. The activity of the PGI on glucose 6-phosphate was determined by measuring the decrease of NADH in a coupled assay with *A. nidulans* mannitol-1-phosphate dehydrogenase (12). The assay mixture contained 0.2 mM NADH, 5 mM glucose 6-phosphate, and 1.4 units of mannitol-1-phosphate dehydrogenase. One unit was defined as the amount of enzyme required to convert 1 μmol of fructose 6-phosphate or glucose 6-phosphate per min. All enzyme assays were performed at 50 °C. At this temperature the yeast and *A. nidulans* enzyme remained active, and the *P. furiosus* enzyme was sufficiently active to measure its activity. The auxiliary enzymes were present in excess, to ensure that the detected NADPH and NADH absorbance at 340 nm (ϵ = 6.3 mM^{-1} cm^{-1}) corresponded to the PGI activity.

Substrate Specificity—Substrate specificity was investigated using purified PGI. The use of fructose 6-phosphate and glucose 6-phosphate as possible substrates for the PGI was tested using the standard enzyme assay. For the determination of mannose 6-phosphate as a possible substrate the standard enzyme assay for glucose 6-phosphate was used. Glucose, fructose, galactose, and mannose were tested as possible substrates by incubating an appropriate amount of PGI with 5 mM substrate for 30–60 min at 50 °C in 100 mM MOPS, pH 7.0. The reactions were stopped on ice/ethanol and the products were analyzed by high performance liquid chromatography. The effect of cations ($MgCl_2$ and $MnCl_2$, 10 mM) and cofactors (ATP, NAD^+, arsenate, and phosphate, 10 mM) on the isomerization of non-phosphorylated monosaccharides was investigated by the standard high performance liquid chromatography assay.

Inhibitors of PGI Activity—Possible inhibitors (mannose 6-phosphate, fructose 1-phosphate, fructose 1,6-bisphosphate, fructose, glucose, mannose, galactose, pyruvate, phosphoenolpyruvate, AMP, ADP, or ATP) were tested on the activity of the *P. furiosus* PGI both in the direction of glucose 6-phosphate and fructose 6-phosphate formation by adding (1.25–10 mM) to the standard enzyme assays at 50 °C.

Kinetic Analysis—Kinetic parameters were determined at 50 °C, in 100 mM MOPS buffer, pH 7.0, by varying the concentration of fructose 6-phosphate (0.05–3.50 mM) or glucose 6-phosphate (0.47–10.0 mM), respectively. 2.0 μg of purified PGI was used for these determinations. Data were analyzed by computer-aided (Program Tablecurve) fit to the Michaelis-Menten curve.

Temperature Optimum and Thermal Inactivation—The temperature optimum was determined in the direction of glucose 6-phosphate formation. Purified PGI (0.0064 mg/ml) was incubated in 1-ml crimp-sealed vials containing 100 mM sodium phosphate buffer, pH 7.0. The vials were submerged in an oil bath at temperatures varying from 30 to 120 °C, pre-heated for 2 min, and the enzyme reaction was started by injecting 20 mM fructose 6-phosphate. After 1, 2, and 3 min the reaction was stopped by transferring the vials on ice/ethanol, and the amount of glucose 6-phosphate formed was determined spectrophotometrically at room temperature by measuring the reduction of NADP (340 nm) in an assay with glucose-6-phosphate dehydrogenase. Corrections were made for the chemical isomerization of fructose 6-phosphate in the absence of PGI.

Thermal inactivation of PGI was determined by incubating the enzyme (1.28 μg) in 200 μl of a pre-heated 100 mM sodium phosphate buffer, pH 7.0, at 60, 70, 80, and 90 °C in crimp-sealed vials, submerged in an oil bath. At certain time intervals, 200-μl aliquots were withdrawn and analyzed for activity in the standard assay. Studies were

TABLE I
Purification of PGI from P. furiosus

Purification step	Total activity	Protein	Specific activity[a]	Purification factor	Recovery
	units	*mg ml^{-1}*	*units mg^{-1}*	*-fold*	*%*
Cell-free extract	295.8	39.7	0.276	1.0	100
Phenyl-Sepharose	93.6	3.54	0.588	2.1	31.6
Q-Sepharose	90.2	0.670	2.99	10.8	30.5
Hydroxyapatite	38.1	0.426	3.58	13.0	12.9
Mono-Q	25.8	9.92	3.86	14.0	8.7
Superdex200	10.1	0.196	14.5	52.5	3.4

[a] Specific activities were determined at 50 °C, with fructose 6-phosphate as substrate.

performed under V_{max} conditions, since substrate concentrations in the assays are ~30-fold higher than the K_m.

pH Optimum—The pH optimum was determined at 50 °C in 200 mM Tris maleate buffer over the pH range 6.0–9.5. Buffer pH values were adjusted at this temperature. Except for buffer and temperature, assay conditions were identical to analyze the enzyme's temperature optimum. In the case of fructose 6-phosphate conversion, glucose-6-phosphate dehydrogenase was used as following enzyme. When glucose 6-phosphate was used as substrate, mannitol-1-phosphate dehydrogenase was used as following enzyme.

Transcript Analysis—RNA was isolated from maltose (10 mM) and pyruvate (40 mM) grown *P. furiosus* cells as described previously (16). For Northern blot analysis 15 μg of total RNA was separated on a 1.5% formaldehyde-agarose gel and transferred to a Hybond N$^+$ membrane. Probes were generated by PCR with the primers BG902 and BG903. The PCR product was purified by Qiaquick (Qiagen) and labeled by nick translation with [α-^{32}P]dATP. The transcription start was determined with a fluorescence (IRD800)-labeled antisense oligonucleotide (5′-CTTTCCATGCCCTTTCATCAAC-3′, position 103–124 of the *pgiA* gene). Primer extension reactions were performed using the Reverse Transcription System (Promega) according to the instructions of the manufacturer with the following modifications. Hybridization of total RNA (15 μg) and oligonucleotide (5 pmol) was performed for 10 min at 68 °C before allowing to cool to room temperature. The reaction (20 μl final volume) was started by addition of dNTPs (1 mM), $MgCl_2$ (5 mM), RNasin (20 units), and avian myeloblastosis virus-reverse transcriptase (22.5 units). After incubation for 30 min at 45 °C the reaction volume was diluted to 50 μl with 10 mM Tris/HCl, pH 8.5, 1 μl of RNase A (5 mg/ml) was added and the sample was incubated for 10 min at 37 °C. cDNA was precipitated with ethanol, dissolved in 3 μl of loading buffer, and 1 μl was applied to a sequencing gel in parallel with the sequencing reactions obtained with the same oligonucleotide.

Multiple Sequence Alignment and Tree Construction—The sequence alignment of homologs of the *P. furious* PGI was generated with T-coffee (17) followed by small, manual refinements. A neighbor joining (18) tree of the aligned sequences was generated with clustalX (19). Bootstrap values above 60 out of 100 are indicated. A secondary structure prediction was generated with Profile-based neural network system from HeiDelberg (20).

RESULTS AND DISCUSSION

Purification of the PGI from P. furiosus—Purification of the *P. furiosus* PGI was performed aerobically at ambient temperature. PGI was purified from a *P. furiosus* cell-free extract using a number of conventional chromatographic steps (Table I). Anion exchange chromatography (Q-Sepharose Fast Flow) and gel filtration (Superdex 200 HR 10/30) resulted in PGI purification to apparent homogeneity as judged from SDS-PAGE analysis (Fig. 1). Additional native PAGE analysis resulted in a single protein band (not shown). The enzyme was purified 52.5-fold from the cell-free extract, suggesting that the PGI accounts for ~2% of the soluble cellular protein in *P. furiosus*. The amino-terminal sequence has been identified by Edman degradation: MYKEPFGVKVNFETGIIEGA. This sequence had a perfect match with the N-terminal part of a 21-kDa hypothetical protein from *P. furiosus* as identified from the genome sequence (www.genome.utah.edu).

Heterologous Production and Purification of PGI—The putative 570-base pair PGI-encoding gene (*pgiA*) was PCR amplified and cloned into pET24d, resulting in plasmid pLUW557.

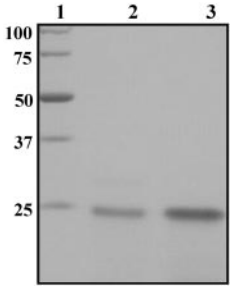

FIG. 1. **SDS-polyacrylamide gel electrophoresis of the purified PGI from *P. furiosus*.** *Lane 1* contained a set of marker proteins with their molecular mass indicated (kDa). *Lane 2* contained the purified PGI from *P. furiosus* cell-free extract. *Lane 3* contained purified recombinant PGI. Proteins were stained with Coomassie Brilliant Blue R-250.

DNA sequence analysis of pLUW557 confirmed that the cloned *pgiA* gene showed the expected sequence. SDS-PAGE analysis of a heat-treated cell-free extract of *E. coli* BL21(DE3) harboring pLUW557 revealed an additional band of 23 kDa which was in good agreement with the calculated molecular mass (21.6 kDa) of the gene product. This band was absent in a heat-treated cell-free extract of *E. coli* BL21(DE3) carrying the pET24d vector without insert, in which no PGI activity was detected (not shown). In a heat-treated cell-free extract of *E. coli* BL21(DE3) harboring pLUW557, a PGI activity of 8.3 units/mg was measured at 50 °C, confirming that the cloned *P. furiosus pgiA* gene indeed encoded a PGI. The recombinant PGI was easily purified by two successive chromatographic steps, *i.e.* anion exchange chromatography and gel filtration. The recombinant enzyme eluted as the native enzyme, and was purified to apparent homogeneity as judged by SDS-PAGE analysis (Fig. 1).

Physical and Biochemical Characterization of PGI—The molecular mass of both the native and recombinant PGI as determined by gel filtration was 49.6 ± 0.3 kDa. SDS-PAGE analysis of the two enzymes resulted in identical bands of 23.5 ± 0.2 kDa, suggesting that the PGI is a homodimer. This homodimeric composition has been observed also for bacterial and eukaryal PGIs, although homotetrameric compositions occur as well. Furthermore, the *P. furiosus* PGI differs from all known PGIs by its subunit molecular mass, which is about half of its canonical counterparts (Table II). Moreover, the *P. furiosus* PGI, the first archaeal PGI described to date, exhibits the lowest pH optimum and highest temperature optimum of all known PGIs (Table II).

The specific activities of the native and the recombinant PGI exhibited similar temperature or pH optima. The *P. furiosus* PGI showed reversible isomerization activity with fructose 6-phosphate and glucose 6-phosphate between pH 6.0 and 8.5, with an optimum at pH 7.0 (not shown). PGI showed maximal activity around 90 °C (Fig. 2). From the Arrhenius plot between 30 and 90 °C, an inactivation energy of 41 kJ/mol was calculated. Thermal inactivation was determined at 60, 70, 80, and

TABLE II
Comparison of PGI from P. furiosus with other PGIs

Domain	Species	T-opt.[a]	pH-opt.	Molecular mass		K_m		Ref.
				Native	Subunit	Fru-6-P	Glu-6-P	
		°C		*kDa*		*mM*		
Archaea								
	P. furiosus	90	7.0	49.3 (α_2)[b]	23.5	0.71	1.57	This work
Bacteria								
	B. caldotenax	77	8–9	202 (α_4)	50.6	ND	2.46	21
	E. coli (I)	ND[b]	8.0	125 (α_2)	59	0.2	ND	22
	E. coli (II)	ND	8.0	230 (α_4)	59	0.2	ND	22
Eucarya								
	A. niger	ND	7.5–10	118 (α_2)	60	0.32	0.48	12
	T. brucei	ND	7.5–9.5	ND	64	0.12	ND	23
	Yeast	ND	7.5–9.5	119 (α_2)	61	0.17	ND	23, 24
	Rabbit	ND	7.5–9.5	125 (α_2)	64	0.12	ND	23, 25

[a] T optimum determined for purified PGI.
[b] The proposed subunit composition of the native enzyme is shown in parentheses.
[c] ND, not determined.

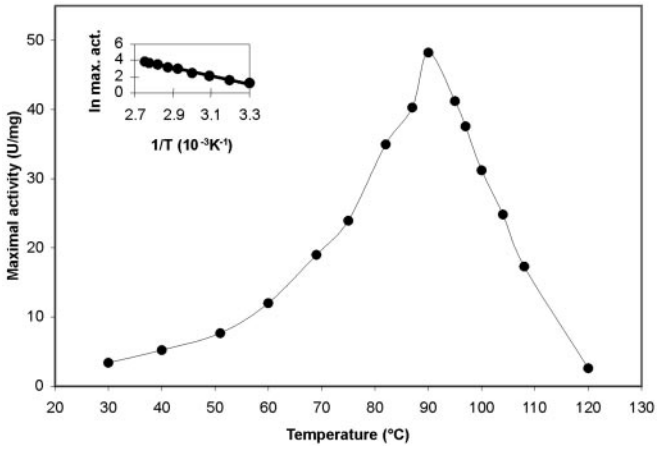

FIG. 2. **Dependence of PGI activity on temperature.** Activity of native PGI was determined by measuring the amount of glucose 6-phosphate formed after incubation for 1, 2, and 3 min at the desired temperature. *Inset*, Arrhenius plot of the data from 30 to 90 °C. Both native and recombinant PGI showed similar behaviors to temperatures (not shown).

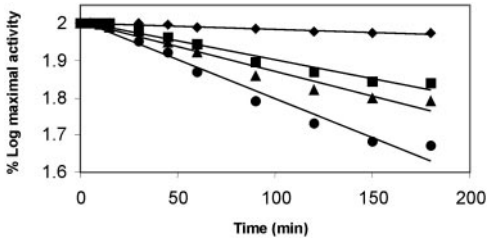

FIG. 3. **Thermal stability of PGI.** The native enzyme (0.0064 mg/ml) was preincubated at 90 °C in 100 mM sodium phosphate buffer, pH 7.0. Residual activity was measured at 50 °C using fructose 6-phosphate as substrate. The 100% activity corresponds to 18.6 units/mg for the native PGI. Thermal inactivation is plotted on logarithmic scale to demonstrate first-order kinetics. The recombinant PGI showed similar inactivation profiles at the respective temperatures as the native PGI (not shown). Half-lifes of 1500, 300, 230, and 143 min were calculated at 60 (♦), 70 (■), 80 (▲), and 90 °C (●), respectively.

90 °C and followed first-order kinetics (Fig. 3). With a half-life of ~2.4 h at 90 °C it is the most thermostable PGI presently known. The second most thermostable PGI is the one from *Bacillus caldotenax*, that exhibits a half-life of ~2 h at 65 °C (21).

The purified enzyme only showed activity in the isomerization of fructose 6-phosphate and glucose 6-phosphate (5 mM), with specific activities at 50 °C of 14.5 and 29.1 units/mg, respectively, pH 7.0. The PGI activity was not affected by addition of cations (Mg^{2+} or Mn^{2+}), nor by addition of 10 mM EDTA to the assay mixture. Under the tested conditions the enzyme did not convert mannose 6-phosphate to fructose 6-phosphate. The PGI from *Escherichia intermedia* has been reported to catalyze the isomerization of non-phosphorylated sugars, like fructose and glucose, but only in the presence of arsenate (26). The purified enzyme from *P. furiosus* was unable to isomerize non-phosphorylated sugars like glucose, fructose, mannose, and galactose both in the absence or presence of cofactors like arsenate and phosphate. This suggests that the phosphoryl group at the C-6 position of fructose 6-phosphate and glucose 6-phosphate plays an important role in substrate recognition of the *P. furiosus* PGI.

The native *P. furiosus* PGI showed Michaelis-Menten kinetics at 50 °C, K_m values of 0.63 ± 0.07 and 1.99 ± 0.11 mM for fructose 6-phosphate and glucose 6-phosphate, respectively, and V_{max} values of 20.1 ± 0.73 and 34.3 ± 0.71 units/mg for fructose 6-phosphate and glucose 6-phosphate, respectively. K_m values and V_{max} values determined for the recombinant PGI were in the same order of magnitude, with K_m values of 0.42 ± 0.03 and 2.00 ± 0.17 mM for fructose 6-phosphate and glucose 6-phosphate, respectively, and V_{max} values of 19.2 ± 0.37 and 47.7 ± 1.40 units/mg for fructose 6-phosphate and glucose 6-phosphate, respectively. The k_{cat}/K_m values for fructose 6-phosphate and glucose 6-phosphate conversion of the native PGI were 11.5 and 6.2 s^{-1} mM^{-1}, and of the recombinant PGI 16.5 and 8.6 s^{-1} mM^{-1}.

The effect of potential inhibitors was tested on the activity of the recombinant PGI (5 mM substrate). The addition of fructose, glucose, mannose, galactose (10 mM), pyruvate, phosphoenolpyruvate (10 mM), AMP, ADP, or ATP (3.5 mM), did not show any effect on the PGI activity neither in the fructose 6-phosphate formation, nor in the glucose 6-phosphate formation. Typical PGI inhibitors like mannose 6-phosphate, fructose 1-phosphate, and fructose 1,6-bisphosphate negatively effected the PGI activity in both directions. Residual activities of 18 and 38% were monitored in the presence of 1.25 mM mannose 6-phosphate, in the direction of fructose 6-phosphate and glucose 6-phosphate formation, respectively. In the presence of 2 mM fructose 1-phosphate residual activities of 50 and 69% were measured, respectively. Finally, the addition of 10 mM fructose 1,6-bisphosphate to the assay mixture resulted in residual activities of 41 and 53%, respectively. Hence, the activity of the *P. furiosus* PGI is inhibited by classical PGI inhibitors (27), and the affinity of the *P. furiosus* enzyme for fructose 6-phosphate and glucose 6-phosphate (determined at 50 °C) was in the same order of magnitude as that of the classical PGIs (Table II). Hence, catalytic properties of the *P. furiosus* PGI resemble that

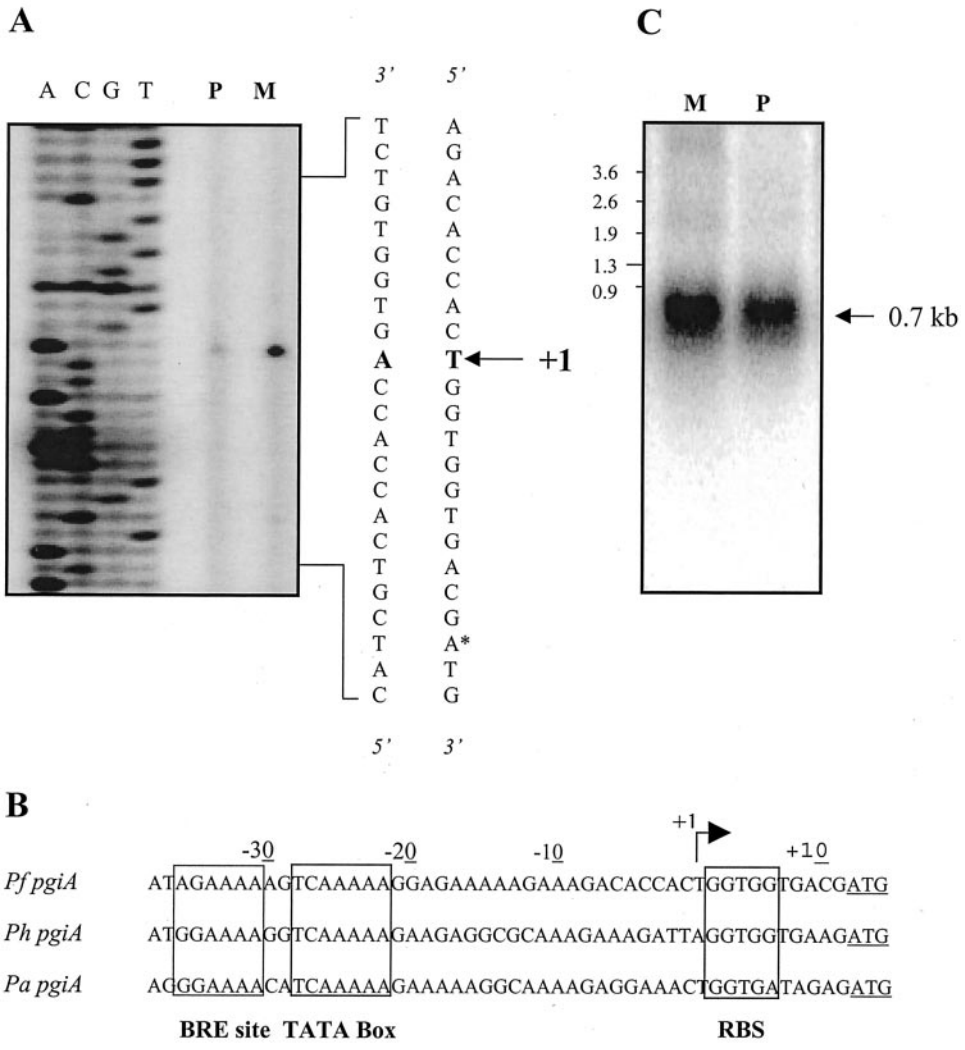

FIG. 4. **Transcript analyses of the *P. furiosus pgiA*.** *A,* mapping of the transcription start. The transcript begins at position +1 (*arrow*), an *asterisk* marks the start codon (ATG) and the sequence ladder (*lanes A, C, G,* and *T*) is shown. *B,* upstream nucleotide sequence of the *P. furiosus pgiA* gene. The transcription factor B recognition element (*BRE site*), putative TATA box element and the ribosome-binding site (*RBS*) is marked. The mapped start site of transcription is marked by an *arrow* and the ATG start codon is *underlined*. Promoter regions of *Ph pgiA* (PH1956) and *Pa pgiA* (PAB1199) are included. *C,* Northern blot analysis. For both primer extension and Northern blot analysis 15 μg of total RNA was used from maltose (*M*) and pyruvate (*P*) grown cells.

of the classical PGIs in most respects. When this paper was being evaluated, Hansen *et al.* (28) independently described a biochemical characterization of the phosphoglucose isomerase from *P. furiosus*, in general revealing features as reported in this study.

Transcript Analysis—For an accurate assignment of the promoter region in *P. furiosus* the transcription start of the *pgiA* mRNA was determined by primer extension. The transcription is initiated at the thymine (T) 11 base pairs upstream of the ATG start codon (Fig. 4*A*). A putative ribosomal binding site was identified at position +2 to +6. A putative TATA box is positioned around −24/−25 of the transcription start, and a clear transcription factor B Recognition Element (BRE site, consensus sequence (A/G)N(A/T)AA(A/T)) (29) is positioned around −33/−34 (Fig. 4*B*).

Northern blot analysis revealed a strong hybridization signal at 0.7 kilobase pairs with the *pgiA* probe, indicating the presence of a monocistronic transcript (Fig. 4*C*). As shown by primer extension (4-fold) and Northern blot analysis (1.5-fold), pgiA transcription is slightly higher under catabolic (maltose) than under anabolic (pyruvate) conditions. Moreover, a 1.7-fold increase of PGI activity was detected when grown on maltose (0.32 units/mg) compared with pyruvate (0.19 units/mg). Similar observations were made for the reversible fructose-1,6-bisphosphate aldolase and phosphoenolpyruvate synthetase from *P. furiosus* (30, 31). This might suggest a different flux through the pathway when used in the anabolic or in the catabolic direction.

Structural Analysis—The amino acid sequence of PGI has full-length homologs with high levels of sequence identity (90 and 91% for *Pyrococcus abyssi* and *Pyrococcus horikoshii*, respectively) in the other two *Pyrococci*, suggesting that these genes most likely also function as PGIs. Homology with other sequences is limited to positions 66 to 152 of the *P. furiosus* PGI (Fig. 5). Using profile based sequence comparisons (PSI-Blast, 9 iterations, $E < 0.002$) this area can be shown to be homologous to a wide range of proteins belonging to the cupin superfamily, that consists of a variety of proteins that are generally involved in sugar metabolism in both prokaryotes and eukaryotes (33). The molecular function of this cupin domain (consensus, $PG(X)_5HXH(X)_4E(X)_7G$ and $G(X)_5PXG(X)_2H(X)_3N$) is generally the binding of carbohydrates, and in some cases apparently to establish an interaction with other proteins (33, 34). Among the homologs are two additional hypothetical proteins from *Pyrococcus* itself (PF_396648 and PF_62346), as well as several type-2 mannose-6-phosphate isomerases, oxalate de-

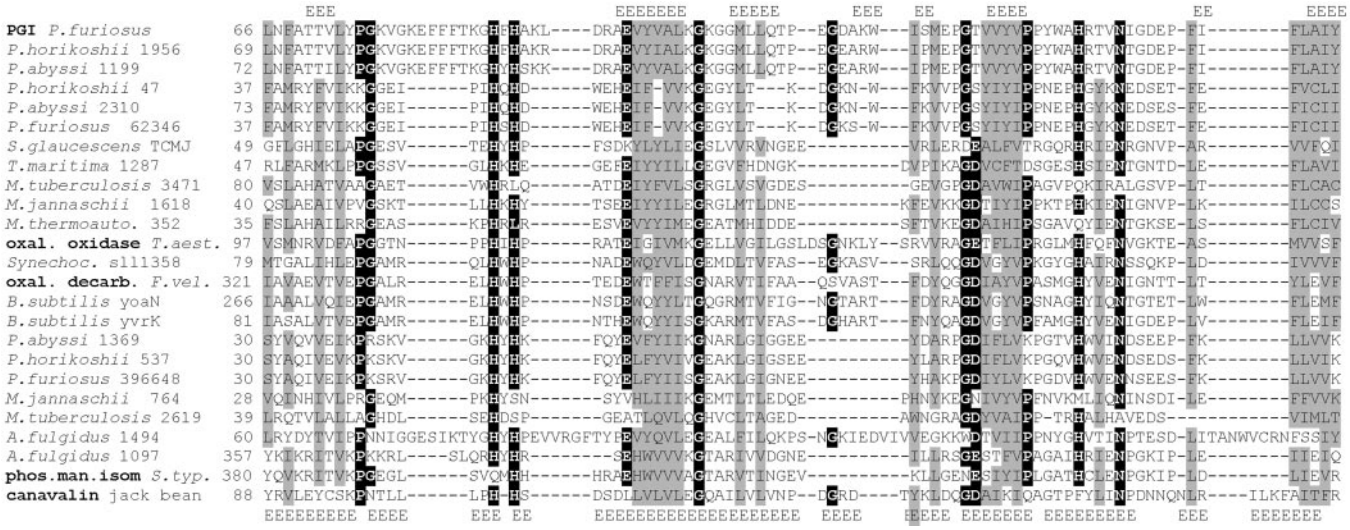

FIG. 5. **Alignment of the PGI of *P. furiosus* with: 1) its most similar homologs (PSI-Blast 5 iterations $E < 0.002$) from completely sequenced genomes, 2) sequences with experimentally determined function, and 3) canavalin of which a three-dimensional structure is available (32).** A secondary structure (above the alignment, *E* denotes β-strand) is consistent with the secondary structure of canavalin (below the alignment). With each sequence is given the number of its gene in the genome. The species abbreviations with the GenBank™ identifiers of the sequences: *P. furiosus* = *P. furiosus* (AF381250); *P. horikoshii* = *P. horikoshii* (g3258400 g3256432 g3256943); *P. abyssi* = *P. abyssi* (g5459164 g5457489 g5458926); *A. fulgidus* = *Archaeoglobus fulgidus* (g2649077 g2649495); *M. jannaschii* = *Methanococcus jannaschii* (g1499583 g1592216); *M. tuberc.* = *M. tuberculosis* = *Mycobacterium tuberculosis* (g2104394 g2113903); *T. aest.* = *Triticum aestivum* (g121129); *B. subtilis* = *Bacillus subtilis* (g2635821 g2634260); *Synechoc.* = *Synechocystis* (g1652630); *F. vel.* = *Flammulina velutipes* (g6468006); *S. typ.* = *Salmonella typhimurium* (g117277); *T. maritima* = *Thermotoga maritima* (g4981845); *S. glaucescens* = *Streptomyces glaucescens* (g153495); *M. thermo* = *Methanobacterium thermoautotrophicum* (g2621410); *oxal. decarb.* = oxalate decarboxylase; *phos.man.isom.* = phosphomannose isomerase; *jack bean* = *Canavalia ensimorfis*. The *P. furiosus* sequences are available from www.genome.utah.edu/sequence.html. Conserved amino acids are shaded *black*, conserved hydrophobic positions are *shaded gray*. The alignment was generated with T-Coffee (17) followed by small, manual refinements.

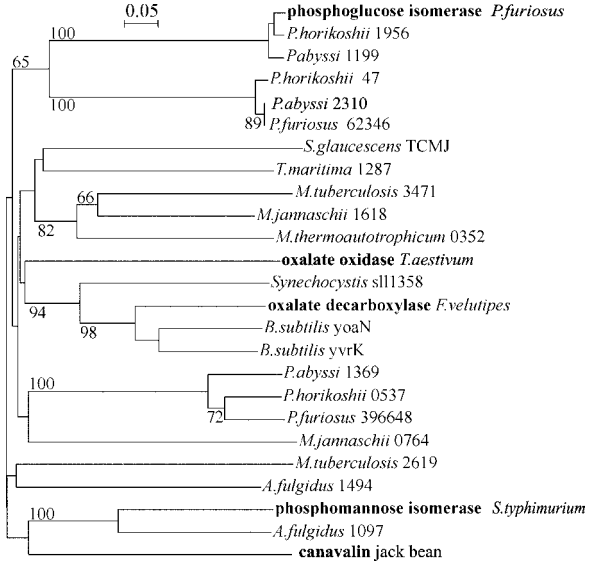

FIG. 6. **Neighbor joining tree of the aligned sequences.** The tree was generated with clustalX. Bootstrap values above 60 out of 100 are indicated. The genes *PH1956* from *P. horikoshii* and *PAB1199* from *P. abyssi* are clearly orthologous to the PGI from *P. furiosus*. No other orthologous are present in currently available genomes.

carboxylases, oxalate oxidases (germin), seed storage protein, canavalin (Figs. 5 and 6), as well as sugar-binding transcriptional regulators of the AraC family (33). No proteins with PGI activity have been reported to belong to this family before.

For a number of sequences in this family a crystal structure has been determined (*e.g.* canavalin), revealing that the cupin domain has a typical double-stranded β-helix, forming a barrel (32, 33). Based on an alignment of PGI with its closest homologs, a secondary structure prediction has been performed using Profile-based neural network system from HeiDelberg (20), confirming that PGI is homologous to canavalin (Fig. 5). The structure of canavalin does not resemble that of the classic PGIs, because it does not contain a sugar isomerase (SIS) domain that is typical for PGI (scop.mrc-lmb.cam.ac.uk/scop/). Comparisons both at the sequence level and at the structure level therefore indicate that the PGI from *Pyrococcus* has evolved independently from the classic PGI from bacteria (Fig. 6), and hence most likely is an example of convergent evolution.

Recruitment of Enzymes in Unique "Top" Glycolysis—The identification of PGI allows a comparison of the nine-enzyme glycolysis in *Pyrococcus* with the classical 10-enzyme glycolysis in bacteria and eucarya. Notably four of the nine pyrococcal enzymes, that were identified experimentally, are non-homologous to their classical counterparts. Here we have shown, based on sequence comparison and on structural data, that the *P. furiosus* PGI (the second step in glycolysis) is not homologous to the bacterial and eukaryal PGI. The other five enzymes (fructose-1,6-bisphosphate aldolase, triose-phosphate isomerase, phosphoglycerate mutase, enolase, and pyruvate kinase) have been predicted on the basis of orthology with bacterial proteins (9). Four of these five are orthologous to their bacterial counterparts in the glycolysis. The fifth, fructose-1,6-bisphosphate aldolase, is not orthologous to the standard bacterial class II aldolase (35). This aldolase has recently been proposed to constitute a new family of aldolases, archaeal type Class I aldolase (Class IA), that is rare in bacteria and abundant in archaea, and only distantly related to Class I fructose-1,6-bisphosphate aldolases (31).

The question remains whether or not a complete glycolytic pathway existed at the time that the non-homologous enzymes evolved in *Pyrococcus*; in other words, was (part of) the glycolytic pathway introduced by these newly evolving enzyme activities, or was it rather a substitution of their classical counterparts. Two patterns in these non-homologous replacements argue for an independent invention of the glycolysis that, made use of enzymes of an incomplete glyconeogenic pathway (from pyruvate to fructose 1,6-bisphosphate) that was already pres-

ent: (i) three of the unique glycolytic steps in *Pyrococcus* are specifically catabolic (ADP-GLK, ADP-PFK, and glyceraldehyde-3-phosphate ferredoxin oxidoreductase); (ii) the first three unique steps (catalyzed by ADP-GLK, PGI, and ADP-PFK) form the part of the pathway that is rather *specific* for glucose degradation, whereas the more conserved part of the pathway (the interconversion of glyceraldehyde 3-phosphate and pyruvate) is made up by a more *general* set of enzymes that are potentially involved in numerous metabolic routes. This would argue for an independent invention of the glycolytic pathway in the lineage leading to *Pyrococcus*. Although nonhomologous displacement of enzymes in *Pyrococcus* central carbohydrate metabolism has been observed before (36), this would be, to our knowledge, the first example of such excessive replacement of enzymes in a pathway, and is a compelling example of convergent evolution.

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The Phosphoglucose Isomerase from the Hyperthermophilic Archaeon*Pyrococcus furiosus* Is a Unique Glycolytic Enzyme That Belongs to the Cupin Superfamily

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