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ORIGINAL PAPER

Characterization of cofactor-dependent and cofactor-independent phosphoglycerate mutases from Archaea

Ulrike Johnsen · Peter Schönheit

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Abstract Phosphoglycerate mutases (PGM) catalyze the reversible conversion of 3-phosphoglycerate and 2-phosphoglycerate as part of glycolysis and gluconeogenesis. Two structural and mechanistically unrelated types of PGMs are known, a cofactor (2,3-bisphosphoglycerate)-dependent (dPGM) and a cofactor-independent enzyme (iPGM). Here, we report the characterization of the first archaeal cofactor-dependent PGM from *Thermoplasma acidophilum*, which is encoded by ORF TA1347. This ORF was cloned and expressed in *Escherichia coli* and the recombinant protein was characterized as functional dPGM. The enzyme constitutes a 46 kDa homodimeric protein. Enzyme activity required 2,3-bisphosphoglycerate as cofactor and was inhibited by vanadate, a specific inhibitor of dPGMs in bacteria and eukarya; inhibition could be partially relieved by EDTA. Histidine 23 of the archaeal dPGM of *T. acidophilum*, which corresponds to active site histidine in dPGMs from bacteria and eukarya, was exchanged for alanine by site directed mutagenesis. The H23A mutant was catalytically inactive supporting the essential role of H23 in catalysis of the archaeal dPGM. Further, an archaeal cofactor-independent PGM encoded by ORF AF1751 from the hyperthermophilic sulfate reducer *Archaeoglobus fulgidus* was characterized after expression in *E. coli.* The monomeric 46 kDa protein showed cofactor-independent PGM activity and was stimulated by Mn^{2+} and exhibited high thermostability up to 70°C. A comprehensive phylogenetic analysis of both types of archaeal phosphoglycerate mutases is also presented.

Keywords Phosphoglycerate mutases · Archaea · *Thermoplasma acidophilum* · *Archaeoglobus fulgidus*

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U. Johnsen · P. Schönheit (✉)
Institut für Allgemeine Mikrobiologie,
Christian-Albrechts-Universität Kiel,
Am Botanischen Garten 1-9, 24118 Kiel, Germany
e-mail: peter.schoenheit@ifam.uni-kiel.de

Introduction

Phosphoglycerate mutase (PGM, EC 5.4.2.1) catalyzes the reversible interconversion of 3-phosphoglycerate and 2-phosphoglycerate in both glycolysis and gluconeogenesis. Two distinct types of PGM have been described, which are structurally and mechanistically unrelated. One type, the cofactor-dependent or dPGM, requires the cofactor 2,3-bisphosphoglycerate for activity, while the other type, the cofactor-independent or iPGM, does not require this cofactor. In vertebrates, yeasts and several bacteria dPGMs are predominant or the only PGM type, whereas iPGMs are found in plants, nematodes and many bacteria (Grana et al. 1995; Fothergill-Gilmore and Watson 1989; Zhang et al. 2004; Jedrzejas 2000). Several bacteria were found to contain both iPGM and dPGM, e.g., in *E. coli* differential expression of both enzymes has recently been demonstrated (Fraser et al. 1999). dPGMs belong to the dPGM superfamily, which comprise, e.g., acid phosphatases and fructose 2,6-bisphosphatases. All dPGM sequences contain a conserved active site histidine, which is phosphorylated during the catalytic cycle (Jedrzejas 2000). A specific property of dPGMs is the reversible inhibition by vanadate in the micromolar range (Bond et al. 2002; Carreras et al. 1980). iPGMs belong to the alkaline phosphatase superfamily, which comprises a variety of metalloenzymes, with diverse function such as phosphopentomutases, alkaline phosphodiesterases and sulfatases (Galperin et al. 1998;

Galperin and Jedrzejas 2001). iPGMs are structurally unrelated to dPGM and thus represent a convergent line of PGM evolution.

Although quite detailed information of PGMs are available from members of Eukarya and Bacteria, the knowledge about PGMs from Archaea is limited. PGM activities have been demonstrated in extracts of various Archaea, involved in the modified glycolytic pathways and in gluconeogenesis (Siebers and Schönheit 2005; Ronimus and Morgan 2003). However, archaeal cofactor-independent iPGMs have been identified only recently, in *Pyrococcus furiosus* (PF1959) and *Methanococcus jannaschii* and *Sulfolobus solfataricus* (Galperin et al. 1998; Van der Oost et al. 2002; Graham et al. 2002; Koonin et al. 1997; Potters et al. 2003). So far, cofactor-dependent dPGMs in archaea have not been analysed. Putative dPGMs encoding genes have been predicted for *Sulfolobus* and *Thermoplasma* species (Van der Oost et al. 2002), but none of them have been shown to code for functional dPGMs.

In this communication we report the biochemical characterization of the first archaeal cofactor-dependent dPGM, the gene product of ORF TA1347 from *Thermoplasma acidophilum*. The recombinant enzyme was characterized as functional dPGM; it showed strict dependence of activity for the cofactor 2,3-bisphosphoglycerate, it was reversibly inhibited by vanadate and contained catalytically essential histidine as demonstrated by site directed mutagenesis studies. Further, a functional cofactor-independent iPGM from the archaeal sulfate reducer *Archaeoglobus fulgidus* was characterized. Finally, comprehensive phylogenetical analyses of both archaeal dPGMs and iPGMs were performed using recent available sequence information from NCBI and SwissProt.

Materials and methods

Cloning of TA1347, overexpression in *E. coli* and purification of recombinant *T. acidophilum* dPGM

The ORF TA1347 from *T. acidophilum* (DSM 1728), annotated as putative dPGM encoding gene, was amplified by PCR and cloned into pET17b via two restriction sites (*Nde*I, *BamH*I) using the primers 5′CCGTATACATATG ATATATAAATTTC′3 and 5′CTGTGAGGATCCGATC TATTG′3 (restriction sites are underlined). The vector pET17b-TA1347 was transformed into *E. coli* BL21 codon plus(DE3)-RIL (Stratagene). For expression, cells were grown in Luria–Bertani medium at 37°C. The expression was initiated by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration 0.4 mM). After 18 h of further growth cells were harvested by centrifugation. The pellet was suspended in 50 mM Tris–HCl, pH 8, containing 2 mM EDTA and passed four times through a French press cell. After centrifugation (48,000×*g*, 4°C, 30 min) the supernatant was heat precipitated at 55°C for 30 min, followed by an additional centrifugation step. An aliquot of the supernatant (18 mg protein) was applied to Superdex 200 HiLoad column equilibrated with buffer a (0.1 M Tris–HCl, pH 7.4, containing 150 mM NaCl). Protein was eluted at a flow rate of 1 ml/min. The fractions containing the highest PGM activity were pooled and applied to an Uno Q1 column previously equilibrated with buffer b (50 mM Tris–HCl, pH 8.5). Protein was eluted at a flow rate of 2 ml/min with a linear NaCl gradient from 0 to 2 M NaCl in buffer b. At this stage fractions containing the highest PGM activity were essentially pure. Purified enzyme was stored at –20°C.

Construction of *T. acidophilum* dPGM mutant

The *dpg*m gene, which has been cloned into vector pET17b, was mutated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). For introduction of the mutation H23A into pET17b-TA1347 the forward primer 5′-ATCGCTATACTTATAAGGGCTGGGGAGAGCGAT ATCAATGTC-3′ and the reverse primer 5′-GACATTGAT ATCGCTCTCCCCAGCCCTTATAAGTATAGCGAT-3′ were used. The mutated bases are underlined. The mutant was expressed in *E. coli* and purified as described for the wild type dPGM.

Cloning of AF1751, overexpression in *E. coli* and purification of recombinant *A. fulgidus* iPGM

ORF AF1751 predicted as putative iPGM encoding gene was amplified from genomic DNA of *A. fulgidus* strain VC16 by PCR and cloned into pET19b, containing a N-terminal His-tag, via two restriction sites (*Nde*I, *BamH*I) created with the primers 5′-GAAAGGCTGAGGTGAT CATATGCCTGTAC-3′ and 5′-CAGTCAAGATAAGGA TCCGTTATGAGGAAG-3′ (restriction sites are underlined). The vector pET19b-AF1751 was transformed into *E. coli* BL21 codon plus(DE3)-RIL. For expression of AF1751 cells were grown in Luria–Bertani medium at 37°C. The expression was initiated by the addition of IPTG (final concentration 1 mM). After 3 h of further growth cells were harvested by centrifugation. The pellet was suspended in buffer c (20 mM Tris–HCl, pH 8.2, containing 0.3 M NaCl and 4 mM imidazole) and passed three times through a French press cell. After centrifugation (48,000×*g*, 4°C, 30 min), the supernatant was heat precipitated at 75°C for 30 min, followed by an additional centrifugation step. The supernatant was applied to Nickel-NTA equilibrated with buffer c. Protein was eluted at a flow rate of 1 ml/min with three increasing imidazole

steps: 4–20 mM, 20–50 mM and 50–500 mM imidazole. The fractions containing the highest enzyme activity were applied to a Superdex 200 HiLoad column equilibrated with buffer a. Protein was eluted at a flow rate of 1 ml/min. At this stage fractions containing the highest phosphoglycerate mutase activity were essentially pure. Purified enzyme was stored at 4°C.

Analytical assays

The purity of enzyme preparations was checked by SDS-PAGE in 12% gels followed by staining with Coomassie Brilliant Blue R250. Protein concentrations were determined by the method of Bradford with BSA as standard. Gel filtration chromatography was carried out with a flow rate of 1 ml/min on a Superdex 200 HiLoad column. The column was equilibrated with buffer a. Cytochrome c (12.4 kDa), carboanhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa) and amylase (200 kDa) were used as the standards (Sigma).

Detection of iPGM transcription in *A. fulgidus* by RT-PCR

For preparation of RNA, *A. fulgidus* strain VC16 (DSM 4304) was grown at 83°C on lactate/sulfate medium as described by Möller-Zinkhan et al. (Möller-Zinkhan and Thauer 1990). At late exponential phase cells were cooled to 4°C, harvested by centrifugation at 10,000×*g* for 20 min. Cells were disrupted by freezing and crushing under liquid nitrogen and passing through a Qiashredder column (Qiagen). RNA was extracted from 5×10^9 cells by using the RNeasy isolation kit (Qiagen). RT-PCR was carried out by the Qiagen oneStep RT-PCR kit. For the RT-PCR, primers of 25 bp, 5′-GTGCCTG-TACTGCTGATTGTTGTTG-3′ (sense) and 5′-CTTCGCAATGTTCAGCAGATCAAGG-3′ (antisense) directed against the ends of the ORF AF1751 were used.

Enzyme assays for PGM activity

The activitity of dPGM from *T.acidophilum* and of iPGM form *A. fulgidus* were determined at 50°C in both reaction directions [3-phosphoglycerate (3PGA) ↔ 2-phosphoglycerate (2PGA)] using the following continuous assays. It was ensured that the auxiliary enzymes were not rate-limiting. One unit (U) of enzyme activity is defined as 1 μmol product formed per min.

dPGM activity

The formation of 2PGA from 3PGA was determined by measuring NADH oxidation at 365 nm in an assay mixture containing 0.1 M HEPES-KOH, pH 7.5, 2 mM 3PGA (purified), 1 mM ADP, 5 mM $MgCl_2$, 0.3 mM NADH, 100 μM 2,3-bisphosphoglycerate (2,3PGA), 0.08 U enolase, 0.4 U pyruvate kinase, 0.5 U lactate dehydrogenase and protein. The formation of 3PGA from 2PGA was determined by measuring NADH oxidation at 365 nm in an assay mixture containing 0.1 M HEPES-KOH, pH 7.5, 1 mM 2PGA, 100 μM 2,3PGA, 5 mM $MgCl_2$, 2 mM ATP, 2 mM phosphoenolpyruvate, 0.3 mM NADH, glycerate kinase, 0.4 U pyruvate kinase, 0.5 U lactate dehydrogenase and protein.

Effect of vanadate and EDTA on dPGM activity

The effect of vanadate on dPGM activity was tested by adding 5–100 μM vanadate to the assay mixture following 3PGA formation from 2PGA (see above). After preincubation (5 min) of the protein with vanadate, the reaction was started by the addition of 2PGA. After 2 min EDTA (2.5 mM) was added.

2,3PGA dependence of dPGM activity

The dependence of dPGM on the cofactor 2,3PGA was tested using 3PGA as substrate, from which contaminating 2,3PGA has been removed. Commercially available 3PGA contained 2,3PGA as contamination and was therefore not suitable for prove 2,3PGA dependence of dPGM activity. 3PGA free of 2,3PGA was prepared according to Towne et al. (1957) as follows: 5 g of barium salt of 3PGA (Sigma) was dissolved in 7 ml H_2O and 3 ml 10 N H_2SO_4. After centrifugation at 5,000×*g* for 10 min the pellet was washed twice with 9 ml H_2O. The supernatant solutions were pooled, diluted to 730 ml (20 mM 3PGA, pH 1.85) in H_2O and applied to a Dowex 1-X8 (400 mesh) column (5 ml). 3PGA was eluted with 40 ml H_2O and was concentrated to one-tenth of its volume (speed vac). After adjustment to pH 4 with KOH and addition of excess of $BaCl_2 \times 2\ H_2O$, two volumes of 95% ethanol were added to the solution. The barium salt of 3PGA was obtained after crystallization at –20°C for several hours by filtration of the crystallization fluid through to a 0.2 μm filter. The crystals were washed with 100 ml 65% ethanol and 50 ml 95% ethanol and dried under vacuum. The crystals were dissolved in 1 M HCl and barium ions were precipitate as $BaSO_4$ with saturated Na_2SO_4 solution. The concentration of purified 3-phosphoglycerate was determined at 37°C by measuring the amount of NADH oxidized at 365 nm in an assay mixture containing 0.1 M Tris–HCl, pH 8, 2 mM ADP, 5 mM $MgCl_2$, 0.3 mM NADH, 0.05 U phosphoglycerate mutase, 0.08 U enolase, 0.4 U pyruvate kinase, 0.5 U lactate dehydrogenase and various limiting amounts of the 3PGA solution.

iPGM activity

The formation of 2PGA from 3PGA was determined by measuring NADH oxidation at 365 nm in an assay mixture containing 0.1 M Tris–HCl, pH 7, 3 mM 3PGA, 5 mM $MgSO_4$, 50 μM $MnCl_2$, 1 mM ADP, 0.3 mM NADH, 0.08 U enolase, 0.4 U pyruvate kinase, 0.5 U lactate dehydrogenase and protein. The formation of 3PGA from 2PGA was determined by measuring NADH oxidation at 365 nm in an assay mixture containing 0.1 M Tris–HCl, pH 7, 2 mM 2PGA, 5 mM $MgSO_4$, 50 μM $MnCl_2$, 1 mM ADP, 1 mM phosphoenolpyruvate, 0.3 mM NADH, glycerate kinase, 0.4 U pyruvate kinase, 0.5 U lactate dehydrogenase and protein. The effect of Mn^{2+} (10 μM to 1 mM) on the activity was tested in the direction of 3PGA formation in an assay system containing 5 mM Mg^{2+} to ensure that the auxiliary enzyme pyruvate kinase was not rate limiting.

pH dependence and thermostability of iPGM

The pH dependence of iPGM was measured between 6 and 7.7 at 50°C using either 0.1 M bis–Tris (pH 6–6.7) or 0.1 M Tris–HCl (pH 7–7.7). The thermostability of the purified enzyme was tested in sealed vials, containing 8 μg protein in 20 μl 0.1 M triethanolamine, pH 7 (at the respective temperature), with or without 50 μM $MnCl_2$ and 5 mM $MgCl_2$. The vials were incubated at temperatures between 60 and 85°C, cooled on ice for 10 min and the remaining enzyme activities were tested at 50°C in the assay of 2PGA formation as described above.

Results

dPGM from *T. acidophilum*

Cloning, functional overexpression and purification of dPGM

ORF TA1347, predicted to encode a putative dPGM in *T. acidophilum*, consists of 642 bp coding for a polypeptide of 241 amino acids with a calculated molecular mass of 23.8 kDa. To prove its coding function TA1347 was cloned into the vector pET17b followed by transformation in BL21(DE3)CodonPlus-RIL. After induction of the cells with IPTG, a polypeptide of 24 kDa was overexpressed. Protein was purified to electrophoretic homogeneity by heat treatment and chromatographic steps using gel filtration and UnoQ1. The purified recombinant enzyme showed dPGM activity of about 12 U/mg (50°C) in the direction of 3PGA formation.

Molecular and catalytic properties of dPGM

The apparent molecular mass of the native protein was determined by gel filtration and was about 46 kDa. SDS-PAGE revealed only one subunit with an apparent molecular mass of 23 kDa, indicating a dimeric α_2 structure of the native dPGM. Kinetic constants of the purified recombinant dPGM for substrates were determined for both directions. Rate dependence (at 50°C) on 3PGA and 2PGA followed Michaelis–Menten kinetics with V_{max} and K_m values of 14 ± 2 U/mg and 3.3 ± 1.1 mM, and 12 ± 0.3 U/mg and 1.1 ± 0.06 mM, respectively.

Proof of cofactor 2,3PGA dependence of dPGM activity

Using commercially available 3PGA, which contains contaminating levels of 2,3PGA, a stimulation of PGM activity by added 2,3PGA was not observed. However, using the purified 3PGA, free of 2,3PGA (for preparation see ''Materials and methods''), a strict dependence of PGM activity on 2,3PGA could be demonstrated thus defining the PGM as dPGM. Rate dependence of PGM activity on the 2,3PGA followed Michaelis–Menten kinetics with an apparent K_m value of about 17 ± 0.7 μM and an apparent V_{max} of 14 ± 0.3 U/mg (Fig. 1).

Inhibition of dPGM activity by vanadate

dPGMs from eukarya and bacteria have been described to be specifically inhibited by vanadate which can be reversed by EDTA (Carreras et al. 1980). The effect of vanadate and of EDTA on the dPGM activity from *T. acidophilum* was tested. Addition of vanadate (up to 100 μM) resulted in a maximal inhibition of dPGM of 90%. Inhibition by vanadate could be partially reversed by EDTA (tested at 2.5 mM); at 20 μM vanadate inhibition was reversed by 40%. Thus, the archaeal dPGM showed similar features as reported for the bacterial and eukaryal counterparts.

Histidine 23 is essential for dPGM activity

Sequence alignment of *T. acidophilum* dPGM with bacterial and eukaryal dPGMs (see Fig. 3; ''Discussion'') revealed that histidine 23 corresponds to the conserved active site histidine proposed to be essential for dPGM catalysis via a phosphohistidine enzyme intermediates. To assess the importance of His23 on *T. acidophilum* dPGM activity the amino acid was exchanged for alanine by site directed mutagenesis. The H23A mutant, purified as homodimeric protein of 23 kDa subunits as the wild type enzyme, did not show any activity indicating that histidine 23 is essential for dPGM catalysis.

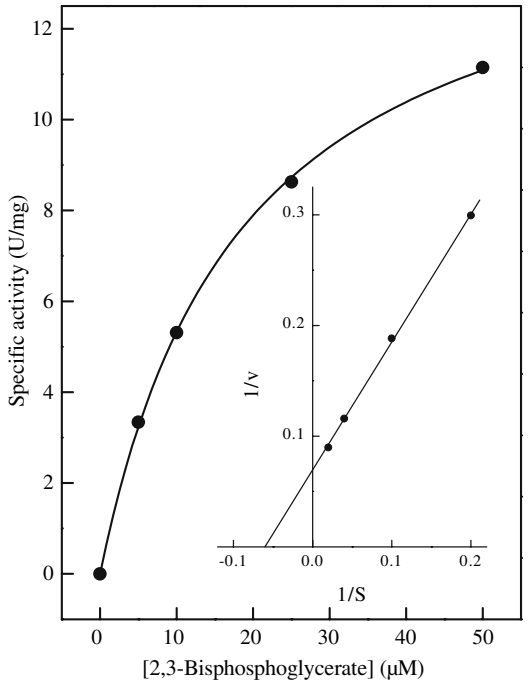

Fig. 1 Effect of 2,3-bisphosphoglycerate on the activity of the dPGM from *T. acidophilum*. Rate dependence on the concentration of 2,3PGA concentration is shown. The *insert* shows the double-reciprocal plot of the rates versus the substrate concentration. The assay mixture contained 0.75 mM 3-phosphoglycerate and 2.6 μg protein

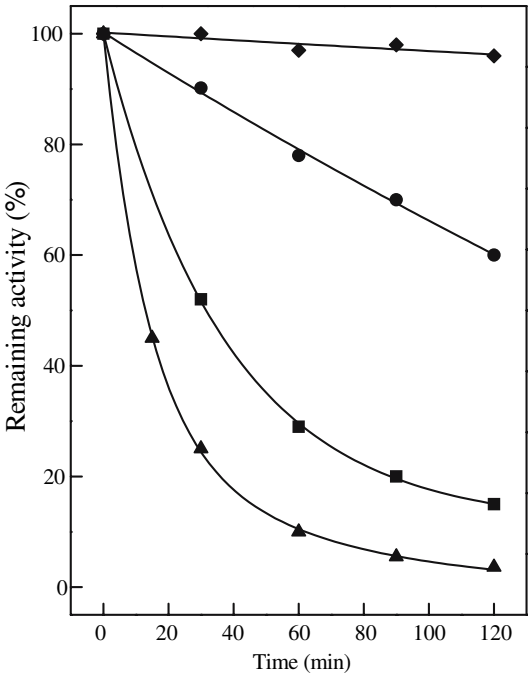

Fig. 2 Thermostability of iPGM from *Archaeoglobus fulgidus* VC16. Enzyme (10.7 μg) was incubated in 30 μl of 100 mM triethanolamine pH 7 containing 50 μM $MnCl_2$ and 5 mM $MgCl_2$, at 60°C (*filled diamond*), 70°C (*filled circle*) and 85°C (*filled triangle*), or without $MnCl_2$ and $MgCl_2$ at 70°C (*filled square*). At the times indicated, 20 μl aliquots were assayed for remaining activity at 50°C in the direction of 2-phosphoglycerate formation. One hundred percent activity corresponded to the specific activity of PGM of 2.7 U/mg

iPGM from *A. fulgidus*

Cloning, functional overexpression and purification of iPGM

The ORF AF1751, proposed to encode a putative iPGM in *A. fulgidus*, contains 1,224 bp coding for a polypeptide of 408 amino acids with a calculated molecular mass of 44.1 kDa. To prove its coding function, ORF AF1751 was overexpressed as His-tagged 48 kDa protein in *E. coli*. The enzyme was purified to homogeneity by heat treatment and two chromatographic steps, and showed iPGM activity of 3.3 U/mg (50°C) in the direction of 2PGA formation.

Molecular and catalytic properties of iPGM

The apparent molecular mass of the native protein was determined by gel filtration and was about 46 kDa. SDS-PAGE revealed only one subunit with an apparent molecular mass of 48 kDa, indicating that iPGM is a monomeric protein. Kinetic constants of the purified recombinant iPGM for substrates were determined for both directions. Rate dependence (at 50°C) on 3PGA and 2PGA followed Michaelis–Menten kinetics with V_{max} and K_m values of 3.3 ± 0.2 U/mg and 0.8 ± 0.1 mM, and 0.8 ± 0.01 U/mg and 0.2 ± 0.01 mM, respectively. iPGM activity was 2–3-fold stimulated by Mn^{2+} (50 μM), in the presence of 5 mM Mg^{2+}. The pH optimum, measured with 2PGA as substrate, was at pH 7.1. About 40 and 70% of activity were found at pH 6 and 7.7, respectively. The temperature stability of iPGM was tested between 60 and 85°C by incubating the enzyme at pH 7 up to 120 min, in a buffer containing 5 mM $MgCl_2$/50 μM $MnCl_2$. After 120 min of incubation at 60°C, the enzyme did not lose activity. At 70°C and at 85°C the enzyme showed a half-life of 150 and 15 min, respectively. For comparison, iPGM was incubated at 70°C in the absence of Mg^{2+}/Mn^{2+}, under these conditions the half life of the enzyme decreased from 150 to 30 min, indicating that these cations significantly stabilize iPGM activity again heat inactivation (Fig. 2).

In vivo transcription of iPGM

The in vivo transcription of ORF AF1751 in lactate/sulfate grown *A. fulgidus* was demonstrated by RT-PCR experiments. A specific cDNA band of the expected length was detected (not shown), indicating an in vivo transcription of iPGM in *A. fulgidus* under gluconeogenetic growth conditions.

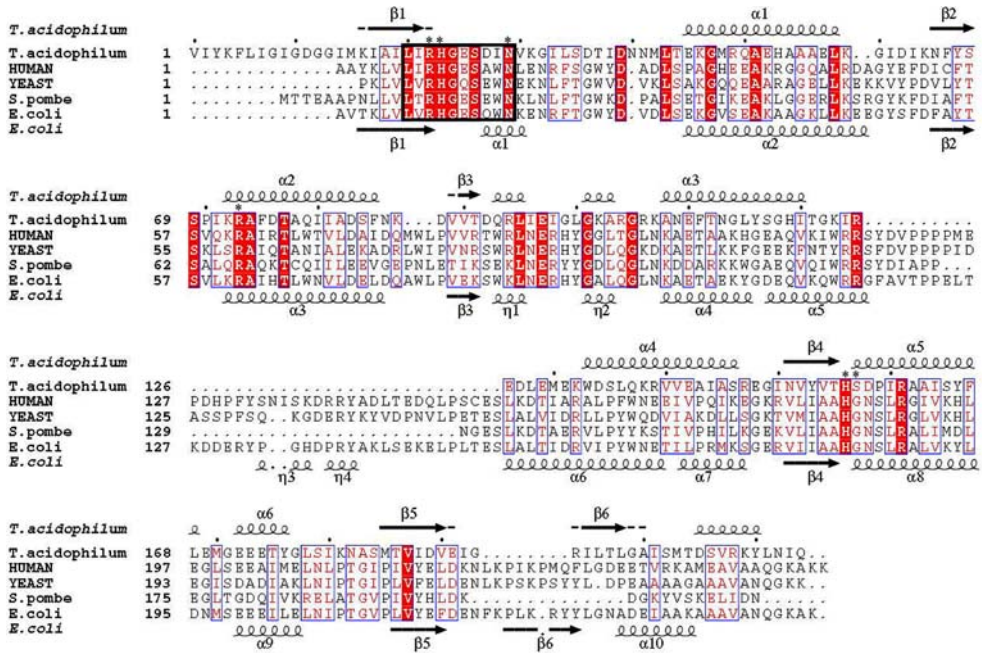

Fig. 3 Multiple amino acid sequence alignment of dPGM from *T. acidophilum* and selected dPGM sequences from bacteria and eukarya. The alignment was generated by ClustalX and ESPript v. 2.2 (Gouet et al. 1999; Thompson et al. 1997). The predicted secondary structure elements (Jones 1999) of *T. acidophilum* dPGM are shown above the alignment (*arrows* for *β*-strands, *coils* for helices) and are in accordance with the structure based secondary structure elements of *E. coli* dPGM (shown below the alignment). Catalytic essential residues are marked with *asterisks* (Bond et al. 2001). The consensus pattern [LIVM]–x–R–H–G–[EQ]–x—{Y}–x—N is indicated by a *box*. NCBI accession numbers of the sequences: *E. coli* P31217; Human P18669; *S. pombe* P36623; *T. acidophilum* CAC12468; Yeast P00950

Discussion

In the present communication we describe the first characterization of an archaeal cofactor-dependent phosphoglycerate mutase (dPGM) from the thermoacidophile *T. acidophilum*. Further, an archaeal cofactor-independent iPGM was characterized from the hyperthermophilic sulfate reducer *A. fulgidus*. The biochemical properties and the phylogeny of both types of archaeal phosphoglycerate mutases will be discussed.

Archaeal dPGM from *T. acidophilum*

Biochemical properties

ORF TA1347 from *T. acidophilum*, annotated as putative dPGM gene, was expressed in *E. coli* and the recombinant protein was characterized as functional dPGM. This first characterized archaeal dPGM showed similar biochemical features as reported for bacterial and eukaryal dPGMs.

(1) The enzyme is a 46 kDa homodimeric protein composed of 23 kDa subunits. Most characterized dPGMs from bacteria and eukarya are also dimeric enzymes of similar size (Jedrzejas 2000). A different oligomeric state was reported for dPGMs in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, which constitute homotetrameric and monomeric proteins, respectively (Fothergill-Gilmore and Watson 1989; Uhrinova et al. 2001; Price and Jaenicke 1982). (2) PGM activity showed strict dependence on the cofactor 2,3-bisphosphoglycerate (2,3PGA) with the apparent K_m of 17 μM**,** which is similar to the K_m values of dPGM, e.g., from yeast (White and Fothergill-Gilmore 1992) (3) dPGM activity was inhibited by micromolar concentrations of vanadate. Inhibition by vanadate could be partially relieved by EDTA. The reversible inhibition by vanadate has been described to be a specific property of dPGMs in bacteria and eukarya (Carreras et al. 1980; Fraser et al. 1999). A competitive effect of the inhibitor on the proposed phosphoryl transfer to active site histidine in the catalytic cycle of dPGM has been discussed (Fothergill-Gilmore and Watson 1989; Fraser et al. 1999; Carreras et al. 1980). (4) Sequence analysis (see sequence alignment, Fig. 3) of *Thermoplasma* dPGM revealed that histidine 23 corresponds to the conserved active site histidine in known bacterial and eukaryal dPGM. These invariant His residues have been proposed to be essential as phosphoryl-acceptor during the catalytic cycle, forming a phosphohistidine-enzyme intermediate. Exchange of His23 for alanine resulted in a complete loss of activity, supporting the essential role of this histidine in dPGM catalysis.

Sequence analysis alignment and phylogenetic affiliation of archaeal dPGM

The archaeal dPGM (TA1347) from *T. acidophilum* shows moderate sequence identities (12–18%) with characterized dPGMs from bacteria and eukarya, e.g., of 16 and 14% with dPGMs from *E. coli* and *S. cerevisia*, respectively. As shown in the sequence alignment in Fig. 3, the archaeal dPGM exhibits a similar secondary structure and most conserved amino acids residues involved in substrate binding and catalysis, as concluded from the crystal structure from *E. coli* dPGM (Bond et al. 2001). TA1347 also contain the typical family consensus pattern: [LIVM]–x–R–H–G–[EQ]–x—{Y}–x—N, which includes the conserved active site histidine of dPGMs proposed to be essential for catalysis involving a phosphohistidine enzyme intermediate (Fothergill-Gilmore and Watson 1989). Evidence for histidine phosphorylation has been demonstrated in *E. coli* dPGM by labelling studies with ^{32}P labelled 2,3-BPG (Rose 1971). In this study, the essential role of active site histidine, His23, of archaeal *Thermoplasma* dPGM was demonstrated by site directed mutagenesis.

Cofactor-dependent phosphoglycerate mutases (dPGM) are members of the phosphoglycerate mutase (like) superfamily (SCOP 53255, Pfam PF00300), which also include histidine acid phosphatases, bifunctional 6-phosphofructo 2 kinase/fructose–2,6-bisphosphatases and bacterial alpha-ribazole 5′-phosphate phosphatase (Jedrzejas 2000). dPGMs and the phosphatase domains of these enzyme families share a similar structural folds, and the consensus pattern (see above) including the conserved active site histidine, suggesting a similar catalytic mechanisms via phosphohistidine enzyme intermediates.

Using *Thermoplasma* dPGM sequence, homologs with high degree of sequence identity (35–66%) or similarity (56–81%) were identified in other thermoacidophilic archaea, including the *T. vulcanii*, *Ferroplasma acidarmanus*, *Picrophilus torridus* and the crenarchaeota *S. solfataricus*, *S. tokodaii* and *S. acidocaldarius*. Due to high sequence identity it is very likely that these archaeal homologs code for functional dPGMs as well, which has to be demonstrated. Putative dPGM homologs with significant lower sequence identity (15–17%) to *Thermoplasma* dPGM were identified in other archaea including *Methanosarcina* species (*M. barkeri*, *M. acetivorans* and *M. mazei*), and the extreme halophiles *Haloarcula marismortui* and *Haloquadratum walsbyi*. The coding function of these genes as dPGMs has to be shown.

The characterization of the first archaeal dPGM from *T. acidophilum* and thus the identification of putative archaeal dPGM homologs allow phylogenetic studies. As shown in the phylogram in Fig. 4) selected dPGM sequences, from the archaea, bacteria and eukarya including both functionally characterized and hypothetical proteins, cluster in three groups: one archaeal group (Archaea) and two bacterial groups: Bacteria I (PGMA), which includes eukaryal sequences, and Bacteria II (PGMB). The overall topology of the tree was achieved by the Neigbor-joining method and is supported by strong bootstrap values. According to high degree of sequence identity, the characterized archaeal *Thermoplasma* dPGM (TA1347) and putative homologs of other thermoacidophilic archaea form a distinct cluster. Besides TA1347 a second dPGM homolog of *T. acidophilum*, TA0823, is present in this cluster, which either represents an orthologous or paralogous enzyme. This has to be verified by biochemical analyses. Putative dPGM sequences from halophilic archaea, *Haloarcula* and *Haloquadratum*, originate very close to the archaeal cluster. The minor deviation of this position might be explained by a specific adaptation of the halophilic proteins to high salt conditions. The putative dPGMs of *Methanosarcina* species form a branch more closely related to bacterial dPGMs. This close phylogenetic relationship might be explained by lateral gene transfer events from bacterial dPGM genes to *Methanosarcina* sp. A frequent lateral gene transfer from bacteria to *Methanosarcina* has recently been proposed on the basis of the complete genome sequence of *Methanosarcina mazei* showing a large number of proteins to be closely related to bacterial homologs (Deppenmeier et al. 2002).

All characterized bacterial dPGMs and a large number of putative homologs with high sequence identity (55–80%) are annotated as members of PGMA-family and constitute a distinct cluster (Bacteria I) clearly separated from the archaeal cluster. Putative dPGM sequences from *Bacillus* species, *B. subtilis* and *B. stearothermophilus*, were not included in the phylogenetic analyses, since these were characterized as broad specific phosphatases rather than as functional dPGMs (Rigden et al. 2001; Pearson et al. 2000). This bacterial cluster also includes all characterized eukaryotic dPGM sequences from vertebrates (human, rat, mouse), e.g., various dPGM isoenzymes from human, and the dPGMs from *S. cerevisiae* and *S. pombe*. The close phylogenetic relationship of eukaryotic dPGMs with bacterial dPGMs might indicate a bacterial origin of eukaryotic dPGMs. A putative dPGM homolog from *Methanospirillum hungatei* (Mhun_2324), recently released in database (August 2006), showed highest sequence identity (50%) to characterize dPGMs from vertebrates and thus belongs to the eukaryal group of the bacterial dPGM cluster. Thus, one might speculate that the putative dPGM in *Methanospirillum* originated from eukarya via lateral gene transfer. Such a gene transfer from eukarya to methanogenic archaea has also been proposed to explain the presence

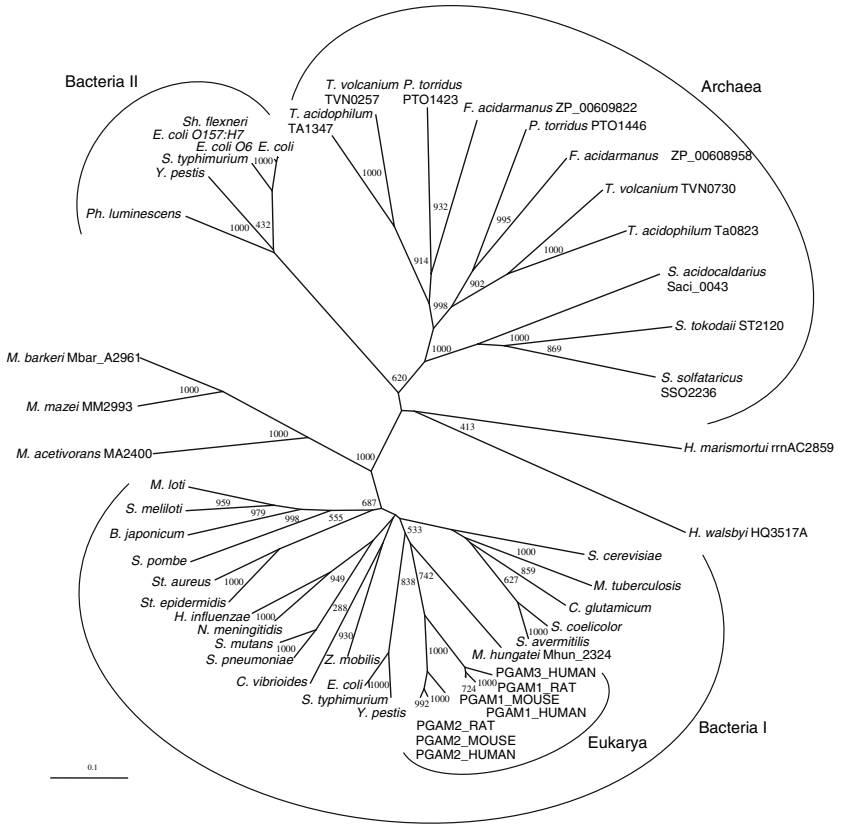

Fig. 4 Phylogenetic relationships of cofactor dependent phosphoglycerate mutases (dPGMs) from bacteria, eukarya and archaea. The numbers at the nodes are bootstrapping values according to neighbor-joining (generated by using the neighbor-joining options of ClustalX). NCBI accession numbers or SwissProt identifiers: *Bradyrhizobium japonicum* Q89WK1, *Corynebacterium glutamicum* Q8NTA5, *Corynebacterium vibrioides* Q9A634, *E. coli* P62707, *E. coli* GPMB_ECOLI P0A7A2, *E. coli* O157:H7 P0A7A3, *E. coli* O6 Q8FA40, *Ferroplasma acidarmanus* ZP_00608958, *Ferroplasma acidarmanus* ZP_00609822, *Haemophilus influenzae* P44865, *Haloarcula marismortui* rrnAC2859, *Haloquadratum walsbyi* HQ3517A, Human PGAM1_HUMAN P18669, Human PGAM2_HUMAN P15259, Human PGAM3_HUMAN Q8N0Y7, *Methanosarcina acetivorans* MA2400, *Methanosarcina acetivorans* MA3008, *Methanosarcina barkeri* Mbar_A2961, *Methanospirillum hungatei* Mhun_2324, *Mesorhizobium loti* Q98DM0, *Methanosarcina mazei* MM2993, *Mycobacterium tuberculosis* P0A5R6, Mouse PGAM1_MOUSE Q9DBJ1, Mouse PGAM2_MOUSE O70250, *Neisseria meningitidis* Q9JYF7, *Picrophilus torridus* PTO1423, *Picrophilus torridus* PTO1446, *Photorhabdus luminescens* Q7N900, Rat PGAM1_RAT P25113, Rat PGAM2_RAT P16290, *Sulfolobus acidocaldarius* Saci_0043, *Streptomyces avermitilis* Q82GB8, *Saccharomyces cerevisiae* P00950, *Streptomyces coelicolor* P33158, *Sinorhizobium meliloti* Q92T25, *Streptococcus mutans* P59161, *Streptococcus pneumoniae* P0A3Y3, *Schizosaccharomyces pombe* P36623, *Sulfolobus solfataricus* SSO2236, *Sulfolobus tokodaii* ST2120, *Salmonella typhimurium* Q8ZQS2, *Salmonella typhimurium* GPMB_SALTY Q8ZJU8, *Shigella flexneri* P0A7A4, *Staphylococcus aureus* P65709, *Staphylococcus epidermidis* Q8CN61, *Thermoplasma acidophilum* Ta0823, *Thermoplasma acidophilum* TA1347, *Thermoplasma volcanium* TVN0257, *Thermoplasma volcanium* TVN0730, *Yersinia pestis* Q8ZGY5, *Yersinia pestis* GPMB_YERPE Q8ZIP0, *Zymomonas mobilis* P30798

of eukaryotic like catalase encoding genes in *Methanobrevibacter* and *Methanosarcina* species (Shima et al. 2001).

A second group of bacterial sequences (Bacteria II) annotated as phosphoglycerate mutase B subfamily (GpmB, UniProtKB/Swiss-Prot family/domain classification) form a distinct phylogenetic cluster with strong bootstrapping support. So far none of these putative dPGM sequences, which all belong to γ-proteobacteria (Enterobacteriaceae), including, e.g., *E. coli* and *Salmonella*, have been functionally characterized.

Archaeal iPGM from *A. fulgidus*

Biochemical properties

ORF AF1751 from *A. fulgidus*, annotated as putative iPGM gene, was expressed in *E. coli* and the recombinant protein was characterized as functional iPGM. This archaeal iPGM was characterized as a 46 kDa monomeric enzyme and thus is similar to characterized iPGMs from bacteria and eukarya, i.e., from plants and nematodes (Jedrzejas 2000). In contrast, the recently characterized archaeal iPGMs from

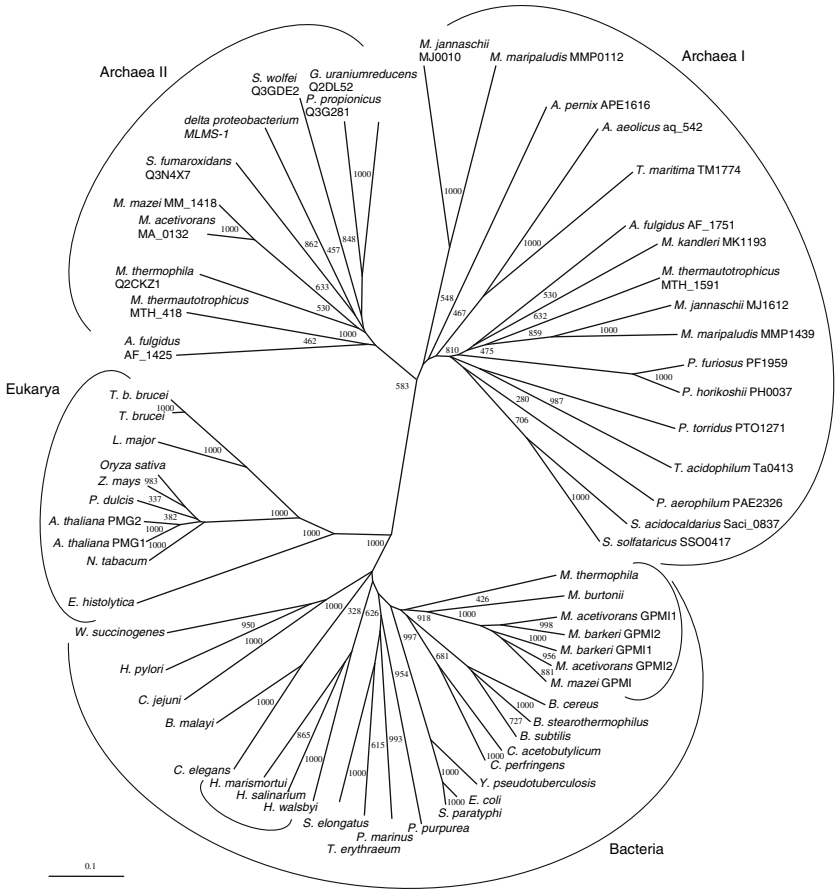

Fig. 5 Phylogenetic relationships of cofactor independent phosphoglycerate mutases (iPGMs) from bacteria, eukarya and archaea. The numbers at the nodes are bootstrapping values according to neighbor-joining (generated by using the neighbor-joining options of ClustalX). NCBI accession numbers or SwissProt identifiers: *Aquifex aeolicus* aq_542 O66820, *Archaeoglobus fulgidus* AF_1425 O28847, *Archaeoglobus fulgidus* AF_1751 O28523, *Aeropyrum pernix* APE1616 Q9YBI2, *Arabidopsis thaliana* PMG1_ARATH, *Arabidopsis thaliana* PMG2_ARATH, *Bacillus cereus* Q815K7, *Brugia malayi* Q4VWF8, *Bacillus stearothermophilus* Q9X519, *Bacillus subtilis* P39773, *Clostridium acetobutylicum* Q97L53, *Caenorhabditis elegans* O44742, *Campylobacter jejuni* Q9PI71, *Clostridium perfringens* Q0STD7, *E. coli* P37689, *Entamoeba histolytica* Q50PX5_ENTHI, *Geobacter uraniumreducens* Q2DL52, *Haloarcula marismortui* Q5UXB9, *Helicobacter pylori* Q9ZKM7, *Halobacterium salinarium* Q9HNY7, *Haloquadratum walsbyi* Q18GK9, *Leishmania major* Q4Q090_LEIMA, *Methanosarcina acetivorans* MA_0132 P58812, *Methanosarcina acetivorans* Q8TIY2 GPMI2, *Methanosarcina acetivorans* Q8TMI6 GPMI1, *Methanosarcina barkeri* Q46AE4 GPMI2, *Methanosarcina barkeri* Q46D52 GPMI1, *Methanococcoides burtonii* Q12UT0, *Methanocaldococcus jannaschii* MJ0010 Q60326, *Methanocaldococcus jannaschii* MJ1612 Q59007, *Methanopyrus kandleri* MK1193 P58813, *Methanococcus maripaludis* MMP0112 Q6M106, *Methanococcus maripaludis* MMP1439 Q6LXB3, *Methanosarcina mazei* MM_1418 Q8PX04, *Methanosarcina mazei* Q8PYF8 GPMI, *Methanothermobacter thermautotrophicus* MTH_1591 O27628, *Methanothermobacter thermautotrophicus* MTH_418 O26518, *Methanosaeta thermophila* Q2CKZ1, *Methanosaeta thermophila* Q2CLC6, MLMS-1 delta proteobacterium MldDRAFT_0629, *Nicotiana tabacum* PMGI_TOBAC, *Oryza sativa* Q5KQH5_ORYSA, *Pyrobaculum aerophilum* PAE2326 Q8ZVE4, *Prunus dulcis* PMGI_PRUDU, *Pyrococcus furiosus* PF1959 P58814, *Pyrococcus horikoshii* PH0037 O57742, *Prochlorococcus marinus* Q7V5U5, *Pelobacter propionicus* Q3G281, *Porphyra purpurea* P51379, *Picrophilus torridus* PTO1271 Q6KZJ6, *Sulfolobus acidocaldarius* Saci_0837 Q4JAH5, *Synechococcus elongatus* P59177, *Syntrophobacter fumaroxidans* Q3N4X7, *Salmonella paratyphi* Q5PBZ2, *Sulfolobus solfataricus* SSO0417 Q980A0, *Syntrophomonas wolfei* Q3GDE2, *Thermoplasma acidophilum* Ta0413 Q9HL27, *Trypanosoma brucei* Q38AH1_9TRYP, *Trypanosoma brucei* Q9NG18_TRYBB, *Trichodesmium erythraeum* Q117D6, *Thermotoga maritima* TM1774 Q9X295, *Wolinella succinogenes* Q7M7W9, *Yersinia pseudotuberculosis* Q66GC2, *Zea mays* PMGI_MAIZE

P. furiosus, ORF PF1959, and from *M. jannaschii*, ORF MJ1612, have been described as homotetrameric (α_4) enzymes of 45 kDa subunits (Van der Oost et al. 2002). Mn^{2+} stimulated iPGM activity from *A. fulgidus* suggesting that the archaeal enzyme require Mn^{2+} as has been shown for the bacterial iPGMs from *B. stearothermophilus* and *E. coli* (Fraser et al. 1999; Jedrzejas et al. 2000). However, the iPGMs from *M. jannaschii* and *P. furiosus* were reported to be slightly stimulated by Mg^{2+} rather than by Mn^{2+} (Van der Oost et al. 2002). iPGM activity for *Sulfolobus*, SSO0417, was stimulated by Co^{2+} and Mn^{2+} (Potters et al. 2003). The iPGM activity from *A. fulgidus* showed a high

thermostability up to 70°C, which is in accordance with the growth temperature of the organism (Stetter 1988). RT-PCR analysis showed that AF1751, encoding *A. fulgidus* iPGM, was transcribed in vivo during growth of the organism on lactate/sulfate suggesting a role in gluconeogenesis.

Phylogenetic analysis of iPGMs

Using a large number of characterized and putative iPGM sequences, recently available from database, a phylogenetic analysis of iPGMs from archaea, bacteria and eukarya was performed (Fig. 5), extending previous phylogenetic analyses (Graham et al. 2002; Van der Oost et al. 2002). In accordance with these studies, iPGM sequences form three main clusters, Archaea (I, II), Bacteria and Eukarya, which are largely congruent with the phylogenetic 16S-rRNA based phylogenetic tree. Several deviations of this pattern were observed which can most likely be explained by lateral gene transfer events taking place between the three domains. The archaeal cluster can be divided into two subgroups. Archaea I contained the characterized archaeal iPGMs from *Archaeoglobus* (AF1751, this work), *Pyrococcus furiosus* (PF1959), *Methanocaldococcus jannaschii* (MJ1612 and MJ0010) and from *S. sulfataricus* (SSO0417) (Potters et al. 2003; Graham et al. 2002; Van der Oost et al. 2002) and a large number of putative iPGM homologs. This subcluster also contains several bacterial sequences from the hyperthermophiles bacteria, *Thermotoga* and *Aquifex*, and from *Deinococcus* (not shown). These might have acquired their genes via lateral gene transfer from archaea. For *Thermotoga* a frequent gene transfer was proposed as concluded from genome sequence analysis (Nelson et al. 1999). The second archaeal subgroup, Archaea II, comprise sequences of several hydrogen consuming methanogens, *M. thermoautotrophicus* and *Methanosarcina* species; in addition a second sequence of the sulfate reducer *A. fulgidus* (AF1425), which might be a paralog of AF1751, is present in this subgroup. Few bacterial sequences, from *Geobacter* and the obligate syntrophs *Pelobacter*, *Syntrophobacter* and *Syntrophomonas* also clusters within this group. Since the obligate syntrophs live in nature in close contact to with H_2 consuming methanogens or sulfate reducers, lateral gene transfer events between these groups are very likely to occur. The eukaryotic cluster contains sequences from plants, e.g., from maize (Grana et al. 1995), *Nicotiana* and two sequences from *Arabidopsis*. This cluster also includes sequences from the protists *Trypanosoma*, *Leishmania* and *Entamoeba* (Saavedra et al. 2005; Guerra et al. 2004; Collet et al. 2001). The bacterial cluster comprises all characterized and putative bacterial iPGMs. Few archaeal sequences, from *Methanosarcina* and extreme halophiles and two putative sequences from *Arabidopsis* (not shown) also cluster within the bacterial group. In case of *Methanosarcina* this position can well be explained by lateral gene transfer events (Deppenmeier et al. 2002).

In summary, with the first proof of a functional dPGM in Archaea and the identification of various close homologs, along with the characterization of several functional archaeal iPGMs, including iPGM from *Archaeoglobus* (this paper), it has now been established that in the domain of archaea both types of phosphoglycerate mutase are present. Thus, the data fill the gap of previously assumed ''missing'' phosphoglycerate mutases in this phylogenetic domain (Galperin et al. 1998; Selkov et al. 1997). Several archaea, including methanogenic and thermoacidophilic archaea contain homologs of both PGM types, as has been reported for few bacterial species, including *E. coli*. In *E. coli* a differential expression of iPGM and dPGM has been demonstrated with a dominant role of dPGM (Fraser et al. 1999). Similar experiments including transcriptional analyses, e.g., in response to glycolytic and gluconeogenetic growth conditions or to growth phases are necessary to define the physiological role of both dPGM and iPGM homologs in archaea.

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