Purification and Characterization of Glycerate Kinase from the Thermoacidophilic Archaeon *Thermoplasma acidophilum*: An Enzyme Belonging to the Second Glycerate Kinase Family

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Abstract *Thermoplasma acidophilum* is a thermoacidophilic archaeon that grows optimally at 59°C and pH 2. Along with another thermoacidophilic archaeon, *Sulfolobus solfataricus*, it is known to metabolize glucose by the non-phosphorylated Entner-Doudoroff (nED) pathway. In the course of these studies, the specific activities of glyceraldehyde dehydrogenase and glycerate kinase, two enzymes that are involved in the downstream part of the nED pathway, were found to be much higher in *T. acidophilum* than in *S. solfataricus*. To characterize glycerate kinase, the enzyme was purified to homogeneity from *T. acidophilum* cell extracts. The *N*-terminal sequence of the purified enzyme was in exact agreement with that of Ta0453m in the genome database, with the removal of the initiator methionine. Furthermore, the enzyme was a monomer with a molecular weight of 49 kDa and followed Michaelis-Menten kinetics with K_m values of 0.56 and 0.32 mM for DL-glycerate and ATP, respectively. The enzyme also exhibited excellent thermal stability at 70°C. Of the seven sugars and four phosphate donors tested, only DL-glycerate and ATP were utilized by glycerate kinase as substrates. In addition, a coupled enzyme assay indicated that 2-phosphoglycerate was produced as a product. When divalent metal ions, such as Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, and Sr^{2+}, were substituted for Mg^{2+}, the enzyme activities were less than 10% of that obtained in the presence of Mg^{2+}. The amino acid sequence of *T. acidophilum* glycerate kinase showed no similarity with *E. coli* glycerate kinases, which belong to the first glycerate kinase family. This is the first report on the biochemical characterization of an enzyme which belongs to a member of the second glycerate kinase family.

Keywords: glycerate kinase, 2-phosphoglycerate, non-phosphorylated Entner-Doudoroff pathway, thermoacidophilic archaea, *Thermoplasma acidophilum*

INTRODUCTION

Thermoacidophilic archaea, such as *Thermoplasma acidophilum* and *Sulfolobus solfataricus*, are known to metabolize glucose via the non-phosphorylated Entner-Doudoroff (nED) pathway, an ED-like pathway in which hexose intermediates are not phosphorylated [1]. The first step of the nED pathway involves the $NAD(P)^+$-dependent oxidation of glucose to gluconate, catalyzed by glucose dehydrogenase [2]. Gluconate is then dehydrated by gluconate dehydratase to 2-keto-3-deoxygluconate (KDG), which undergoes an aldolate cleavage to pyruvate and glyceraldehyde, catalyzed by KDG aldolase. Glyceraldehyde dehydrogenase then oxidizes glyceraldehyde to glycerate, which is phosphorylated by glycerate kinase to 2-phosphoglycerate. A second molecule of pyruvate is produced from 2-phosphoglycerate by the actions of enolase and pyruvate kinase.

This modified pathway, involving gluconate dehydratase and KDG aldolase, that metabolizes glucose to glyceraldehyde and pyruvate was found experimentally in *S. solfataricus* [3]. Subsequent studies with *T. acidophilum* have confirmed that the glyceraldehyde formed via this non-phosphorylative route is converted by glyceraldehyde dehydrogenase to glycerate, which is then phosphorylated by glycerate kinase to form 2-phosphoglycerate [4]. Most enzymes involved in the upper portion of the nED pathway, those that transform glucose to pyruvate and glyceraldehyde, have been identified and characterized. These include glucose dehydrogenase from *T. acidophilum* [5] and *S. solfataricus* [6], gluconate dehydratase from *S. solfataricus* [7,8], KDG aldolase from *S. solfataricus* [9], and glyceraldehyde dehydrogenase from *T. acidophilum* [10]. However, glycerate kinases in the downstream portion of the nED pathway in thermoacidophilic archaea have not yet been characterized.

It is known that glycerate kinases fall into two separate families with no significant sequence similarity between

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the two groups [11]. One family contains glycerate kinases from bacteria such as *Neisseria meningitidis*, whereas the second family consists of proteins from eukarya and archaea, in addition to some bacterial species such as *Thermotoga maritima*. This suggests that glycerate kinase from the thermoacidophilic archaeon *T. acidophilum* is probably a member of the second glycerate kinase family. However, unlike the first glycerate kinase family, the annotation for the second glycerate kinase family is not based on the biochemical function of the enzyme. Rather, the annotation for these putative glycerate kinases is based on a gene that is apparently responsible for complementation in *Methylbacterium extorquens* AM1 mutants lacking glycerate kinase activity (*gckA* gene) [12]. Moreover, some other family members are annotated as putative hydroxypyruvate reductases (also known as glycerate dehydrogenases) based on genetic analysis of the tartrate utilization pathway in *Agrobacterium vitis* (*ttuD* gene) [13]. Therefore, as noted by Cheek *et al.* [11], the exact biochemical function of the second glycerate kinase family remains to be resolved.

In this study, we first examined the activities of enzymes involved in the conversion of glyceraldehyde to 2-phosphoglycerate and of various sugar kinases in the crude extracts of two thermoacidophilic archaea, *T. acidophilum* and *S. solfataricus*. Based on the activity data, glycerate kinase was purified from *T. acidophilum*, and its *N*-terminal sequence identified. Out of the seven sugars and four phosphate donors tested, the purified enzyme utilized only DL-glycerate and ATP. The amino acid sequence analysis showed that *T. acidophilum* glycerate kinase is homologous to *gckA* of *M. extorquens* and *ttuD* of *A. vitis*. To our knowledge, this is the first report on the biochemical characterization of a glycerate kinase belonging to the second glycerate kinase family.

MATERIALS AND METHODS

Strains, Media, and Culture Conditions

T. acidophilum (JCM9062) was obtained from the Japan Collection of Microorganisms. The growth media composition was (per liter): 1.0 g yeast extract, 3.0 g casamino acid, 1.3 g $(NH_4)_2SO_4$, 0.3 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and 0.25 g $CaCl_2 \cdot 2H_2O$. The final pH was adjusted to 2.0 using H_2SO_4. The strain was cultured aerobically in a 3-liter fermentor at 59°C for 2 days with stirring at 50 rpm. *S. solfataricus* P2 (DSM1617) was obtained from Deutsche Sammlung von Microorganismen, and GYM medium [14-17] was used for cultivation. The final pH was adjusted to 3.0 using 10 N H_2SO_4. The strain was cultured aerobically in screw-capped flasks at 78°C for 3 days with stirring at 150 rpm.

Glyceraldehyde Dehydrogenase and Glyceraldehyde 3-Phosphate Dehydrogenase Assays

The activities of glyceraldehyde dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase in *T. acidophilum* and *S. solfataricus* crude extracts were determined for comparison purposes. Cells were harvested by centrifugation (4,000 g, 20 min), cell pellets were resuspended in 50 mM Tris-HCl (pH 8), and cells were broken by sonication. Cell debris was eliminated by low-speed centrifugation (4,000 g, 20 min, 4°C), and the supernatant was clarified by high-speed centrifugation (20,000 g, 1 h, 4°C) and then dialyzed against 50 mM Tris-HCl (pH 8).

Assays were performed at 55°C for *T. acidophilum* and 75°C for *S. solfataricus*. The reaction mixtures contained 25 mM DL-glyceraldehyde or DL-glyceraldehyde 3-phosphate, 1 mM $NADP^+$ as coenzyme, and an appropriate amount of crude extract solution in 100 mM Tris-HCl (pH 8). Reactions were initiated by adding crude extract solution. After incubating for 30 min, the reaction mixtures were cooled on ice to stop the reaction. Increases in absorption at 340 nm due to $NADP^+$ reduction were monitored. Enzyme activities were calculated using an NADPH molar absorption coefficient at 340 nm of 5,841 $M^{-1}cm^{-1}$.

Glycerate Kinase Assay

The consumption of DL-glycerate was measured spectrophotometrically at 340 nm as NADH oxidation in the presence of coupling enzymes. The glycerate kinase reaction mixture was comprised of 100 mM Tris-HCl (pH 8), 5 mM EGTA, 1 mM DL-glycerate, 1 mM ATP, 10 mM $MgCl_2$ and an enzyme solution. EGTA was included in the assay because Ca^{2+}, which is present in commercial preparations of DL-glycerate, was found to inhibit glycerate kinase. The reaction was initiated by adding the enzyme solution and was carried out at 55°C. After a 30-min incubation period, reaction mixtures were cooled on ice to stop the enzyme reaction. Then, 1 mM phosphoenolpyruvate, 1 mM NADH, 10 mM KCl, 5.4 U rabbit muscle pyruvate kinase, and 3 U porcine heart lactate dehydrogenase were added to the reaction mixture to a final volume of 1 mL. The coupled enzyme reactions were carried at 25°C, and reductions in absorption at 340 nm (due to the oxidation of NADH) were monitored. Glycerate kinase activities were calculated using an NADH molar absorption coefficient of 5,841 $M^{-1}cm^{-1}$ at 340 nm. Enzyme activity (1 unit; U) was defined as the amount of enzyme producing 1 μmol of product per min under standard assay conditions.

Purification and Identification of Glycerate Kinase

Dialyzed crude extract was loaded onto a DEAE-Sepharose column (2.5 × 16 cm) equilibrated with 50 mM Tris-HCl (pH 8). The column was washed with 2 volumes of the same buffer, and the enzyme was eluted using a stepwise gradient of NaCl (0.25~1 M) in the same buffer. Enzyme activity was found in 0.25 M NaCl fractions, which were pooled and dialyzed against 50 mM Tris-HCl (pH 8). The DEAE-Sepharose active fraction was loaded onto a Q-Sepharose column (2.5 × 14 cm) equilibrated with 50 mM Tris-HCl (pH 8). After washing,

a stepwise gradient of NaCl (0.05~0.25 M) in the same buffer was applied. Fractions containing enzyme activity were pooled, dialyzed using the same buffer, and concentrated using a concentrator membrane. The Q-Sepharose active fraction was then loaded onto a Phenyl-Sepharose column (1.0 × 12 cm) equilibrated with 50 mM Tris-HCl (pH 8) containing 0.5 M $(NH_4)_2SO_4$. After washing with equilibrated buffer, the enzyme was eluted using a decreasing salt gradient (0.25~0.05 M $(NH_4)_2SO_4$). Enzyme activity was found in 0.1 M $(NH_4)_2SO_4$ fractions, which were pooled, dialyzed against 50 mM Tris-HCl (pH 8), and concentrated using a concentrator membrane. Finally, the Phenyl-Sepharose active fraction was loaded onto an Affi-gel blue column (0.5 × 14 cm) equilibrated with 50 mM Tris-HCl (pH 8). The column was washed with 3 volumes of the same buffer, and active enzyme was pooled, dialyzed against the same buffer, and concentrated using a concentrator membrane.

SDS-PAGE was performed in 10% polyacrylamide slab gels using a Tris-glycine system. Proteins were detected by Coomassie Blue staining. For amino-terminal sequence analysis, purified glycerate kinase was transferred to a polyvinylidene difluoride (PVDF) membrane. Protein sequencing was performed at the Korean Basic Science Institute (Daejeon, Korea).

Molecular Mass Determination

Purified glycerate kinase (100 μg) was chromatographed at a flow rate of 0.4 mL/min on a Sephacryl S-200 column (2.5 × 100 cm) using a gel filtration calibration kit (Amersham Pharmacia Biotech, Sweden). The buffer used for column equilibrium and elution was 50 mM Tris-HCl buffer (pH 8) containing 150 mM NaCl. The molecular weight markers used were catalase (232 kDa), aldolase (158 kDa), BSA (67 kDa), and ovalbumin (43 kDa). The absorbance of the eluent was monitored at 280 nm, and glycerate kinase activity was measured by the method described above. The molecular weight of native glycerate kinase was calculated by interpolation on a plot of the log of molecular mass against the K_{av} values [K_{av} = (eluate volume – void volume)/(total volume – void volume)].

Characterization of Glycerate Kinase

The kinetic parameters of glycerate kinase were determined by measuring enzyme activity at various DL-glycerate concentrations. The apparent V_{max} and K_m values were calculated by fitting the initial rate data to the Michaelis-Menten equation using a non-linear regression analysis program (Sigma Plot, ver. 7.0).

The dependence of enzyme activity on temperature was examined using the assay method mentioned above at temperatures between 20 and 90°C. Enzyme thermostability was determined at 70, 75, 78, and 80°C by incubating the enzyme (65 μg/mL) in 50 mM Tris-HCl (pH 8). At various time intervals, samples were withdrawn, cooled on ice, and examined for residual enzyme activities.

Substrate specificity was determined by measuring the concentration of 2-phosphoglycerate formed as a result of kinase activity. Each of the following compounds was checked as a possible substrate for glycerate kinase: D-gluconate, DL-glyceraldehyde, glycerol, L-tartrate, 2-phospho-D-glycerate, and 3-phospho-D-glycerate. Its specificity for phosphate donors was also examined by substituting ATP with GTP, CTP, or UTP. The amount of 2-phosphoglycerate present in the reaction mixture was measured by omitting phosphoenolpyruvate and adding baker's yeast enolase (7 U) to the standard enzyme assay reaction mixture mentioned above.

The effects of divalent ions (Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, and Sr^{2+}) on glycerate kinase were examined by measuring enzyme activities in the presence of these divalent ions. Reactions were carried out using the standard assay conditions mentioned above, except that 10 mM of the chloride salts of these metal ions were added instead of $MgCl_2$. In this series of investigations, all enzyme activities were determined in triplicate.

Table 1. Comparison of $NADP^+$-dependent glyceraldehyde dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase activities in the crude extracts of *T. acidophilum* and *S. solfataricus*

Enzyme	Specific activity (U/mg)	
	T. acidophilum	*S. solfataricus*
Glyceraldehyde dehydrogenase	0.053 ± 0.001	<0.002
Glyceraldehyde 3-phosphate dehydrogenase	0.021 ± 0.001	0.026 ± 0.001

RESULTS AND DISCUSSION

Glyceraldehyde Dehydrogenase and Sugar Kinase Activities

The activities of $NADP^+$-dependent glyceraldehyde dehydrogenase and sugar kinases in *T. acidophilum* and *S. solfataricus* were examined using crude cell extracts (Tables 1 and 2). For comparison purposes, glyceraldehyde 3-phosphate dehydrogenase activity was also determined using $NADP^+$ as a coenzyme.

Unexpectedly, *S. solfataricus* showed no appreciable glyceraldehyde dehydrogenase activity (<0.002 U/mg), whereas *T. acidophilum* did (0.053 U/mg). The absent, or barely detectable, glyceraldehyde dehydrogenase activity in *S. solfataricus* was confirmed by repeating the experiments. On the other hand, levels of glyceraldehyde 3-phosphate dehydrogenase activity were similar in both archaea (*T. acidophilum*, 0.021 U/mg; *S. solfataricus*, 0.026 U/mg). The absence of $NADP^+$-dependent glyceraldehyde dehydrogenase activity in *S. solfataricus* cell-free extracts suggests that the conversion of glyceraldehyde to glycerate in the Crenarchaeota is catalyzed by some enzyme other than glyceraldehyde dehydrogenase.

To examine the sugar phosphorylation activities in

Table 2. Various sugar kinase activities of *T. acidophilum* and *S. solfataricus*

Substrate	Kinase activity (U/mg)	
	T. acidophilum	*S. solfataricus*
DL-glycerate	0.135 ± 0.005	0.030 ± 0.001
KDG	0.126 ± 0.014	0.112 ± 0.011
D-glucose	<0.001	0.045 ± 0.004
D-gluconate	<0.001	0.020 ± 0.007
glycerol	<0.001	0.018 ± 0.003
D-fructose	<0.001	0.014 ± 0.007
DL-glyceraldehyde	<0.001	<0.001
D-ribose	<0.001	<0.001
D-xylose	<0.001	<0.001

Table 3. Purification of glycerate kinase from *T. acidophilum*

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Crude extract	1,680.0	265.5	0.16	1.0	100
DEAE-Sepharose	880.0	191.3	0.22	1.4	72
Q-Sepharose	43.0	184.1	4.30	27.0	69
Phenyl-Sepharose	9.1	130.1	14.40	91.0	49
Affi-gel blue	0.47	55.2	118.00	747.0	21

these two closely related thermoacidophilic archaea, the existence of various sugar kinase activities was examined in both *T. acidophilum* and *S. solfataricus* (Table 2). Among the nine sugars tested, *T. acidophilum* was found to have kinase activity only for DL-glycerate and KDG. As can be seen from Table 2, glycerate kinase activities were markedly different: glycerate kinase activity in *S. solfataricus* (0.030 U/mg) was only 22% of that in *T. acidophilum* (0.135 U/mg).

Both *T. acidophilum* and *S. solfataricus* had kinase activity for KDG. This indicates the presence of another modified ED pathway in these archaea, because KDG can be phosphorylated to 2-keto-3-deoxy-6-phosphogluconate by KDG kinase. Recently, we identified and characterized KDG kinases from *T. acidophilum* and *S. solfataricus* [18,19]. *S. solfataricus* also showed weak, but appreciable, kinase activities towards D-glucose (0.045 U/mg), glycerol (0.018 U/mg), and D-gluconate (0.020 U/mg). On the other hand, kinase activities towards D-glyceradehyde, D-ribose, and D-xylose were negligible in both *T. acidophilum* and *S. solfataricus*.

Identification of Glycerate Kinase

Since glycerate kinase activities were significantly higher in *T. acidophilum* than in *S. solfataricus*, glycerate kinase was purified using cell extracts of *T. acidophilum*. The enzyme was purified 747-fold using a four-step procedure (Table 3). The most effective step was ion-exchange chromatography on Q-Sepharose, which yielded a 19.5-fold increase in specific activity over the DEAE-Sepharose step. The final purified fraction showed homogeneity on SDS gels (Fig. 1), and the molecular mass of the denatured enzyme was approximately 45 kDa.

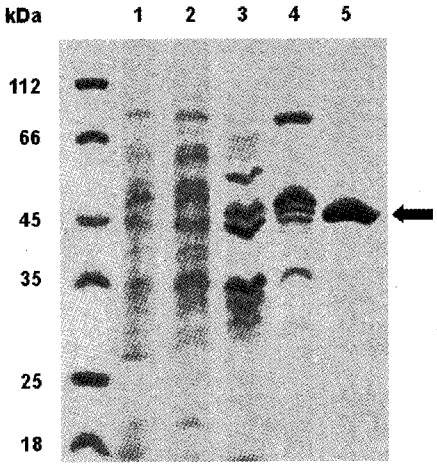

Fig. 1. SDS-PAGE of purified glycerate kinase from *T. acidophilum*. Lane 1, crude extract; lane 2, DEAE-Sepharose active fraction; lane 3, Q-Sepharose active fraction; lane 4, Phenyl-Sepharose active fraction; lane 5, Affi-gel blue active fraction.

To determine the *N*-terminal amino acid sequence of glycerate kinase, the purified enzyme was loaded on SDS-PAGE, blotted onto a PVDF membrane, and excised. The *N*-terminal sequence of glycerate kinase purified from *T. acidophilum* was determined to be VFT-FKNAKDI. This amino acid sequence was in exact agreement with that of Ta0453m in the *T. acidophilum* DSM 1728 genome database (Genbank: NC_002578), with the removal of the initiator methionine at the *N*-terminus of the protein. The molecular mass deduced from its amino acid sequence (45,820 Da) agreed well with the molecular mass of the purified glycerate kinase in denaturing gel (45 kDa). Consequently, it was confirmed that the protein purified from *T. acidophilum* was glycerate kinase and that its ORF corresponds to that of Ta0453m. We named this ORF *glyK*, as it encodes *gly*cerate *k*inase.

Characterization of Glycerate Kinase

The molecular mass of the native enzyme was estimated to be 49.3 kDa using a calibrated Sephacryl S-200 column. Since the molecular mass of the denatured enzyme determined by SDS-PAGE was approximately 45 kDa, it appears that *T. acidophilum* glycerate kinase is a monomer.

The effect of temperature on its activity was examined from 20 to 90°C (Fig. 2A). The purified enzyme displayed optimal activity at 70°C, which is somewhat higher than the optimum temperature for cell growth

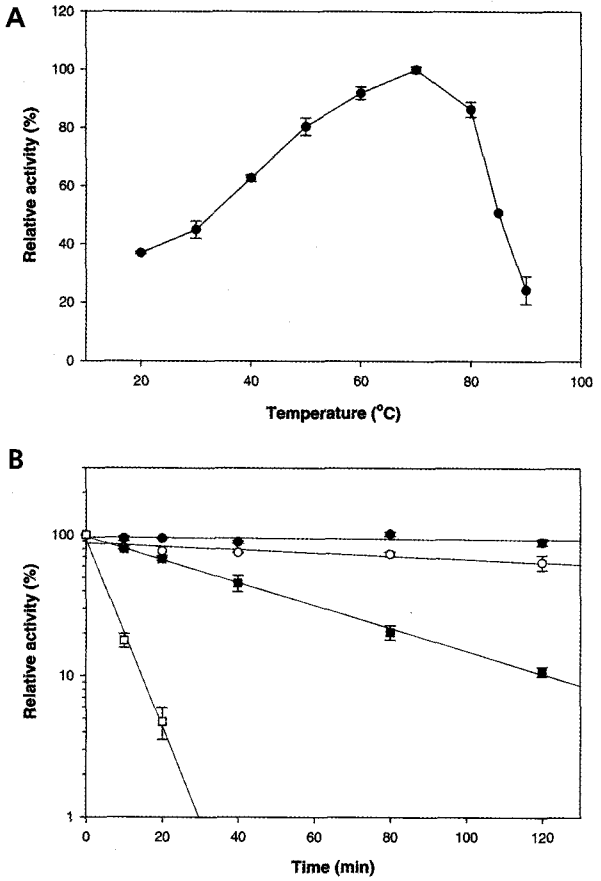

Fig. 2. (A) Temperature profile of purified glycerate kinase from *T. acidophilum*. (B) Thermostability of purified glycerate kinase from *T. acidophilum*. ●, incubation at 70°C; ○, 75°C; ■, 78°C; □, 80°C.

(59°C). Its activity decreased rapidly at temperatures above 80°C, but slowly at temperatures below 70°C. The thermostability of purified glycerate kinase was also examined between 70 and 80°C (Fig. 2B). At temperatures up to 75°C, glycerate kinase was stable for over 2 hr, but at 78°C its activity was reduced to less than 50% in one hour, and at 80°C its activity diminished rapidly, with less than 5% of its initial activity remaining after 20 min.

The ability of glycerate kinase to phosphorylate sugar compounds other than DL-glycerate was examined under standard assay conditions by substituting the following compounds for DL-glycerate: D-gluconate, DL-glyceraldehyde, glycerol, L-tartrate, 2-phospho-D-glycerate, or 3-phospho-D-glycerate (Table 4). Substrate specificity was examined by measuring the concentration of 2-phosphoglycerate formed during the reaction using a coupled enzyme assay. *T. acidophilum* glycerate kinase specifically phosphorylated DL-glycerate, and showed specificity for ATP as a phosphate donor. GTP, CTP, and UTP exhibited only 0~4% of the activity of ATP, a range that lay within that of the error.

The effects of substrate concentrations on purified glycerate kinase activity were also tested. Kinetic constants were deduced by nonlinear regression, and the K_m values for DL-glycerate and ATP were found to be 0.56 and 0.32 mM, respectively.

Table 4. Substrate specificity of the purified glycerate kinase

Substrate	Relative activity (%)
DL-glycerate	100
D-gluconate	5 ± 2
glycerol	3 ± 1
DL-glyceraldehyde	<1
2-phospho-D-glycerate	<1
3-phospho-D-glycerate	<1
L-tartrate	<1

In addition, the effects of various divalent metal ions on glycerate kinase activity were examined. Mn^{2+} and Co^{2+} were found to substitute for Mg^{2+}, but activities were reduced to 10 and 8% of that for Mg^{2+}, respectively. The other metal ions did not substitute for Mg^{2+}.

Homology with Other Sequences

It is known that *E. coli* produces two types of glycerate kinase: *glxK* (*b0514*) and *garK* (*b3124*) [20-24]. The former catalyzes the conversion of glycerate to 3-phosphoglycerate and the latter its conversion to 2-phosphoglycerate. However, the amino acid sequence of *T. acidophilum* glycerate kinase showed no similarity to the *E. coli* glycerate kinases, which both belong to the first glycerate kinase family [11]. Homologs of *E. coli* glycerate kinases could only be found in bacterial genomes (*Salmonella typhimurium*, 80% identity; *Klebsiella pneumoniae*, 71%; *Yersinia pestis*, 60%; *Bacillus subtilis*, 53%; *Pseudomonas putida*, 50%; *Clostridium tetani*, 48%; *Zymomonas mobilis*, 45%; *Burkholderia cepacia*, 41%; *Corynebacterium glutamicum*, 35%) and fungal genomes (*Aspergillus nidulans*, 42%; *Gibberella zeae*, 38%).

On the other hand, homologs of *T. acidophilum* glycerate kinase were identified in many archaeal genomes (*Thermoplasma volcanium*, 63% identity; *Ferroplasma acidarmanus*, 51%; *Picrophilus torridus*, 50%; *Pyrobaculum aerophilum*, 37%; *Pyrococcus horikoshii*, 32%; *Aeropyrum pernix*, 31%; *S. solfataricus*, 30%; *Sulfolobus tokodaii*, 29%; *Thermoproteus tenax*, 26%), bacterial genomes (*Thermotoga maritima*, 31%; *Agrobacterium vitis*, 30%; *Thermus thermophilus*, 28%; *Mesorhizobium loti*, 28%; *Methylobacterium extorquens*, 28%), and eukaryote genomes (*Homo sapiens*, 30%; *Mus musculus*, 31%; *Caenorhabditis elegans*, 30%; *Gallus gallus*, 27%; *Drosophila melanogaster*, 26%). Interestingly, glycerate kinases from thermophilic bacteria (*T. maritima*, *T. thermophilus*) and soil bacteria (*A. vitis*, *M. loti*) are homologous to archaeal enzymes. This may indicate a lateral gene transfer event between these bacteria and archaea, which share their environment. It is also interesting that human glycerate kinase, which catalyzes the conversion of glycerate to 2-phosphoglycerate [25], shares 45% homology with the glycerate kinases in hyperthermophilic archaea.

Fig. 3. Amino acid sequence alignment of *T. acidophilum* glycerate kinase orthologs. The sequences aligned are: Ta0453m (*T. acidophilum*, GenBank accession number NP_393931); TVN0783 (*T. volcanium*, GenBank accession number BAB 59939); Fac0418 (*F. acidarmanus*, GenBank accession number ZP_00609886), PTO1442 (*P. torridus*, GenBank accession number YP_024220); and Tm1585 (*T. maritima*, GenBank accession number NP_229385). Invariant residues are indicated by dark gray and putative active sites are highlighted in black with white letters.

Recently, the 3-D structure of putative glycerate kinase from *T. maritima* (PDB 2B8N; Joint Center for Structural Genomics, Crystal structure of glycerate kinase (EC 2.7.1.31) (Tm1585) from *Thermotoga maritima* at 2.70 Å resolution) has been determined. Based on 3-D structure, the presumed active site of the *T. maritima* glycerate kinase has also been proposed [11]. The presumed active site is in the cleft between the two α/β domains, and six highly conserved polar or charged residues are found with side chains pointing into the presumed active site. Sequence comparison of the *T. acidophilum* glycerate kinase (Ta0453m) and *T. maritima* putative glycerate kinase (Tm1585) shows that the six amino acids of the active site are all conserved (Fig. 3).

Finally, the sequences of putative hydroxypyruvate dehydrogenase (TtuD) of *A. vitis* [13] and the experimentally verified hydroxypyruvate dehydrogenase of *Hyphomicrobium methylovorum* [26] were compared with several glycerate kinase and hydroxypyruvate dehydrogenase sequences (results not shown). Glycerate kinases and TtuD clustered together, whereas hydroxypyruvate dehydrogenase sequences clustered separately. TtuD protein was more closely related to bacterial glycerate kinases than to archaeal glycerate kinase. This indicates that TtuD is not a hydroxypyruvate dehydrogenase, but an enzyme which belongs to the second glycerate kinase family.

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