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NOTE

A unique highly thermostable 2-phosphoglycerate forming glycerate kinase from the hyperthermophilic archaeon *Pyrococcus horikoshii*: gene cloning, expression and characterization

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Abstract A glycerate kinase (GK) gene (PH0495) from the hyperthermophilic archaeon *Pyrococcus horikoshii*, was cloned and expressed in *Escherichia coli*. The recombinant protein was purified to homogeneity by affinity chromatography and ion exchange chromatography. The enzyme was likely a homodimer based on SDS-PAGE (47 kDa) and gel filtration chromatography (100 kDa) analysis. A radioisotope-labeling examination method was initially used for the enzymatic activity detection, and the enzyme (GK_{ph}) was found to catalyze the formation of 2-phosphoglycerate using D-glycerate as the substrate. The enzyme exhibited unique phosphoryl donor specificity with maximal activity towards pyrophosphate. The temperature and pH optima of the enzyme were 45°C and 7.0, respectively, and about half of the maximal activity remained at 100°C. The enzyme was highly thermostable with almost no loss of activity at 90°C for 12 h. Based on sequence alignment and structural comparison it was assigned to group I of the trichotomy of GKs.

Keywords Glycerate kinase · Thermostability · *Pyrococcus horikoshii* · *Archaea*

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Abbreviations

GK	Glycerate kinase
PGA	Phosphoglycerate
LB	Luria–Bertani
IPTG	Isopropyl-ß-D-thiogalactopyranoside
DMSO	Dimethyl sulfoxide
PMSF	Phenylmethyl sulfonylfluoride
DTT	Dithiothreitol
ED	Entner–Doudoroff

Introduction

Glycerate kinases catalyse the reaction in which one phosphate group is transferred from ATP to the substrate glycerate to produce 2-phosphoglycerate (2-PGA) or 3-PGA (Black and Wright 1956). Studies have shown that GKs play metabolic roles in many organisms, such as serine cycle (Chistoserdova et al. 1997), glycolate metabolism (Hansen et al. 1962), tartrate utilization (Crouze et al. 1995) and proposed ED glycolytic pathway in *Archaea* (Verhees et al. 2003). The enzymatic product 2-PGA, is a useful compound in biomedicine and biotechnology. It is reported to serve as a transport component or energy source in some microorganisms (Saier et al. 1975; Thompson and Thomas 1977) and function as a stimulator for phosphorylation of a protein that is involved in insulin secretion in eukaryotes (Pek et al. 1990). Additionally, it is a valuable substrate and can be used in crystallization studies of some glycolytic enzymes (Parthasarathy et al. 2003). However, this compound is not available commercially due to the disadvantages of production methods, such as the time-consuming steps in organic synthesis or unfavorable equilibrium or purification in enzymatic conversions via

PGA mutase and enolase (Meyerhof and Oesper 1949; Sims and Reed 2005). Recent report has shown that GKs could be used to optimize the enzymatic synthesis of 2-PGA (Sims and Reed 2005).

To date, GKs have been reported from *Bacteria* and *Eukarya* (Ornstoni et al. 1969; Katayama et al. 1980; Chistoserdova et al. 1997). However, report about the characterization of GK from *Archaea* has been quite limited with only two recent studies on the 2-PGA forming kinases from the thermoacidophilic euryarchaeon *Picrophilus torridus* and *Thermoplasma acidophilum* (Noh et al. 2006; Reher et al. 2006). A hyperthemophilic archaeon *Pyrococcus furiosus*, belonging to the euryarchaeal order *Thermococcales*, also harbored the GK activity, but the encoding gene has not been identified and the enzymatic properties have not been characterized (Schäfer and Schönheit 1992). *Pyrococcus horikoshii* OT3, an anaerobic hyperthermophilic archaeon, grows from 85 to 105°C producing many thermophilic or thermostable enzymes which have been used as models to study extremozymes, and some have potential commercial applications (Ando et al. 2002). In this paper, we report the cloning, expression and characterization of the unique hyperthermostable 2-PGA forming glycerate kinase (GK) from the hyperthemophilic archaeon *P. horikoshii*.

Materials and methods

Chemicals, enzymes, bacterial strains and plasmid

AMP, ADP, ATP, pyruvate, phosphoenolpyruvate, L-serine, glycine, citric acid, DTT and ß-mercaptoethanol were purchased from BioBasic Inc. (Canada). D-(+)-glycerate (hemicalcium salt), hydroxypyruvate, NADH, GTP, CTP, UTP, enolase, pyruvate kinase and lactate dehydrogenase, 3-PGA kinase, glyceraldehyde-3-phosphate dehydrogenase and thrombin were from Sigma-Aldrich (USA). Chitotriose, *N*-acetylchitotriose, cellotriose and glucosamine were purchased from Seikagaku (Japan). TLC plastic sheets (PEI cellulose F) were from Merck (Germany). Restriction enzymes, *Pyrobest* DNA polymerase, DNA ligation kit ver. 2.1 and *Escherichia coli* DH5α were obtained from Takara Bio. (Dalian, China). *Escherichia coli* BL21-CodonPlus (DE3)-RIL and plasmid pET15b were from Novagen (Madison, WI, USA).

Cloning of the glycerate kinase gene

P. horikoshii OT3 genomic DNA was kindly provided by Dr. Ikuo Matsui (AIST, Japan). The nucleotide sequence of ORF PH0495 under accession number BAA29583 and annotated as a gene for a hypothetical protein, was identified and extracted from GenBank database. Two primers [sense (5′-ATTCGCTGATTCATATGATTGCCATGGATATTAGGGAG-3′) and antisense (5′-GTGAACAGTCGACTTAAGTACGGCCTCGTTTCGATGTGAC-3′), underlined nucleotides indicate *Nde*I and *Sal*I restriction enzyme sites, respectively)] were designed to amplify the gene by PCR with 100 µl reaction mixture subjected to 30 cycles of amplification (30 s at 94°C, 30 s at 55°C, 1 min at 72°C). The amplified DNA was digested with *Nde*I and *Sal*I and then cloned into plasmid pET15b using *E. coli* DH5α as the host. The resulting plasmid contained the GK gene was designated pET15b-GK_{ph}. The host containing pET15b-GK_{ph} was grown at 37°C in LB medium supplemented with 100 µg/ml ampicillin. The recombinant plasmid was checked by DNA sequencing analysis.

Overexpression of the gene and purification of GK_{ph}

For overexpression of the recombinant protein, *E. coli* BL21-CodonPlus (DE3)-RIL were transformed with pET15b-GK_{ph}. An overnight culture in LB medium at 37°C was diluted 1:100 and grown until the OD_{600} reached 0.5, and then induced with 1 mM IPTG for 4 h. Cells were harvested by centrifugation (6,000*g* for 15 min at 4°C), resuspended in buffer A (50 mM Tris–HCl and 50 mM NaCl, pH 8.0), and then disrupted by sonication. The disrupted cells were incubated at 80°C for 30 min and then centrifuged (14,000*g* for 30 min) to obtain heat-stable enzymes. The supernatant was loaded on a nickel column (Novagen). The resulting elute was dialyzed in buffer A and then applied on a HiTrap Q anion exchange column (Amersham). The peak fractions eluting at 0.3 M NaCl were collected and analyzed by SDS-PAGE and gel filtration with Sephacryl^{TM} S-200 HR column (Amersham) equilibrated in 50 mM Tris–HCl, 100 mM NaCl buffer (pH 8.0) at 1 ml/min. Protein concentration was determined according to the Bradford method. The His-tag sequence was removed by thrombin to check its probable effect on the recombinant enzyme activity.

Determination of glycerate kinase acitivity and product identification

The following three assays were used to determine GK activity. A 20 µl reaction mixture containing 1 mM D-glycerate, 1 mM ATP, 10 mM $MgCl_2$, 50 mM potassium phosphate (pH 7.0), 0.2 μCi [γ-^{32}P]ATP and increasing amount of purified GK_{ph} (assay 1). Samples of each reaction were run on a TLC plastic sheets (PEI cellulose F) in the chromatography buffer containing 1 M formic acid and 0.5 M lithium chloride and visualized on phosphorstorage screen using Typhoon 9410 (Amersham). The extent of ATP hydrolysis was calculated by IamgeQuant version 5.2.

The reaction mixture (1 ml) contained 1 mM D-glycerate, 1 mM ATP, 10 mM $MgCl_2$, 0.3 mM NADH, 1 mM phosphoenolpyruvate, 10 nM purified GK_{ph}, 3.5 units pyruvate kinase and 5 units lactate dehydrogenase in 50 mM potassium phosphate (pH 7.0) and was incubated at 45°C for 10 min (assay 2). The reactions without GK_{ph} were used as blanks. One unit of the enzyme activity was defined as the amount of the enzyme, which transferred 1 μmol phosphate group per minute. Each sample was measured in triplicates and the data were averaged.

The GK activity was assayed in another reaction mixture (1 ml) contained 1 mM D-glycerate, 1 mM ATP, 10 mM $MgCl_2$, 0.3 mM NADH, 10 nM purified GK_{ph}, 2 units of enolase, 3.5 units pyruvate kinase and 5 units lactate dehydrogenase in 50 mM potassium phosphate (pH 7.0) and was incubated at 45°C for 10 min (assay 3).

The enzymes 3-PGA kinase and glyceraldehyde-3-phosphate dehydrogenase, which are able to convert 3-PGA to glyceraldehyde-3-phosphate by coupling the oxidation of NADH were used to determine whether 3-PGA was produced by GK_{ph}. The product from GK activity was determined using the following procedure. GK_{ph} was initially incubated in 1 ml 50 mM potassium phosphate (pH 7.0), 1 mM D-glycerate, 1 mM ATP and 10 mM $MgCl_2$ at 45°C for 10 min. After the reaction, the mixture was cooled, and 100 μl was then transferred into a 1 ml reaction mixture which contained 50 mM potassium phosphate (pH 7.0), 1 mM ATP, 0.3 mM NADH, 8 units of glyceraldehyde-3-phosphate dehydrogenase and 9 units of 3-PGA kinase. The reaction was performed at 25°C for 10 min.

Effects of pH and temperature

The optimal temperature was determined at temperatures ranging from 20 to 100°C (assay 1 or 2). The optimal pH was determined in various pHs of 50 mM buffer as follows (assay 1): Na_2HPO_4–citric acid (pH 2.5–5.0), MES–NaOH (pH 5–7) and 50 mM Tris–HCl (pH 7–9). The highest activity was defined as 100% level.

Thermostability

The enzyme was preheated in 50 mM potassium phosphate (pH 7.0) at 70, 80 and 90°C for up to 12 h, respectively. Samples were taken at various times (2 h once) and the residual activities were measured (assay 2 or 3) and expressed as the percentage of the initial activity.

Substrate specificity

To study the substrate specificity, the following compounds were substituted for D-glycerate: glycerol, lactate, malonate, tartarate, hydroxypyruvate, pyruvate, 1,3-propanediol, L-serine, glycine, citric acid, chitotriose, *N*-acetylchitotriose, cellotriose, glucose and glucosamine (assay 1 or 2). Alternative phosphate donors GTP, CTP, UTP ADP, AMP and pyrophosphate (1 mM) were substituted for ATP, respectively (assay 3). Various divalent metals Mn^{2+}, Co^{2+}, Ca^{2+}, Sr^{2+} and Ni^{2+} (10 mM) were added instead of Mg^{2+}, respectively (assay 2).

Effects of monovalent metal ions and various additives

To investigate the effect of monovalent metal ions on the enzyme activity, the reaction was carried out except that 50 mM Tris–HCl (pH 7.0) was used instead of potassium phosphate, and 50 mM KCl, NaCl, NH_4Cl and LiCl were added, respectively (assay 3). The following additives were added in the reaction mixture (assay 1) to study their effect on the enzymatic activity: urea, NaN_3 (50 mM), PMSF (10 mM), ethanol, 2-propanol, *n*-butanol, DMSO (10%, v/v), H_2O_2, $MnCl_2$, $CoSO_4$, $CaCl_2$, $SrCl_2$, $NiCl_2$, $CuCl_2$, $HgCl_2$ (1 mM). Reducing agents DTT or ß-mercaptoethanol was also added at 1 mM to study their effects on the enzyme activity (assay 3).

Kinetic properties

Kinetic studies towards D-glycerate and ATP were measured using six different substrate concentrations (0–1 mM) according to assay 2, and the kinetic parameters were determined from the rates by means of respective Lineweaver-Burk plots.

Results and discussion

Sequence analysis of GK_{ph}

The GK_{ph} gene PH0495 consists of 1,323 bp, encoding a protein of 440 amino acids with a predicted molecular mass of 47.4 kDa (http://www.expasy.org/tools/pi_tool.html). By sequence comparison, the deduced amino acids showed high overall sequence identity to its archaeal homologues in *Pyrococcus* species: the hypothetical protein, PAB1021 from *P. abysii* (82%, CAB50451) and the putative GK, PF0024 from *P. furiosus* (64%, AAL80148). There were many other homologues from *Archaea*, such as PAE1309 from *Pyrobaculum aerophilum* (41%, AAL63393), APE0996 from *Aeropyrum pernix* (44%, BAA79980) and PTO1442, a recently characterized thermoacidophilic 2-PGA forming GK (31%, AAT44027) from *Picrophilus torridus* (Reher et al. 2006). In addition, it showed homology with some bacteria homologues such as the putative GK, TM1585 from *Thermotoga maritime* (45% identity, AAD36652) and GK from *Methylobacterium*

extorquens (34% identity, AAB66496). Multiple alignment of the sequence of GK_{Ph} with some of its homologous proteins was shown in Fig. 1. Many conserved amino acids were present among the sequences. GK_{Ph} showed high sequence identity to archaeal GKs (such as *P. torridus* and *P. furiosus*) and structural similarity with the putative GK from *T. maritime* (see follows), which all belonged to group I GKs as a second clade (Boldt et al. 2005; Reher et al. 2006). Moreover, a MOFRL (multi-organism fragment with rich leucine) domain, which has been reported as characteristic for the archaeal GKs in this group (Boldt et al. 2005; Ahmed et al. 2006), was presented at the C-terminal of GK_{Ph} (Fig. 1). Thus, GK_{Ph} should be classified into group I GKs of the proposed trichotomy of GKs (Boldt et al. 2005; Reher et al. 2006).

Cloning, expression of the gene and purification of GK_{ph}

The purified recombinant protein was estimated to have a size of 47 kDa as evidenced by SDS-PAGE electrophoresis (Fig. 2, lanes 1–5). However, gel filtration showed that the molecular mass was approximately 100 kDa (Supplementary Fig. 1), suggesting that the native structure was likely to be a homodimer.

GK enzymatic studies

A radioisotope-labeling examination method was initially used for the detection of kinase activity as described in ''Materials and methods'' (assay 1). When increasing amount of GK_{ph} was added, ATP hydrolysis increased accordingly. A fast-moving band appeared, presumably to be the phosphoryled glycerate (Fig. 3, lanes 2–6), suggesting the presence of kinase activity of GK_{ph}.

To confirm that the enzyme is indeed a GK, the enzymatic activity of GK_{ph} was assayed as NADH oxidation at 340 nm by the spectrophotometer method in the presence of coupling enzymes (assay 2, 3) (Yoshida et al. 1992). No decrease in absorbance was observed at 340 nm without GK_{ph}. After addition of GK_{ph} to the reaction mixture, an obvious decrease was observed (data not shown), indicating the presence of 2-PGA. These results also confirmed that the [γ-^{32}P]-labeled product in assay 1 was phosphoryled glycerate.

To rule out the production of 3-PGA, another coupled method was employed. When 3-PGA kinase and glyceraldehyde-3-phosphate dehydrogenase were added, no oxidation of NADH was detected after the reaction, indicating the absence of 3-PGA. These results showed that GK_{ph} is a 2-PGA forming GK (rather than 3- PGA forming GK).

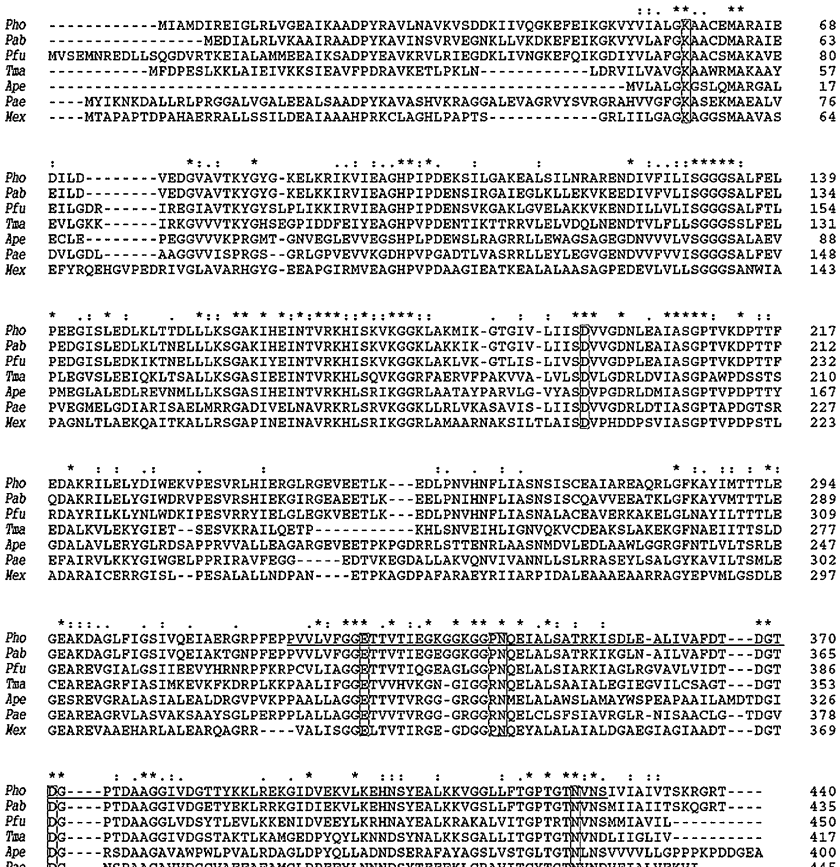

Fig. 1 Alignment of the amino acid sequences of GK_{Ph} with some of its homologous proteins. Numbering of the amino acids is indicated on the *right*. Conserved residues are indicated by an *asterisk* above the alignment, *single* and *double dots* represent amino acids with semi-conservative and conservative substitutions. Gaps introduced during the alignment process are indicated as *dashes*. Highly conserved amino acids of the presumed active site are *boxed*. The residues of conserved glycine rich loop are in *shadow*. The MOFRL domain of GK_{Ph} was *underlined*. *Pho P. horikoshii*; *Pab P. abyssi*; *Pfu P. furiosus*; *Tma Thermotoga maritima*; *Ape Aeropyrum pemix*; *Pae Pyrobaculum aerophilum*; *Mex Methylobacterium extorquens*. Accession numbers for these sequences are stated in ''Sequence analysis of GK_{ph}'' in Results

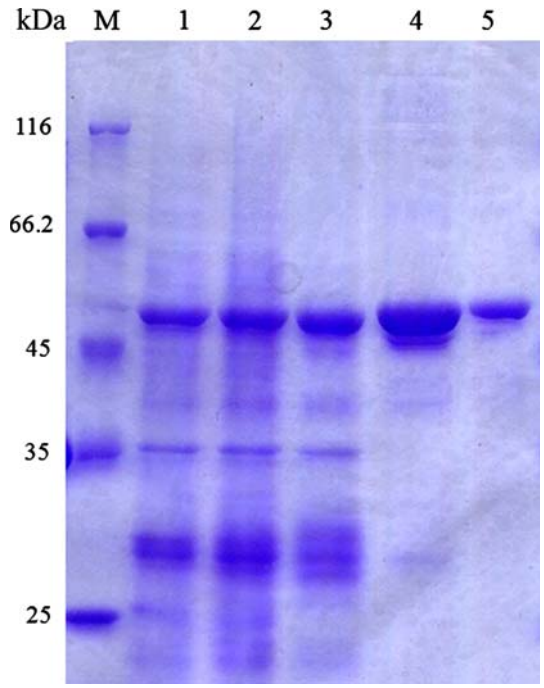

Fig. 2 SDS-PAGE of GK_{Ph}. *Lanes M* protein markers, *1* crude cell extract, *2* soluble fraction, *3* supernatant after heat-treatment for 30 min at 80°C, *4* the partially purified enzyme after nickel column, *5* the purified enzyme after ion exchange chromatography

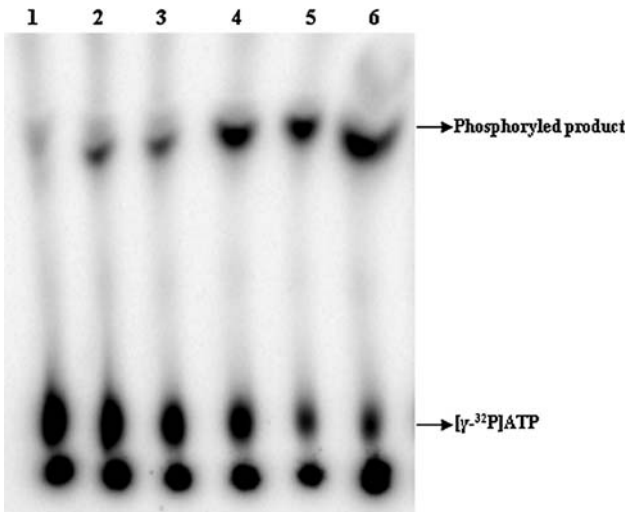

Fig. 3 Detection of the glycerate kinase activity using radioisotope-labeling examination method (as described in ''Materials and methods''). Different concentrations of GK_{Ph} used in each lane were (from the *left* to the *right*): 0, 2, 4, 16, 32 and 160 nM, respectively

The optimal temperature and thermostability of GK_{ph} were determined with optimal activity at 45°C. It had strong activity at moderate temperature 30–50°C and about half of the maximal activity retained at 100°C (Supplementary Fig. 2b). Compared with the enzyme having no heat treatment, most of activity (>90%) remained after incubation for 12 h at 70, 80 or 90°C (details not shown), indicating that the enzyme was a highly thermostable GK. GK_{ph} was shown to be optimal at pH 7.0 and half of the maximum activity remained at pH 6–10 (Supplementary Fig. 2a). Removal of the His-tag sequence had no effect on the activity of the recombinant enzyme (data not shown).

Table 1 Phosphate donor specificity of GK_{ph}

Phosphate donor	Relative activity (%)
ATP	100
GTP	64
CTP	73
UTP	29
ADP	32
AMP	0
Pyrophosphate	112

Table 2 Effects of monovalent metal cations on GK_{ph} activity

Monovalent metal ions	Relative activity (%)
None	100
KCl	794
NaCl	349
NH_4Cl	783
LiCl	228

Substrate specificity, effects of metal ions and additives

GK_{ph} was shown to be specific to phosphorylate glycerate among the compounds tested. No activity was observed in the absence of divalent metal ion and maximal activity was observed in the presence of Mg^{2+}. When Mn^{2+}, Co^{2+}, Ca^{2+}, Sr^{2+} and Ni^{2+} was substituted for Mg^{2+}, respectively, Mn^{2+}, Co^{2+} and Ni^{2+} showed 76, 68 and 11% activity of that for Mg^{2+}. The phosphate donor specificity was shown in Table 1. To our surprise, pyrophosphate exhibited the maximal phosphate donor activity and ADP could also serve as the phosphate donor, which has not been reported before for GKs.

The effects of monovalent metal cations on the enzyme activity were shown in Table 2. The addition of K^+ stimulated the enzyme activity (about 8 times), which was similar to that of GK from *Hyphomicrobium methylovorum* GM2 (Yoshida et al. 1992), but different from GK from *P. torridus* (Reher et al. 2006). The enzyme was inhibited by $HgCl_2$ (11%) and $CuCl_2$ (61%), and resistant to denaturing agent urea and organic solvents ethanol, 2-propanol, *n*-butanol and DMSO (>90%). NaN_3, PMSF, H_2O_2 and other divalent metal compounds tested had no obvious effect on the enzymatic activity (>95%). Similar to GK

Table 3 Kinetic properties of GK_{ph}

Substrate	K_m (mM)	V_{max} (U/mg)	K_{cat} (s^{-1})	K_{cat}/K_m (M^{-1} s^{-1})
Glycerate	0.044 ± 0.0074	624.46 ± 10.726	489.16 ± 8.399	1.1×10^7
ATP	0.102 ± 0.0199	639.01 ± 16.189	500.56 ± 12.689	4.89×10^6

from maize leaf, reducing agents DTT and ß-mercaptoethanol could slightly increased the enzyme activity (128 and 114% of the original activity, respectively) (Kleczkowski and Randall 1985).

Kinetic properties

Kinetic experiments were performed as described in ''Materials and methods''. As shown in Table 3, GK_{ph} showed lower K_m, higher V_{max} values than the reported GKs from *E. coli*, *P. torridus*, *T. acidophilum* and *H. methylovorum* GM_2 (Yoshida et al. 1992; Hubbard et al. 1998; Noh et al. 2006; Reher et al. 2006), suggesting its advantages for enzymatic catalysis in application.

During our previous study on the characterization of two enzymes involving in chitin degradation in *P. horikoshii*, an ORF (PH0495) was found adjacent to the exo-ß-D-glucosaminidase (PH0511) and a diacetylchitobiose (PH0499) (Liu et al. 2006a, b). A radioisotope-labeling examination method was introduced to detect the enzyme activity (PH0495) and used to test some enzymatic properties, which can help avoid the probable effect of the auxiliary enzymes in the spectrophotometer assays with coupling enzymes. Considering the biotechnological advantages of thermophilic enzymes such as avoiding contamination, allowance of higher substrate concentrations, and lower viscosity and improvement of the rate of reaction (Egorova and Antranikian 2005), GK_{ph} thus could have potential applications for the enzymatic catalysis of glycerate to synthesize 2-PGA (Sims and Reed 2005).

Recently, the crystal structure of GK_{ph} has been solved (PDB: 1X3L). By comparing the structure with that of the putative GK from *Thermotoga maritime* (TM1585, PDB: 2B8N), which was denoted as a new fold GK (Schwarzenbacher et al. 2006), striking similarities of the overall folds and regions of the presumed active sites between the two enzymes were revealed (Supplementary Fig. 3). The seven putative catalytic amino acids and glycine loop of GK_{ph} are conserved and arranged identically on the structure of TM1585 except that Pro343 is substituted for Arg325 of TM1585 (Fig. 1, Supplementary Fig. 3). Site directed or deleted mutation analysis is needed to elucidate the catalytic properties of these new fold enzymes (Cheek et al. 2005; Schwarzenbacher et al. 2006).

Until now, 2-PGA forming GKs from *E. coli*, *P. torridus*, *T. acidophilum* and *H. methylovorum* GM_2 have been well characterized (Yoshida et al. 1992; Hubbard et al. 1998; Noh et al. 2006; Reher et al. 2006). The unique phosphate donor dependency and the high thermostability of GK_{ph} were distinctive to the formerly reported GKs, possibly implying its unique metabolic role. It was reported that 2-PGA forming GK is a key enzyme involved in the non-phosphorylative ED (nED) pathway in *Archaea* (Ahmed et al. 2006; Reher et al. 2006), which has been reported in several thermpacidophilic organisms involving both groups of the ancient domain (Reher et al. 2006). The presence of enzymes in maltose degradation of *P. furiosus* also indicated the operative role of 2-PGA forming GK in the nED pathway of this organism (Schäfer and Schönheit 1992). However, the function of GK_{ph}, the first characterized GK from the order *Thermococcales* in Euryarchaeota, remains to be investigated. Although it was reported that the hyperthermophilic archaeon was likely to harbor nED pathway (Selig et al. 1997), Gonzalez et al. (1998) reported that *P. horikoshii* could not grow on sucrose, glucose or maltose as sole carbon sources, which is different to *P. furiosus*. The different living ecosystems and evolution divergence between *P. furiosus* and *P. horikoshii* (Maeder et al. 1999) and the unique enzymatic properties suggest the unknown metabolic role of this enzyme. Further studies of this issue should be informative.

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