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Identification and Characterization of *Thermoplasma acidophilum* 2-Keto-3-Deoxy-D-Gluconate Kinase: A New Class of Sugar Kinases

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Abstract The thermoacidophilic archaeon *Thermoplasma acidophilum* has long been known to utilize D-glucose *via* the non-phosphorylated Entner-Doudoroff (nED) pathway. We now report the identification of a gene encoding 2-keto-3-deoxy-D-gluconate (KDG) kinase. The discovery of this gene implies the presence of a glycolysis pathway, other than the nED pathway. It was found that Ta0122 in the *T. acidophilum* genome corresponded to KDG kinase. This enzyme shares no similarity with known KDG kinases, and belongs to a novel class of sugar kinases. Of the five sugars tested only KDG was utilized as a substrate.

Keywords: 2-keto-3-deoxy-D-gluconate kinase, *Thermoplasma acidophilum*, glycolysis, non-phosphorylated Enter-Doudoroff pathway, phosphoprotein, Euryarchaeota

INTRODUCTION

Thermoacidophilic archaea, such as *Thermoplasma acidophilum* and *Sulfolobus solfataricus*, are known to metabolize glucose *via* the modified Entner-Doudoroff (ED) pathway, which produces non-phosphorylated intermediates such as D-gluconate, 2-keto-3-deoxy-D-gluconate (KDG), and D-glyceraldehyde [1-4]. The first step of the non-phosphorylated ED (nED) pathway involves the $NAD(P)^+$-dependent oxidation of D-glucose to D-gluconate, catalyzed by glucose dehydrogenase [5,6]. D-Gluconate is then dehydrated by gluconate dehydratase to KDG [7,8], which undergoes an aldolate cleavage to D-glyceraldehyde and pyruvate, catalyzed by KDG aldolase [9]. Glyceraldehyde dehydrogenase then oxidizes D-glyceraldehyde to D-glycerate, which is phosphorylated by glycerate kinase to 2-phosphoglycerate. One additional molecule of pyruvate is produced from 2-phosphoglycerate by the actions of enolase and pyruvate kinase.

Recently, it was found that both *T. acidophilum* and *S. solfataricus* had KDG kinase activities in their crude cell extracts [10]. Of the eight sugars tested, *T. acidophilum* had kinase activities toward KDG and glycerate, whereas *S. solfataricus* showed kinase activities toward KDG, glycerate, and other sugars such as glucose, fructose, gluconate, and glycerol. This indicates the presence of another modified ED pathway in these archaea, because KDG can be phosphorylated to 2-keto-3-deoxy-6-phosphogluconate (KDPG) by KDG kinase as in bacteria [11,12]. Interestingly, it has been reported that the *S. solfataricus* has a gene (SSO3195) encoding a putative KDG kinase [13,14]. However, no gene that shares homology with SSO3195 or other KDG kinases could be found in the genome of *T. acidophilum*. This is surprising because KDG kinase activity in *T. acidophilum* is higher than that in *S. solfataricus*.

We now report that *T. acidophilum* possesses a KDG kinase that belongs to a new class of sugar kinases. Moreover, this enzyme shares no similarity with previously reported KDG kinases. The discovery of an enzyme that phosphorylates KDG supports the presence of an alternative glycolysis pathway, i.e., other than the nED pathway in *T. acidophilum*.

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MATERIALS AND METHODS

Strains, Media and Culture Conditions

T. acidophilum (JCM9062) was grown in media composed of (per liter) 1.0 g yeast extract, 3.0 g glucose, 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. Cells were cultured aerobically in a 3-liter fermentor (KLF 2000, Bioengineering AG, Wald, Switzerland) at 59°C for 60 h with stirring at 50 rpm.

KDG Kinase Assay

Crude extracts were prepared by sonicating cell pellets suspended in 50 mM Tris-HCl (pH 8.0). KDG kinase ac-

tivities were determined spectrophotometrically at 340 nm. Unless specified otherwise, reaction mixtures comprised 50 mM Tris-HCl (pH 8.0), 5 mM EGTA, 1 mM KDG, 1 mM ATP, 10 mM $MgCl_2$, and an enzyme solution. EGTA was included in the assay because KDG kinase activities were significantly enhanced in its presence. After carrying out reactions at 50°C for 20 min, 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. The coupled enzyme reactions were carried out at 25°C, and reductions in absorption at 340 nm were monitored. All enzyme activities were determined in triplicate.

Purification of KDG Kinase

Dialyzed crude extracts were loaded onto a DEAE-Sepharose column (2.5 × 16 cm) equilibrated with 50 mM Tris-HCl (pH 8.0; buffer), and then enzymes were eluted using a stepwise gradient of NaCl (0.0~1.0 M). Fractions containing enzyme activity were pooled, dialyzed in buffer and concentrated using a Vivaspin™ concentrator membrane. The DEAE-Sepharose active fraction was loaded onto a Q-Sepharose column (2.5 × 14 cm). After washing, a stepwise gradient of NaCl (0.0~1.0 M) was applied. Selected fractions were loaded onto an Affi-gel blue column (0.5 × 14 cm), and the enzyme was eluted using a stepwise gradient of NaCl (0.0~1.0 M). Active enzyme fractions were pooled and concentrated, and further purified using a Sephadex G-75 column (1.5 × 150 cm). Fractions containing enzyme activity were pooled and concentrated, and the Sephadex G-75 active fraction was loaded onto a Mono Q HR 5/5 column (1.0 × 2.5 cm). The enzyme was then eluted using a stepwise gradient of NaCl (0.0~1.0 M). Selected fractions were dialyzed against buffer and stored at 4°C.

Molecular Mass Determination

Purified KDG kinase was chromatographed on a Sephacryl S-200 column (1.5 × 150 cm) using a gel filtration calibration kit. The buffer used for column equilibrium and elution was 50 mM Tris-HCl (pH 8.0). The molecular weight markers used were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), albumin (67 kDa), and ovalbumin (43 kDa).

Identification of KDG Kinase

SDS-PAGE was performed using 12% polyacrylamide slab gels. Proteins in the gels were excised, sequentially digested in gel with trypsin, and then submitted for matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Peptide mass fingerprints were acquired on a MALDI-TOF mass spectrometer (4700 Proteomics Analyzer, Applied Biosystems). Monoisotopic peptide masses obtained by MALDI-TOF were queried against entries in the NCBInr protein databases using Mascot.

Characterization of KDG Kinase

The dependence of enzyme activity on temperature was examined at temperatures between 30 and 80°C. The effect of pH on the KDG kinase activity was determined at 50°C in 50 mM citric acid-NaOH (pH 3.0~6.0), 50 mM Tris-HCl (pH 5.0~9.0), or in 50 mM glycine-NaOH (pH 9.0~11.0).

Substrate specificity was determined using the following compounds as substrates: KDG, D-gluconate, 2-keto-D-gluconate, 5-keto-D-gluconate, N-acetylglucosamine, and D-xylulose.

Dephosphorylation of KDG Kinase

To identify whether KDG kinase is a phosphoprotein, purified proteins were dephosphorylated with either PAP (potato acid phosphatase) or BAP (bacterial alkaline phosphatase). Dephosphorylation by PAP involved incubation at 37°C for 12 h in mixture containing 20 mM MES/NaOH (pH 6.5) and 100 mM NaCl. Dephosphorylation by BAP was performed similarly, except that the reaction mixtures contained 50 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ instead of MES/NaOH and NaCl.

RESULTS

Identification of KDG Kinase

KDG kinase was purified 143-fold using a five-step chromatography procedure. The molecular mass of the native enzyme was estimated to be 260 kDa using a calibrated Sephacryl S-200. Since the molecular mass of the denatured enzyme determined by SDS-PAGE was approximately 34 kDa, it appears that KDG kinase is a homo-octamer.

To identify the gene encoding KDG kinase, purified enzyme was loaded on SDS-PAGE, run, excised, and spots were analyzed by MALDI-TOF MS. The MS data were searched for in the NCBI database using Mascot. Monoisotopic peptide masses obtained from mass spectra matched that of Ta0122 (GenBank: NC_002578). The Mascot score and sequence coverage were 305 and 65%, respectively. We named the ORF of Ta0122 *kdgK*, because it encodes *KDG k*inase. The molecular mass of Ta0122 deduced from its amino acid sequence (36,391 Da) was similar to the molecular mass of the purified KDG kinase in denaturing gel (34 kDa).

Characterization of KDG Kinase

The effect of temperature on KDG kinase activity was examined from 30 to 80°C. The purified enzyme displayed optimal activity at 70°C, which is somewhat higher than the optimum temperature for cell growth (59°C). KDG kinase showed maximal activities at pH's between 4.0 and 5.0, which is lower than intracellular pH of *T. acidophilum* (pH=5.5) [15].

Table 1. Substrate specificity of *T. acidophilum* KDG kinase

Substrate	Enzyme activity (U/mg)	Relative activity (%)
KDG	15.6	100.0
2-keto-D-gluconate	0.2	1.3
5-keto-D-gluconate	0.2	1.3
D-gluconate	0.1	0.6
N-acetylglucosamine	~0	~0
D-xylulose	~0	~0

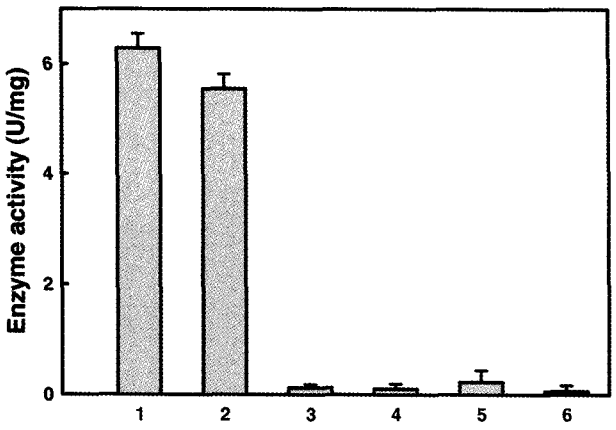

Fig. 1. Activities of *T. acidophilum* KDG kinase after phosphatase treatment: 1, KDG kinase preincubated without PAP; 2, KDG kinase preincubated without BAP; 3, KDG kinase preincubated with PAP; 4, KDG kinase preincubated with BAP; 5, PAP in a buffer solution; 6, BAP in a buffer solution.

The abilities of KDG kinase to phosphorylate sugar compounds were examined under standard assay conditions using KDG, D-gluconate, 2-keto-D-gluconate, 5-keto-D-gluconate, N-acetylglucosamine, or D-xylulose as substrates (Table 1). *T. acidophilum* KDG kinase specifically phosphorylated KDG, and showed no or very little activity toward the other compounds tested in this study.

Deactivation of KDG Kinase by Dephosphorylation

KDG kinase activity was lost after incubation with phosphatase, whereas no such decrease in activity was observed when it was incubated in the same reaction mixtures without phosphatase (Fig. 1). These results indicate that the KDG kinase of *T. acidophilum* is a phosphoprotein. The loss of activity by dephosphorylation indicates that the phosphoamino acid residues are located in the catalytic domain of the enzyme and that phosphorylation and dephosphorylation control the activity of KDG kinase.

Homologs of KDG Kinase

Based on the amino acid sequence of *T. acidophilum* KDG kinase, BLAST searches were performed against the NCBI database. These searches showed that the amino acid sequence of *kdgK* is highly homologous with those of proteins in other thermoacidiphilic archaea. Calculated sequence identities were 60% and 33% for the protein in *Thermoplasma volcanium* (GenBank NP_110718) and *Ferroplasma acidarmanus* (EAM94328), respectively. There were two homologs of Ta0122 in *Picrophilus torridus* with sequence identities of 39% (AAT42596) and 33% (AAT43679) for PTO0011 and PTO1094, respectively. On the other hand, Ta0122 shares no homology with any known KDG kinases.

DISCUSSION

Although many kinds of bacteria and archaea metabolize D-glucose via the modified ED pathway [16], little is known about the biochemical properties of KDG kinases. Up to now, KDG kinases from *E. coli* [17] and *Thermus thermophilus* [18] have been characterized in detail, but the biochemical properties of archaeal KDG kinases have not yet been reported. In this study, we purified, identified, and characterized the KDG kinase from the thermoacidophilic archaeon *T. acidophilum*. By MALDI-TOF/MS peptide mass fingerprinting of the purified enzyme, we were able to determine that the gene encoding KDG kinase corresponded to Ta0122 in the *T. acidophilum* genome database. *T. acidophilum* KDG kinase showed strict specificity toward KDG, but no activity toward xylulose or N-acetylglucosamine.

Currently, the amino acid sequences of many KDG kinases are available in the NCBI database. Phylogenetic analysis indicated that all currently known KDG kinases belong to the PfkB carbohydrate kinase family (Pfam 00294; COG0524), whereas the KDG kinases from the Euryarchaeota belong to the BadF/BadG/BcrA/BcrD ATPase family (Pfam01869; COG2971). The amino acid sequence of *T. acidophilum* KDG kinase showed no similarity with any previously reported KDG kinases, which may be the reason why this enzyme has not been previously identified in the Euryarchaeota.

On the other hand, the amino acid sequences of euryarchaeal KDG kinases were found to be similar to N-acetylglucosamine kinase. Recently, Berger *et al.* [19] proposed a model of the three-dimensional structure of N-acetylglucosamine kinase based on the known structure of glucokinase. They found that N-acetylglucosamine kinase shares all five subdomains of the ATP-binding domain of sugar kinases with high similarity. However, in the euryarchaeal KDG kinases, the connect-2 domain is not conserved (Fig. 2), indicating that the euryarchaeal KDG kinases belong to a new class of sugar kinases.

It has been postulated that *T. acidophilum* utilizes D-glucose via the nED pathway [4]. However, the identification of the gene encoding KDG kinase in *T. acidophilum* strongly suggests that D-glucose can be metabolized *via* the '*partially* non-phosphorylated' ED (pnED) pathway. In this modified ED pathway [16], D-glucose is converted into D-gluconate and KDG as in the nED pathway, but the KDG produced by D-gluconate dehy-

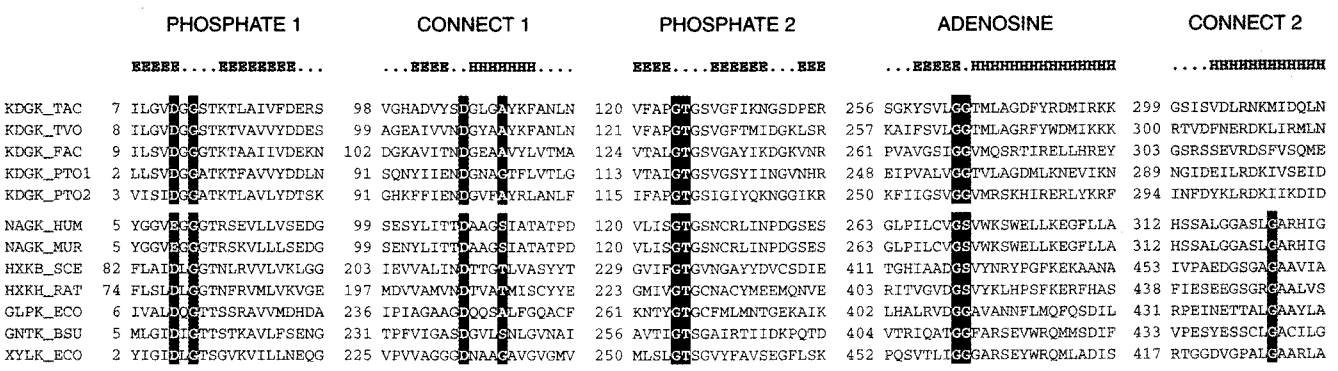

Fig. 2. Multiple alignment of sequence motifs involved in ATP binding. The ATP binding subdomains (Phosphate 1, Connect 1, Phosphate 2, Adenosine, Connect 2) and secondary structure of *T. acidophilum* KDG kinase are given, where E is β-strand and H is α-helix. The residues conserved in the five subdomains of the ATP-binding domain [20] are highlighted by black with white letters. The prediction of the secondary structure was performed using PredictProtein [21]. The sequences aligned are: KDGK_TAC, *T. acidophilum* KDG kinase (GenBank accession number NC_002578); KDGK_TVO, *T. volcanium* KDG kinase (NP_110718); KDGK_PTO1, *P. torridus* KDG kinase homolog 1 (AAT42596); KDGK_PTO2, *P. torridus* KDG kinase homolog 2 (AAT43679); KDGK_FAC, *F. acidarmanus* KDG kinase (EAM94328); NAGK_HUM, human N-acetylglucosamine kinase (CAB61848); and NAGK_MUR, murine N-acetylglucosamine kinase (Q9QZ08). Amino acid sequences of HXKB_SCE (yeast hexokinase), HXKH_RAT (rat glucokinase) GLPK_ECO (*E. coli* glycerokinase), GNTK_BSU (*B. subtilis* gluconokinase), and XYLK_ECO (*E. coli* xylulokinase) were from the literature [20].

dratase is then phosphorylated by KDG kinase to KDPG. KDPG is then cleaved by KDPG aldolase to pyruvate and D-glyceraldehyde 3-phosphate. This latter intermediate is then oxidized to pyruvate by D-glyceraldehyde-3-phosphate dehydrogenase, D-phosphoglycerate kinase, D-phosphoglycerate mutase, enolase, and pyruvate kinase. All of these genes can be found in the *T. acidophilum* genome database, which suggests that the pnED pathway is operative in this microorganism.

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