Characterization of a Novel Glucosamine-6-Phosphate Deaminase from a Hyperthermophilic Archaeon

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A key step in amino sugar metabolism is the interconversion between fructose-6-phosphate (Fructose-6-phosphate (Fr6P)) and glucosamine-6-phosphate (GlcN6P). This conversion is catalyzed in the catabolic and anabolic directions by GlcN6P deaminase and GlcN6P synthase, respectively, two enzymes that show no relationship with one another in terms of primary structure. In this study, we examined the catalytic properties and regulatory features of the glmD gene product (GlmD) present within a chitin degradation gene cluster in the hyperthermophilic archaeon Thermococcus kodakaraensis KOD1. Although the protein GlmD was predicted as a probable sugar isomerase related to the C-terminal sugar isomerase domain of GlcN6P synthase, the recombinant GlmD clearly exhibited GlcN6P deaminase activity, generating Fr6P and ammonia from GlcN6P. This enzyme also catalyzed the reverse reaction, the ammonia-dependent amination/isomerization of Fr6P to GlcN6P, whereas no GlcN6P synthase activity dependent on glutamine was observed. Kinetic analyses clarified the preference of this enzyme for the deaminase reaction rather than the reverse one, consistent with the catabolic function of GlmD. In T. kodakaraensis cells, glmD was polycistronically transcribed together with upstream genes encoding an ABC transporter and a downstream exo-β-glucosaminidase gene (glmM) within the gene cluster, and their expression was induced by the chitin degradation intermediate, diacetylchitobiose. The results presented here indicate that GlmD is actually a GlcN6P deaminase functioning in the entry of chitin-derived monosaccharides to glycolysis in this hyperthermophile. This enzyme is the first example of an archaeal GlcN6P deaminase and is a structurally novel type distinct from any previously known GlcN6P deaminase.

Amino sugars, such as N-acetylgalactosamine (GalNAc), N-acetylgalactosamine (GalNAc), and N-acetylmuramic acid, are important building blocks for structural polysaccharides or sugar chains in several organisms. In the metabolism of these sugars, the conversion between fructose-6-phosphate (Fr6P) and glucosamine-6-phosphate (GlcN6P) is a key step in both anabolic and catabolic directions. The anabolic reaction is catalyzed by GlcN6P synthase, while catabolism is mediated by GlcN6P deaminase (Fig. 1A). GlcN6P synthase catalyzes the irreversible formation of GlcN6P and glutamate from Fr6P and glutamine and is classified in a glutamine-dependent amidotransferase family (18) comprised of an N-terminal glutamine:fructose-6-phosphate amidotransferase, while catabolism is mediated by GlcN6P deaminase (Fig. 1A). GlcN6P synthase catalyzes the irreversible formation of GlcN6P and glutamate from Fr6P and glutamine and is classified in a glutamine-dependent amidotransferase family (18) comprised of an N-terminal glutamine amide transfer (GAT) domain joined to a C-terminal sugar isomerase domain (Fig. 1B). The former domain produces ammonia from glutamine, and the generated ammonia is utilized for amination of Fr6P accompanied by isomerization to GlcN6P in the latter domain. Unlike other glutamine-dependent amidotransferases displaying ammonia-dependent activity, GlcN6P synthase cannot utilize free ammonia as the nitrogen donor in place of glutamine (19).

On the other hand, GlcN6P deaminase catalyzes the deamination-isomerization reaction from GlcN6P to Fr6P and ammonia and can also catalyze its reverse reaction under the presence of high concentrations of ammonia (7, 20). Although GlcN6P synthase and GlcN6P deaminase catalyze similar reactions, there is no relation between the primary structures of these two enzymes. There have been many studies on GlcN6P synthase (3, 5, 13, 24) and GlcN6P deaminase (1, 8, 15–17, 20) from Eucarya and Bacteria due to the importance of these enzymes in the regulation of amino sugar metabolism. In contrast, corresponding enzymes from Archaea have not been reported so far. Most intriguingly, archaeal genomes do not harbor any genes homologous to known GlcN6P deaminases.

We have previously found that the hyperthermophilic archaeon Thermococcus kodakaraensis KOD1 (2) has an ability to degrade chitin, a β-1,4-linked linear homopolymer of GlcNAc, and successfully identified a novel chitin catabolic pathway. Namely, chitin is first degraded into the disaccharide GlcNAc2, by a unique extracellular chitinase from T. kodakaraensis (ChiA) possessing endo- and exo-type catalytic domains (25, 28). The GlcNAc2 is probably translocated across the cell membrane by an ABC transport system and then deacetylated by a deacetylase (Dac) with nonreducing end specificity. The partially acetylated disaccharide GlcN-GlcNAc is hydrolyzed into GlcN and GlcNAc by an exo-β-glucosaminidase (GlmA) and the generated GlcNAc is further deacetylated to GlcN by Dac (26, 27), resulting in the complete
conversion of chitin into GlcN monomers. The genes for these enzymes are highly clustered on the \textit{T. kodakaraensis} genome, whose features have recently been reported (accession no. AP006878) (12). Here, we focused on a gene within this cluster, encoding a probable sugar isomerase related to the isomerase domain of GlcN6P synthase, and clearly demonstrated that this probable sugar isomerase exhibited GlcN6P deaminase activity. This report identifies not only the first archaeal GlcN6P deaminase involved in chitin degradation but also a novel type of GlcN6P deaminase distinct from previously known enzymes.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and media.** \textit{T. kodakaraensis} KOD1 was grown anaerobically at 85°C in a screw-cap bottle with MA medium (4.8 g and 26.4 g of Marine Art SF agents A and B, respectively [Senju Seiyaku, Osaka, Japan], 5 g of yeast extract, and 5 g of tryptone in 1 liter of deionized water) supplemented with 5 g Marine Art SF agents A and B, respectively [Senju Seiyaku, Osaka, Japan], 5 g of H9252-D-thiogalactopyranoside at the mid-exponential growth phase and incubated for 4 h at 37°C. The cells were harvested by centrifugation (5,000 × g for 10 min at 4°C), resuspended in buffer A (50 mM Tris-Cl [pH 8.0]), and then disrupted by sonication. The supernatant after centrifugation (15,000 × g for 10 min) was incubated at 85°C for 10 min and centrifuged (15,000 × g for 10 min) to obtain a heat-stable protein solution. The solution was applied to an anion-exchange Resource Q column (6 ml) (Amersham Biosciences, Piscataway, NJ) equilibrated with buffer A. The proteins were eluted with a linear gradient of 0 to 0.5 M NaCl, and the peak fractions eluted at 0.3 M NaCl were concentrated using an Ultrafree-MC centrifugal filter unit Biomax-10 (Millipore, Bedford, MA). This was applied to a gel filtration Superdex-200 HR 10/30 column (Amersham Biosciences) equilibrated with buffer A containing 0.15 M NaCl. Protein concentration was determined with the Bio-Rad protein assay (Bio-Rad, Hercules, CA), with bovine serum albumin as a standard.

**Enzyme assays.** A qualitative assay for GlmD\textsubscript{Tk} toward various monosaccharides was performed with silica gel thin-layer chromatography (TLC) as described previously (25), with a modification in the developer to methanol-chloroform-acetic acid-water (30:20:1:1 vol/vol/vol/vol). For detection of the products, aniline-diphenylamine reagent and ninhydrin reagent were applied.

GlcN6P deaminase activity was determined by a coupled enzymatic assay with phosphoglucosone isomerase from baker’s yeast (Nacalai Tesque, Kyoto, Japan) and glucose-6-phosphate (Glc6P) dehydrogenase from \textit{Leuconostoc mesenteroides} (Sigma, St. Louis, Mo.). The reaction mixture (30 μl), containing 3.33 mM GlcN6P and 50 ng GlmD\textsubscript{Tk} in 50 mM CHES [2-[N-cyclohexylethylamino]-ethanesulfonic acid] buffer (pH 8.3 at 60°C), was incubated for 1 min at 60°C. After terminating the reaction by rapid cooling, 270 μl of coupling reaction mixture (0.56 mM NAD\textsuperscript{+}, 0.6 U phosphoglucomerase, isozyme 0.25 U Glc6P dehydrogenase in 47.8 mM Tris-Cl [pH 8.0]) was added and was then incubated for 30 min at 25°C. Absorbance at 340 nm derived from NADH formation was measured spectrophotometrically. To determine the optimal temperature, the first reaction was performed at various temperatures (25 to 100°C). The optimal temperature was determined by measuring the activity using the following buffers: MES (pH 5.5 to 7.0), Tris-Cl (pH 7.0 to 8.5), bicine-NaOH (pH 7.5 to 9.0), and CHES-NaOH (pH 8.5 to 10.5). The pH values were adjusted at room temperature, and the values at higher temperatures were calculated according to the temperature coefficients for the respective buffers (9). To investigate the thermostability of GlmD\textsubscript{Tk}, the enzyme solution (50 ng GlmD\textsubscript{Tk} in 20 μl of 75 mM CHES-NaOH [pH 8.3 at 60°C]) was incubated at 80°C or 90°C from 5 to 100 min before the first reaction, and the resulting activity was assessed by the method described above. The activity observed prior to incubation was 100%.

The reverse reaction of GlcN6P deaminase was determined by measuring the generated GlcN6P. The reaction mixture (100 μl), containing 5 mM Fructose-1, 10 mM NH\textsubscript{4}Cl, and 150 ng GlmD\textsubscript{Tk} in 50 mM CHES-NaOH (pH 8.3 at 60°C), was incubated for 1 min at 60°C. The reaction was terminated by cooling and followed by filtration with a Microcon YM-10 (Millipore) to remove the enzyme. The amount of GlcN6P in the filtrate was determined by a modified method of Morgan and Elson (15).

**RNA experiments.** For the isolation of RNA from \textit{T. kodakaraensis} KOD1, cells grown in MA medium with or without 0.1% GlcNAC\textsubscript{2} or maltose were harvested at the early exponential growth phase when \(A_{600}\) was around 0.18. Total RNA was isolated using the RNeasy Midi kit and Rnase-Free DNase set (QIAGEN). For reverse transcription (RT)-PCR, 40 ng of total RNA from the cells grown with GlcNAC\textsubscript{2} was used with the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA). Oligonucleotides RT1, RT2, and RT3 were used for the RT reaction, and pairs of oligonucleotides, F1-R1, F2-R2, and F3-R3, were used for successive PCR amplification. The sequences are as follows: RT1, 5′-CCACCTCTGCTAGAAAG-3′; RT2, 5′-GCTAAACTACCTACCTTCC-3′; RT3, 5′-CTTCAAGGAGTTAAGCG-3′; F1, 5′-GTTGGAGTAACACCACACGGC-3′; R1, 5′-CTTCACTGCGGGCTGG-3′; F2, 5′-GGAGGGTGGAATTAGTGCCG-3′; R2, 5′-CATACGCTTGAGGACG-3′; F3, 5′-GGTCTGATGCAAGAAGA-3′; R3, 5′-GTCATACCCAGCTTTCC-3′. For Northern blot analysis, 30 μg of total RNA from the cells grown with GlcNAC\textsubscript{2} was separated by denaturing agarose gel electrophoresis and transferred to positively charged nylon membranes (Roche Diagnostics, Basel, Switzerland) by capillary blotting. For RNA dot blot hybridization analysis, 30 μg of total RNA (3 μl) was dropped onto the membrane and was immobilized by UV cross-linking. DNA fragments of approximately 600 bp within the coding regions of \(glmD\textsubscript{Tk}, gluS\textsubscript{Tk},\) and a DNA ligase gene (\(ligA\)) were amplified by PCR and used as a template for probe preparation. The sequences of the primer pairs were as follows: F4, 5′-GTTCCTAAGATGTCCGCTTGC-3′ and R4, 5′-GATGGCCTATATGATGAG-3′; for \(glmD\textsubscript{Tk}, F5, 5′-GTCGAAAGGAGCCGAGCG-3′,\) and R5, 5′-GCTGGCTGGTTATATGAC-3′; for \(gluS\textsubscript{Tk}, F6, 5′-GAAGAGCTTCTTCTCAGGCC-3′;\) and R6, 5′-CAAGTATTTGTCCTCTGCG-3′ for \(ligA\). Digoxygenin-label-
ing of DNA fragments, hybridization, and detection of signals were performed according to the instructions of the manufacturer (Roche Diagnostics).

Western blot analysis. T. kodakaraensis KOD1 was cultivated in 10 ml of MA medium supplemented with various kinds of saccharides (final concentration, 0.5%) and 20 μl of polysulfide solution (20% elemental sulfur in 3 M Na2S) in place of elemental sulfur. The cells were harvested, disrupted by sonication in buffer A containing protein inhibitor mix (Complete Mini; Roche Diagnostics) and then centrifuged (15,000 × g for 30 min) to obtain soluble fractions. Each fraction was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and successive Western blot analysis using specific antiserum (rabbit) against the recombinant GlmDtk. A protein A-peroxidase conjugate was used to visualize the specific protein together with 4-chloro-1-naphthol and hydrogen peroxide.

Nucleotide sequence accession number. The nucleotide sequence data of the glmDtk gene reported here (TK1755) have been included within the T. kodakaraensis KOD1 genome (accession number AB006878) in the EMBL, GenBank, and DDBJ nucleotide sequence databases.

RESULTS

A probable sugar isomerase gene within the chitin degradation gene cluster in T. kodakaraensis. We have previously reported that the genes for chitin catabolic enzymes chitinase (ChiA
tk encoded by TK1765) (25, 28), GlcNAc2 deacetylase (DacTk encoded by TK1764) (27), and β-glucosaminidase (GlmA
tk encoded by TK1754) (26) are clustered at the 1,553-to 1,569-kbp region of the T. kodakaraensis KOD1 genome (12), as shown in Fig. 2. In this cluster, one gene (TK1755) was identified between the glmA
tk and putative ABC transporter genes (TK1756 to TK1760) in the same orientation. As these seven genes were overlapping or separated by short interval regions (from −8 bp to 47 bp), they were supposed to be transcribed into a single mRNA. The TK1755 gene, designated glmDtk, consisted of 978 bp encoding a protein of 326 amino acids with a predicted molecular mass of 36,749 Da. The deduced amino acid sequence displayed high overall homologies to the proteins from the closely related hyperthermophilic archaea Pyrococcus furiosus (PF0362), Pyrococcus abyssi (PABI348), and Pyrococcus horikoshii (PH0510) (61 to 63% identical at the amino acid level). GlmDtk also showed weak but notable similarities to the C-terminal isomerase domain of GlcN6P synthases from various sources (25 to 32% identities), although it lacks an N-terminal GAT domain (Fig. 1C). The GlmDtk-related proteins and domains commonly contained a tandem repeat of two sugar isomerase subdomains (SIS domain, Pfam01380). Previous X-ray crystallographic analyses for GlcN6P synthase from Escherichia coli (GlmSEc) have identified catalytically important amino acid residues, Glu-488, His-504, and Lys-603, for the amination and isomerization of Fru6P (30, 31). We found that the corresponding residues were also conserved in GlmDtk as Glu-214, His-230, and Lys-322, suggesting a sugar isomerization activity in GlmDtk with a catalytic mechanism similar to that of GlcN6P synthase. It should be noted that the T. kodakaraensis genome harbors a separate gene (designated glmSTk) encoding a protein entirely homologous to GlcN6P synthase (Fig. 1C), composed of GAT and isomerase domains, at a different locus (TK0809). The C-terminal isomerase domain showed 27% identity to GlmDtk.

As described above, T. kodakaraensis degrades chitin to GlcN by the functions of three catabolic enzymes, ChiA
tk, DacTk, and GlmA
tk, but the metabolic fate of the resulting GlcN was unclear. Bacterial and eucaryal pathways for GlcN metabolism include GlcN6P deaminase for the generation of the glycolytic intermediate Fru6P from GlcN6P. A representative is NagB from E. coli, which belongs to the GlcN6P isomerase/6-phosphogluconolactonase family (Pfam01182). However, no obvious ortholog for NagB and the related enzymes has been identified on the T. kodakaraensis genome or on other known archaeal genomes. These facts raised a possibility that glmDtk within the gene cluster for chitin degradation might participate in catabolism of the amino sugar, so we investigated the catalytic properties and regulatory features of GlmDtk, as described below.

Production and purification of recombinant GlmDtk. To investigate the function of the protein product of glmDtk, the gene was expressed in E. coli with the pET expression system. The recombinant protein was obtained in a soluble form and was purified to apparent homogeneity in SDS-PAGE by heat treatment and column chromatography (Fig. 3A). The molecular mass of the recombinant GlmDtk was estimated to be 37.3 kDa by SDS-PAGE and 71.8 kDa by gel filtration chromatography.
shown in Fig. 3B. GlmD
tk
tkodakaraensis recombinant GlmD lytic ability of GlmD the presence of ammonia (Fig. 4, right). This demonstrates the catalytic reverse reaction producing GlcN6P from Fru6P in the presence of a GAT domain in GlmD
tk
tkodakaraensis. We therefore examined the possibility of heteromeric association of GlmD
tk
tkodakaraensis with other proteins in T. kodakaraensis cells by native PAGE Western blot analysis, as shown in Fig. 3B. GlmD
tk
tkodakaraensis was actually expressed in the cells grown in the presence of GlcNAc2, and the mobility of native GlmD
tk
tkodakaraensis grown in 0.1% GlcNAc 2-containing medium for 24 h (50 µg); lane M, molecular mass marker. In lanes 3 to 6, 5 µg of proteins was applied. (B) Western blot analysis results after native PAGE of recombinant GlmD
tk
tkodakaraensis and cell extracts of T. kodakaraensis. For native PAGE, a 5 to 20% gradient gel was used. Lane 1, recombinant GlmD
tk
tkodakaraensis purified from E. coli (50 ng); lane 2, cell extracts of T. kodakaraensis grown in 0.1% GlcNAc2-containing medium for 24 h (30 µg); lane M, molecular mass marker.

FIG. 3. (A) SDS-PAGE results for samples through the purification steps of recombinant GlmD
tk
tkodakaraensis from E. coli. Lane 1, cell extract of E. coli before induction; lane 2, cell extract after induction for 4 h; lane 3, soluble fraction after sonication; lane 4, thermostable protein fraction after heat treatment at 85°C for 10 min; lane 5, peak fraction after anion-exchange chromatography; lane 6, peak fraction after gel filtration chromatography; lane M, molecular mass marker. In lanes 3 to 6, 5 µg of proteins was applied. (B) Western blot analysis results after native PAGE of recombinant GlmD
tk
tkodakaraensis and cell extracts of T. kodakaraensis. For native PAGE, a 5 to 20% gradient gel was used. Lane 1, recombinant GlmD
tk
tkodakaraensis purified from E. coli (50 ng); lane 2, cell extracts of T. kodakaraensis grown in 0.1% GlcNAc2-containing medium for 24 h (30 µg); lane M, molecular mass marker.

for the amination reaction in the presence of ammonia (data not shown).

GlcN6P deaminase activity levels of GlmD
tk
tkodakaraensis were determined by quantifying the generated Fru6P with an enzyme-coupled assay using phosphoglucose isomerase and Glc6P dehydrogenase. In this assay, no activity was detected in a control experiment without phosphoglucose isomerase. This fact verified the production of Fru6P, but not Glc6P, from GlcN6P by GlmD
tk
tkodakaraensis, as shown by the TLC analysis where the discrimination of Fru6P and Glc6P was somewhat difficult due to similar mobilities. This also implied that the enzyme does not exhibit phosphoglucose isomerase activity. The optimal pH of GlmD
tk
tkodakaraensis was 8.0 to 8.5. This enzyme was thermostable as expected, the optimal temperature was 95°C to 100°C, and the half-lives at 80°C and 90°C were determined to be 103 and 19 min, respectively. We then performed kinetic analysis of GlmD
tk
tkodakaraensis for both forward (GlcN6P deamination/isomerization) and reverse (Fru6P amination/isomerization) reactions. The reverse activity was determined by measuring the amount of produced GlcN6P with a modified Morgan and Elson method (13). As the optimal pH for the reverse reaction was determined to be the same as that for the forward one, assays were commonly performed at pH 8.3. The enzyme followed typical Michaelis-Menten kinetics for both directions, indicating no homotropic allosteric properties. The kinetic constants obtained are summarized in Table 1. The values for the deaminase reactions of GlmD
tk
tkodakaraensis were comparable to those of the GlcN6P deaminase from E. coli (kcat, 158 to 160 s−1; Km, 0.55 to 0.75 mM; and kcat/Km, 2.1 to 2.9 × 105 M−1 s−1) (6, 15). The value of kcat in the forward reaction was two times higher than that in the reverse reaction, and the Km value for GlcN6P was one and two orders lower than those for Fru6P and ammonia, respectively. As a result, the relative kcat/Km values for Fru6P and ammonia in the amination reaction were much lower (14.0% and 0.801%, respectively) than that for GlcN6P in the deamination reaction. These results clearly indicated that GlmD
tk
tkodakaraensis kinetically favored deamination/isomerization of GlcN6P rather than its reverse reaction. This preference was the same as those of classical GlcN6P deaminases and was consistent
with the function of GlmD<sub>Tk</sub> in chitin catabolism in <i>T. kodakaraensis</i>. 

**Expression profiles of GlmD<sub>Tk</sub> in <i>T. kodakaraensis</i>.** As described above, the <i>glmD<sub>Tk</sub></i> gene was assumed to be cotranscribed with the upstream ABC transporter genes and the downstream <i>glmA<sub>Tk</sub></i>, which corresponds to a transcript size of approximately 9,200 bp. Northern blot analysis using a specific probe against <i>glmD<sub>Tk</sub></i> resulted in smeared signals of lower sizes (1,500 to 2,500 bp), probably due to degradation of the long mRNA (data not shown). We therefore performed RT-PCR for three overlapping segments of the transcript (Fig. 2) and confirmed the amplification of all three fragments as shown in Fig. 5A. This result supported a single transcriptional unit for the genes of the ABC transporter, <i>glmD<sub>Tk</sub></i> and <i>glmA<sub>Tk</sub></i>. Then, RNA dot blot hybridization was performed using the <i>glmD<sub>Tk</sub></i> probe. In this analysis, a probe against <i>glmSTk</i> resulted in smeared signals of lower sizes (27). As shown in Fig. 5B, almost no transcription of <i>glmD<sub>Tk</sub></i> was detected in the absence of GlcNAC<sub>2</sub>, whereas transcription was strongly induced in GlcNAc<sub>2</sub>-containing medium, as expected. In contrast, <i>glmSTk</i> was constitutively transcribed in the three growth conditions (no sugar, or addition of GlcNAc<sub>2</sub> or maltose) as in the case of the control DNA ligase gene (<i>ligTk</i>). The expression of GlmD<sub>Tk</sub> in <i>T. kodakaraensis</i> was also examined at the protein level by Western blot analysis. Cells grown in media containing various sugars were used for the analysis, and the result is shown in Fig. 5C. The expression of GlmD<sub>Tk</sub> was not observed under the basal culture condition (Fig. 5C, lane 1), while it was induced by the addition of GlcNAc<sub>2</sub> (lane 2). Chitobiose (GlcN<sub>2</sub>Ac<sub>2</sub>) and maltose did not act as inducers for GlmD<sub>Tk</sub> at all (Fig. 5C, lanes 3 and 4). This profile coincided with that of GlmA<sub>Tk</sub> and reflected the same tendency as that seen in the transcription analysis mentioned above. Clearly, the chitin-catabolic operon consisting of <i>glmA<sub>Tk</sub></i>, <i>glmD<sub>Tk</sub></i>, and ABC transporters was specifically regulated at the transcriptional level by GlcNAc<sub>2</sub>.

**DISCUSSION**

In this study, we revealed that one gene, TK1755, in the gene cluster for chitin degradation on the <i>T. kodakaraensis</i> genome, encoded a GlcN6P deaminase structurally distinct from previously known deaminases. The protein product (GlmD<sub>Tk</sub>) displayed similarity to the sugar isomerase domain of GlcN6P synthases. GlmD<sub>Tk</sub> kinetically preferred the deamination of GlcN6P rather than the reverse ammonia-dependent amination of Fru6P (Table 1), supporting the function of this enzyme in amino sugar catabolism in vivo. Based on these facts together with our previous results (25–28), we can now clearly envision the chitin metabolism in this hyperthermophile in its near entirety, as summarized in Fig. 6. GlmD<sub>Tk</sub> was estimated to have a role in the final step to allow entrance of the chitin-derived catabolites into glycolysis. The highly clustered genes for this pathway were transcriptionally induced by GlcNAc<sub>2</sub>.

![Image](http://example.com/image.png)

**FIG. 5.** (A) Three overlapped segmental RT-PCR analyses of the region for the genes of the ABC transporter, <i>glmD<sub>Tk</sub></i> and <i>glmA<sub>Tk</sub></i>. Annealing sites for oligonucleotides used for the RT reaction and PCR are indicated in Fig. 2. Lanes 1 and 4, amplification with RT3, F3, and R3 (3,899 bp); lanes 2 and 5, amplification with RT2, F2, and R2 (3,874 bp); lanes 3 and 6, amplification with RT1, F1, and R1 (3,513 bp); lane M, molecular mass marker. Lanes 4 to 6 are the results of negative controls without the RT reactions. (B) Dot blot analysis of RNA from <i>T. kodakaraensis</i> grown with or without 0.1% GlcNAc<sub>2</sub> or maltose using specific probes against <i>ligTk</i>, <i>glmD<sub>Tk</sub></i>, and <i>glmSTk</i> genes. Each spot contains 30 μg of total RNA. (C) Western blot analysis of the cell extracts of <i>T. kodakaraensis</i> KOD1 grown in media containing various sugars (0.5%) with elemental sulfur at 85°C for 24 h. The amount of protein applied was 20 μg (T. kodakaraensis cell extract) or 50 ng (recombinant GlmD<sub>Tk</sub>, lane C).

**TABLE 1. Kinetic properties of GlmD<sub>Tk</sub>**

| Direction<sup>a</sup> | Substrate                    | <i>k<sub>a</i></sub> (s<sup>-1</sup>) | <i>K<sub>a</i></sub> (mM)<sup>b</sup> | <i>k<sub>a</i>/<i>K<sub>a</i></sub> (M<sup>-1</sup> s<sup>-1</sup>)<sup>c</sup> |
|----------------------|-------------------------------|------------------|-----------------|-------------------------------|
| Deamination          | GlcN6P (at 170 mM ammonia)    | 97.5 ± 2.1       | 0.339 ± 0.031   | 2.88 × 10<sup>7</sup> (100)   |
|                      | Amination                     | 44.2 ± 1.3       | 1.10 ± 0.09     | 4.02 × 10<sup>4</sup> (14.0)   |
|                      | Ammonia (at 8 mM Fru6P)       | 39.6 ± 2.7       | 17.0 ± 2.3      | 2.33 × 10<sup>3</sup> (0.801)  |

<sup>a</sup> Reactions of both directions were assayed at 60°C in 50 mM CHES-NaOH (pH 8.3 at 60°C) as described in Materials and Methods.

<sup>b</sup> Values shown are ± standard deviations.

<sup>c</sup> Values in parentheses indicate a relative value of <i>k<sub>a</i>/<i>K<sub>a</i></sub> (%).
FIG. 6. Proposed chitin catabolic pathway in *T. kodakaraensis* KOD1 leading to a glycolytic intermediate.

generated from chitin. Although this gene cluster lacks genes for the phosphorylation of GlcN, it is likely that this step can be mediated by an ADP-dependent glucokinase encoded at a different locus (TK1110), as the orthologs from the closely related archaea *P. furiosus* and *Thermococcus littoralis* have been reported to be capable of phosphorylating GlcN as well as glucose (14).

Previous studies have indicated that GlcN6P deaminase and the isomerase domain of GlcN6P synthase share some general similarities despite the lack of significant homology between their primary structures (21, 31). Both proteins have related nucleotide-binding folds, although GlcN6P deaminase has a dehydrogenase-like six-stranded fold, whereas the fold of GlcN6P synthase is a five-stranded flavodoxin type. They commonly catalyze 2R aldose-ketose isomerization by abstracting the C1 pro-R hydrogen of a substrate to form cis-enolamine, followed by reprotonation of C2 at the same re face of the intermediate. Therefore, the catalytic property of GlmD*TK* as a GlcN6P deaminase is feasible. However, an intriguing difference between GlmD*TK* and the isomerase domain of GlcN6P synthase is the ability of the former to accept exogenous ammonia. GlcN6P synthase lacks ammonia-dependent activity unlike other amidotransferases as described above, and in addition, it has been reported that the isomerase domain alone, prepared by limited proteolysis, did not show ammonia-dependent GlcN6P synthesis activity (10). Recent tertiary structural analysis of GlmSeEc has proposed that the binding of glutamine to the GAT domain promotes a conformational change of the protein that results in the opening of an intramolecular channel, through which the ammonia derived from glutamine migrates to the isomerase active site (29). The unique structure of the intramolecular channel seems to be the reason why this enzyme cannot gain access to free ammonia. In contrast, the reversible catalytic property of GlmD*TK* implies that free ammonia is easily accessible to the active site of this enzyme. However it should be noted that the C-terminal decapeptide of GlmSeEc, playing a central role in the migration of ammonia, is highly conserved in GlmD*TK* as well as in the GlcN6P synthases (29, 31). In GlmD*TK*, this region might have a function distinct from those in the usual GlcN6P synthases, such as in the recognition and incorporation of exogenous ammonia.

Besides the isomerase domain of GlcN6P synthase, GlmD*TK* shares overall homology against proteins existing in a subset of *Archaea* and *Bacteria*. These homologs are also comprised of tandem SIS domains and classified as COG2222 in the Cluster of Orthologous Group of proteins database. Among them, the *Pyrococcus* homologs not only showed high homology to glmD*TK* but also were commonly located within the completely conserved gene cluster from *dac* to *glmA* for GlcNAC2 catabolism. Although separated into two open reading frames at a different locus, *P. furiosus* harbors chitinase genes similar to ChiA*TK* and *P. horikoshii* also harbor this gene cluster despite the absence of a chitinase ortholog. We have previously reported that Gly*TK* displays broad specificity to various β-1,4-glycosides (11), is encoded in this cluster (Fig. 2). The enzyme does not seem to participate in GlcNAC2 metabolism (26), but the induction behavior was the same as those of the other clustered genes for chitinolysis (27). Although the physiological role of this β-glycosidase will have to be clarified, the common presence of the gene cluster, containing *gly* in *P. abyssi* and *P. horikoshii*, suggests an additional role of this cluster among members of the *Thermococcales* order. The cluster may be involved in the degradation of other cellular β-1,4-linked heteropolysaccharides. The proteins classified into COG2222 include AgaS encoded within the GalNAC catabolic cluster in *E. coli*. It has been reported that the bacterial metabolism of GalNAC was similar to that of GlcNAC (4, 22). In this pathway, galactosamine-6-phosphate (GalN6P) was predicted to be converted to tagatose-6-phosphate by the function of AgaI corresponding to the classical GlcN6P deaminase NagB. Although the catalytic property of AgaS has not been investigated yet, our results raise the possibility that AgaS might be functional as an additional GalN6P deaminase to achieve efficient processing of GalNAC.

In this study, we identified and characterized a new type of GlcN6P deaminase from the hyperthermophilic archaeon *T. kodakaraensis* and hereby provided an overview of the chitin catabolic pathway to glycolysis in this organism. As the enzymes in this pathway, ChiA*TK*, GlmD*TK*, Dac*TK*, and GlmA*TK*, were all novel enzymes with high thermostability, it is expected that these enzymes can be useful catalysts for future conversion of the unusued biomass chitin.

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