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NOTES

Identification and Characterization of an ATP-Dependent Hexokinase with Broad Substrate Specificity from the Hyperthermophilic Archaeon *Sulfolobus tokodaii*

Hiroshi Nishimasu, Shinya Fushinobu, Hirofumi Shoun, and Takayoshi Wakagi*

Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

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As a new member of the glucose-phosphorylating enzymes, the ATP-dependent hexokinase from the hyperthermophilic crenarchaeon *Sulfolobus tokodaii* was purified, identified, and characterized. Our results revealed that the enzyme differs from other known enzymes in primary structure and its broad substrate specificity for both phosphoryl donors and acceptors.

Phosphorylation of glucose to glucose-6-phosphate (G6P) is important for both energy metabolism and biosynthesis in the cell. Generally, ATP-dependent hexokinases (ATP-HKs, EC 2.7.1.1) catalyze this reaction in eukaryotes (4), whereas in many bacteria phosphoenolpyruvate-dependent phosphotransfer systems transport glucose into the cell with concomitant phosphorylation (24), and ATP-dependent glucokinases (ATP-GKs, EC 2.7.1.2) phosphorylate free intracellular glucose derived through disaccharide hydrolysis. ATP-HKs from eukaryotes generally show broad substrate specificity for hexoses including mannose and fructose as well as glucose (4). On the other hand, ATP-GKs from bacteria usually show high specificity for glucose. Based on primary structure, they can be divided into two groups: (i) ATP-GKs belonging to the ROK family (repressors, open reading frames [ORFs] of unknown function, and sugar kinases), which is characterized by two alternative signature motifs (32), and (ii) ATP-GKs without the ROK signature motifs.

In archaea, two types of glucose-phosphorylating enzymes have been reported so far: (i) ADP-dependent glucokinases (ADP-GKs) from hyperthermophilic euryarchaea *Pyrococcus furiosus* (17–19, 33), *Thermococcus litoralis* (19), *Archaeoglobus fulgidus* (20), and *Methanococcus jannaschii* (27), and (ii) ATP-dependent glucose-phosphorylating enzymes from hyperthermophilic crenarchaea *Aeropyrum pernix* (11) and *Thermoproteus tenax* (8). Most ADP-GKs show high specificity for glucose, although the *M. jannaschii* enzyme shows both glucokinase and phosphofructokinase activities (27). ADP-GKs constitute a novel sugar kinase family, and they are structurally distinct from ATP-HK/GKs but similar to the ATP-dependent ribokinase family (14, 15). On the other hand, the ATP-dependent glucose-phosphorylating enzymes from *A. pernix* (11) and *T. tenax* (8) belong to the ROK family, like bacterial glucokinases, but they show broad specificity for hexoses, like eukaryotic hexokinases. To avoid confusion, we here refer to them as ATP-HKs. The genes of putative homologs of archaeal ATP-HKs have also been identified in several archaeal genomes, although they have not been biochemically characterized and their functions remain unclear.

ATP-dependent glucose-phosphorylating activity has been detected in cell extracts of hyperthermophilic crenarchaeon *Sulfolobus solfataricus* (7), but the enzyme responsible for the activity has been neither isolated nor biochemically characterized. Moreover, the genes of homologs of known glucose-phosphorylating enzymes have not been found in the completely sequenced genome of either *S. solfataricus* (28), *Sulfolobus tokodaii* (16), or *Sulfolobus acidocaldarius* (6). Here we report the purification of a protein responsible for ATP-dependent glucose-phosphorylating activity from *S. tokodaii*, identification of the encoding gene, and characterization of the recombinant enzyme.

Purification of ATP-dependent glucose-phosphorylating activity from *S. tokodaii* cells. *S. tokodaii* cells were grown aerobically at 75°C in 9 liters of medium comprising (per liter) 1 g of glucose, 1 g of yeast extract, 1 g of Casamino Acid, 0.3 g of KH_2PO_4, 0.2 g of NaCl, 0.13 g of ammonium sulfate, 0.25 g of

TABLE 1. Purification of native StoHK from *S. tokodaii*

Purification step	Protein (mg)	Activity (U)[a]	Specific activity (U mg^{-1})[a]	Yield (%)[b]	Purification (fold)[b]
Cell extracts	8,160	5.6[c]	0.0007[c]		
DEAE-Sepharose	1,089	11	0.010	100	1
Butyl-Toyopearl	87	6.8	0.078	62	7.8
Mono Q	2.0	3.0	1.5	27	1.5×10^2
Resource PHE	0.10	1.6	17	15	1.7×10^3
Superdex 200	0.013	0.33	26	3.1	2.6×10^3

[a] The enzyme activity was measured at 50°C using 5 mM glucose and 2 mM ATP as substrates in the presence of 4 mM Mg^{2+}.
[b] Yield and purification were calculated with respect to the DEAE-Sepharose chromatography step.
[c] The enzyme activity was determined by subtracting the GDH activity from the value obtained with a G6PDH-coupled assay.

* Corresponding author. Mailing address: Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. Phone: 81-3-5841-5152. Fax: 81-3-5841-5152. E-mail: atwakag@mail.ecc.u-tokyo.ac.jp.

$MgSO_4 \cdot 7H_2O$, 0.07 g of $CaCl_2$, 0.02 g of $FeSO_4 \cdot 7H_2O$, 4.5 mg of $Na_2B_4O_7 \cdot 10H_2O$, 1.8 mg of $MnCl_2 \cdot 4H_2O$, 0.17 mg of $ZnSO_4 \cdot 7H_2O$, 0.07 mg of $CuSO_4 \cdot 5H_2O$, 0.03 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.03 mg of $VOSO_4 \cdot 2H_2O$, and 0.01 mg of $CoSO_4 \cdot 6H_2O$, adjusted to pH 3 with H_2SO_4. Additional glucose, yeast extract, and Casamino Acid (1 g each per liter) were added to the culture medium at the mid-exponential phase (optical density at 600 nm = 1.5). Cells were harvested at the late-exponential-growth phase (optical density at 600 nm = 2.3).

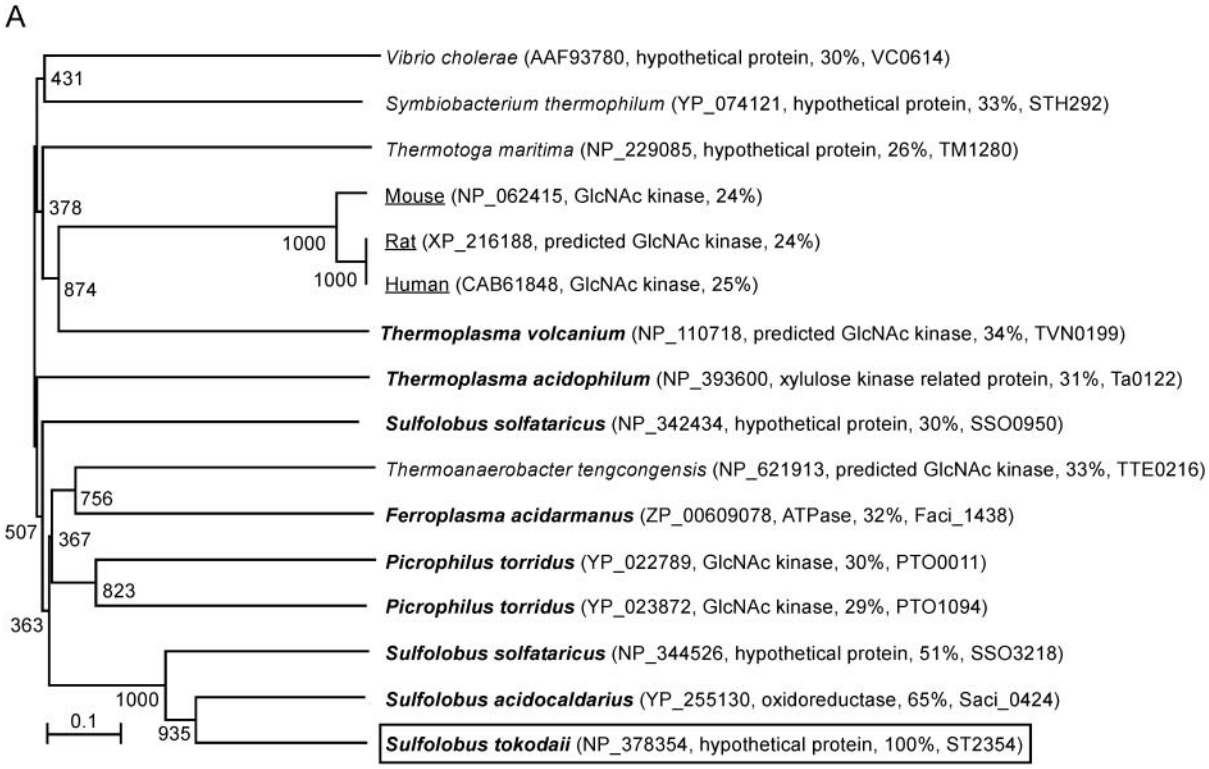
ATP-dependent glucose-phosphorylating activity was purified to homogeneity from *S. tokodaii* cell extracts by sequential column chromatography involving DEAE-Sepharose Fast Flow (Amersham Biosciences), Butyl-Toyopearl 650 M (Tosoh), Mono Q 10/10 (Amersham Biosciences), RESOURCE 15PHE (Amersham Biosciences), and HiLoad 16/60 Superdex 200 (Amersham Biosciences) (Table 1). We refer to the enzyme as StoHK (*S. tokodaii* hexokinase) due to its broad substrate specificity for hexoses described below. The purified StoHK appeared as a single band corresponding to a molecular mass of 32 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown), whereas it was eluted at a volume corresponding to a molecular mass of 64 kDa from a Superdex 200 gel filtration column. These results indicate that StoHK is dimeric in solution. About 0.013 mg of purified StoHK was obtained from 124 g (wet weight) of *S. tokodaii* cells (Table 1). The phosphorylation of glucose was measured spectrophotometrically at 50°C by coupling the production of G6P to the reduction of $NADP^+$ using G6P dehydrogenase (G6PDH) from yeast (Oriental) as an auxiliary enzyme (G6PDH-coupled assay). The assay mixture (500 μl) comprised 100 mM Tris (pH 7.5), 5 mM glucose, 2 mM ATP, 4 mM $MgCl_2$, 0.2 mM $NADP^+$, 1 Unit of G6PDH, and an appropriate amount of enzyme. The reaction was started by the addition of 10 μl of the enzyme solution and followed by monitoring the increase in absorbance at 340 nm ($\varepsilon_{340\ nm}$ = 6.22 $mM^{-1}\ cm^{-1}$). Before the measurements, we confirmed that G6PDH is not rate-limiting, and no increase in absorbance at 340 nm was observed when the reaction mixture was incubated without StoHK. One unit (U) is defined as the amount of the enzyme that catalyzes the reduction of 1 μmol of $NADP^+$ per minute at 50°C. The specific activity of the purified StoHK was determined to be 26 U mg^{-1} at 50°C by a G6PDH-coupled assay. The ATP-dependent glucose-phosphorylating activity was not detected in other fractions under our assay conditions, indicating that StoHK is the sole enzyme responsible for the phosphorylation of glucose in vivo.

Sulfolobus degrades glucose to pyruvate via the nonphosphorylative Entner-Doudoroff pathway (7), in which glucose dehydrogenase (GDH) catalyzes the oxidation of glucose to gluconate using $NADP^+$ or NAD^+ as an electron acceptor (9, 21, 23). The purified recombinant GDH from *S. tokodaii* has been reported to catalyze the oxidation of glucose at 50°C with specific activity of 32 U mg^{-1} and a K_m value for glucose of 0.17 mM (23). Thus, the reduction of $NADP^+$ to NADPH at a rate of 0.0067 U mg^{-1} was observed when the cell extracts were incubated at 50°C in the presence of 0.2 mM $NADP^+$, 5 mM glucose, and 4 mM Mg^{2+}. On the other hand, the reduction of $NADP^+$ to NADPH at a rate of 0.0074 U mg^{-1} was observed when the cell extracts were incubated at 50°C in the presence of 0.2 mM $NADP^+$, 5 mM glucose, 4 mM Mg^{2+}, 2 mM ATP, and 1 Unit of G6PDH. Therefore, the activity of StoHK in the cell extracts was estimated to be 0.0007 U mg^{-1} at 50°C by subtracting the former value from the latter one. StoHK was separated from GDH by DEAE-Sepharose chromatography. The activity of StoHK was about 10-fold lower than that of GDH in cell extracts of *S. tokodaii*, suggesting that intracellular glucose is predominantly metabolized to pyruvate for energy production, whereas a small proportion of glucose is phosphorylated to G6P for biosynthesis. Little is known about the biosynthetic pathways in archaea, although *S. solfataricus* has been reported to contain glycogen (29). However, ORFs probably involved in biosynthesis in the *S. tokodaii* genome suggest the presence of biosynthetic pathways as in other organisms. Of these ORFs, ST0452 has been reported to show both glucose-1-phosphate thymidylyltransferase and GlcNAc-1-phosphate uridylyltransferase activities (35). Moreover, phosphohexomutase (SSO0207) from *S. solfataricus* (25) and glycogen synthase from *S. acidocaldarius* (5) have been biochemically characterized.

The total activity of StoHK after the DEAE-Sepharose chromatography was about twofold higher than that in the cell extracts. To determine whether or not the activity of StoHK is inhibited by any substance (e.g., ADP) in the cell extracts, we measured the enzyme activity in cell extracts that had been desalted with a centrifugal filter device. The enzyme activity in the desalted cell extracts was similar to that in the untreated ones. Therefore, it remains unclear why an increase in the total activity was observed after the first purification step.

Identification of the gene encoding StoHK. To identify the gene encoding StoHK, the single protein band corresponding to the ATP-dependent glucose-phosphorylating activity was excised from the SDS-PAGE gel, digested with trypsin, and then analyzed with a Voyager-DE STR matrix-assisted laser desorption ionization–time of flight mass spectrometer (Applied Biosystems). By comparing the peptides observed with those in the NCBI database using MS-Fit (available at http://prospector.ucsf.edu), we identified an ORF, ST2354, annotated as a gene encoding a hypothetical protein in the genome of *S. tokodaii*. At least 10 of the 17 predicted tryptic peptides were detected, and they covered 58% of ST2354. ORF ST2354 comprises 900 bp and encodes a protein of 299 amino acids with a calculated molecular mass of 32.1 kDa, consistent with the molecular mass of the purified enzyme determined on SDS-PAGE.

With ST2354 as a query sequence, there were more than 100 BLAST hits including weakly related sequences (E value of less than 10) from various organisms such as acidophilic archaea, thermophilic or mesophilic bacteria, and eukaryotes. However, StoHK did not show significant sequence similarity to any known glucose-phosphorylating enzymes. Glucokinase from *Streptomyces coelicolor* A3(2) (22) was found in the BLAST search, but similarity of it to StoHK was barely detectable (E value = 1.3). To clarify the evolutional relationship between StoHK and sequences exhibiting significant homology (E values of less than 2×10^{-8}), a phylogenetic tree was constructed (Fig. 1A). Putative close homologs of the enzyme have been identified in the published genomes of all *Sulfolobus* species, and they form a cluster in the phylogenetic tree. *S. acidocaldarius* contains the closest homolog, Saci_0424 (65% identity), which is annotated as an oxidoreductase in the pub-


A
Vibrio cholerae (AAF93780, hypothetical protein, 30%, VC0614)
Symbiobacterium thermophilum (YP_074121, hypothetical protein, 33%, STH292)
Thermotoga maritima (NP_229085, hypothetical protein, 26%, TM1280)
Mouse (NP_062415, GlcNAc kinase, 24%)
Rat (XP_216188, predicted GlcNAc kinase, 24%)
Human (CAB61848, GlcNAc kinase, 25%)
Thermoplasma volcanium (NP_110718, predicted GlcNAc kinase, 34%, TVN0199)
Thermoplasma acidophilum (NP_393600, xylulose kinase related protein, 31%, Ta0122)
Sulfolobus solfataricus (NP_342434, hypothetical protein, 30%, SSO0950)
Thermoanaerobacter tengcongensis (NP_621913, predicted GlcNAc kinase, 33%, TTE0216)
Ferroplasma acidarmanus (ZP_00609078, ATPase, 32%, Faci_1438)
Picrophilus torridus (YP_022789, GlcNAc kinase, 30%, PTO0011)
Picrophilus torridus (YP_023872, GlcNAc kinase, 29%, PTO1094)
Sulfolobus solfataricus (NP_344526, hypothetical protein, 51%, SSO3218)
Sulfolobus acidocaldarius (YP_255130, oxidoreductase, 65%, Saci_0424)
Sulfolobus tokodaii (NP_378354, hypothetical protein, 100%, ST2354)
431
378
1000
1000
874
756
507
367
823
363
1000
0.1
935
B
ST2354
Saci_0424
SSO3218
Mouse
Rat
Human

lished genome sequence. However, this annotation seems incorrect because Saci_0424 does not exhibit any sequence similarity with any oxidoreductase. *S. solfataricus* contains two putative homologs, SSO3218 (51% identity) and SSO0950 (30% identity), each of which is annotated as a hypothetical protein. Based on sequence similarity, SSO3218, rather than SSO0950, is likely responsible for the ATP-dependent glucose-phosphorylating activity (0.115 U mg^{-1} at 70°C) partially purified from *S. solfataricus* (7). Relatively distant homologs (29 to 34% identity) have also been identified in the genomes of all members of the archaeal order *Thermoplasmatales*, i.e., *Thermoplasma volcanium*, *Thermoplasma acidophilum*, "*Ferroplasma acidarmanus*" (proposed name), and *Picrophilus torridus* (Fig. 1A). The members of the *Thermoplasmatales* have been proposed to share many homologs with *Sulfolobus* due to the common acidic biotopes, although they are phylogenetically distant from *Sulfolobus* (26). The members of the *Thermoplasmatales* also contain putative homologs of the archaeal ATP-HK, although none of them have been biochemically characterized. Therefore, it is unclear whether relatively distant homologs from the *Thermoplasmatales* group exhibit enzyme activity similar to that of StoHK or whether they are physiologically relevant. Because homologs other than Saci_0424 and SSO3218 share relatively low similarity (~30% identity) with StoHK, only three *Sulfolobus* homologs are expected to exhibit the unique hexokinase activity described below.

StoHK shows low but significant sequence similarity (24 to 26% identity) to the biochemically characterized *N*-acetylglucosamine kinases (GlcNAc kinases, EC 2.7.1.59) from mouse, rat, and human, which catalyze the ATP-dependent phosphorylation of GlcNAc to GlcNAc-6-phosphate (12, 13, 34). Figure 1B shows comparison of the amino acid sequence of StoHK with those of Saci_0424 from *S. acidocaldarius*, SSO3218 from *S. solfataricus*, and the characterized mammalian GlcNAc kinases. Two typical ATP-binding motifs (2), (D/E)XGG (X represents any amino acid residue) and GTGS, are conserved among them. Six cysteine residues are strictly conserved among the GlcNAc kinases. In the mouse enzyme, Cys^{131} and Cys^{143} were found to be important for the binding of GlcNAc and of ATP, respectively, in site-directed mutagenesis experiments (1). In contrast, StoHK does not contain these cysteine residues, although it contains three other cysteine residues (Cys^{21}, Cys^{193}, and Cys^{291}). All three cysteine residues are conserved in Saci_0424, whereas that corresponding to Cys^{193} is not present in SSO3218 (Fig. 1B).

Cloning and expression of ORF ST2354 in *Escherichia coli* and purification of recombinant StoHK. ORF ST2354 was amplified by PCR using *S. tokodaii* genomic DNA as a template with *Pfu* Turbo DNA polymerase (Stratagene). The following primers were used: 5′-AAAGTTTTCATATGATGATTATAGTTGGTGTTG-3′ (forward) and 5′-GTGAAGGGAATTCTTTAGTCGCTAATTAAC-3′ (reverse). The NdeI and EcoRI restriction sites, respectively, are underlined. The PCR product was digested with NdeI and EcoRI and then ligated into pET-17b (Novagen) to construct expression vector pET-StoHK. The sequence of the insert was confirmed by DNA sequencing.

E. coli BL21-CodonPlus(DE3)-RIL (Stratagene) cells harboring pET-StoHK were grown in 30 ml of Luria-Bertani medium containing 100 μg ml^{-1} ampicillin and 30 μg ml^{-1} chloramphenicol overnight at 37°C. The cell culture (20 ml) was inoculated into 2 liters of the same medium and then grown for 9 h at 37°C without induction. The recombinant enzyme was purified to homogeneity by heat treatment at 80°C for 30 min followed by sequential column chromatography involving Q-Sepharose Fast Flow and HiLoad 16/60 Superdex 200. The purified enzyme appeared as a single band corresponding to a molecular mass of 32 kDa on SDS-PAGE (data not shown), consistent with the calculated molecular mass of 32.1 kDa. It was eluted at a volume corresponding to a molecular mass of 64 kDa from a gel filtration column, indicating that the recombinant enzyme is dimeric like the native enzyme. About 50 mg of the purified enzyme was obtained from 2 liters of *E. coli* culture. The specific activity of the purified recombinant enzyme was 62 U mg^{-1} at 50°C, comparable to that of the native enzyme (26 U mg^{-1} at 50°C).

Biochemical characterization of recombinant StoHK. The purified recombinant enzyme was used for the following experiments, and the enzyme activity was measured by a G6PDH-coupled assay. The effects of divalent cations on the enzyme activity were examined at 50°C using 5 mM glucose and 2 mM ATP as substrates. No activity was detected without a divalent cation. StoHK showed 100, 153, and 80% relative activity in the presence of Mg^{2+}, Co^{2+}, and Mn^{2+}, respectively, at 4 mM, whereas it showed no activity in the presence of Zn^{2+}. These results demonstrate that StoHK requires divalent cations such as Mg^{2+} for catalysis, like other kinases. The effect of pH on the enzyme activity was examined at 50°C, using the following buffers: morpholineethanesulfonic acid (MES)-NaOH (pH 6.0 to 7.0) and Tris-HCl (pH 7.0 to 9.0), each 100 mM. StoHK showed relatively high activity (more than 60% of the maximal activity) in the pH range of 6.5 to 9.0, with maximal activity

FIG. 1. Phylogenetic relationship and multiple sequence alignment of StoHK and related sequences. (A) Phylogenetic relationship of StoHK and selected related sequences. The phylogenetic tree was constructed by means of the neighbor-joining method using ClustalX (30). Bootstrap values were calculated from 1,000 replicates and are given at each node. All of the archaeal sequences are shown in boldface, and selected bacterial and eukaryotic sequences are also shown. StoHK (ST2354) is shown in a box. The biochemically characterized GlcNAc kinases are underlined. Accession numbers, annotations, sequence identity to StoHK, and locus names are given in parentheses. The bar indicates 10% amino acid changes between close relatives. (B) Multiple sequence alignment of StoHK, putative homologs Saci_0424 from *S. acidocaldarius* and SSO3218 from *S. solfataricus*, and the characterized GlcNAc kinases from mouse, rat, and human. The sequences were aligned using ClustalW (31). Strictly conserved residues are highlighted in black. Highly conserved residues (five out of six sequences are identical) are shown in boxes. Putative ATP-binding motifs are indicated by black lines below the sequences. Cysteine residues conserved among the mammalian GlcNAc kinases are indicated by open or filled triangles below the sequences. Open triangles indicate the cysteine residues involved in the binding of GlcNAc and ATP. Cysteine residues in StoHK are indicated by filled circles above the sequences. The figure was prepared using ESPript (10).

TABLE 2. Substrate specificity and kinetic parameters of StoHK[a]

Substrate	K_m (mM)	V_{max} (U mg^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Glucose	0.050 ± 0.005	67 ± 1.5	717
Mannose	0.13 ± 0.009	102 ± 1.9	420
Glucosamine	0.23 ± 0.01	106 ± 1.2	247
GlcNAc	0.32 ± 0.006	60 ± 0.3	100
2-Deoxyglucose	0.44 ± 0.01	140 ± 1.1	170
Fructose	30 ± 0.7	78 ± 0.7	1.4
ATP	0.12 ± 0.007	58 ± 1.1	259
CTP	0.52 ± 0.05	51 ± 1.5	52

[a] The enzyme activity was measured at 50°C in the presence of 4 mM Mg^{2+}. Substrate specificity for sugars was determined using 2 mM ATP as a phosphoryl donor, and that for phosphoryl donors was determined using 5 mM glucose as a sugar substrate. Kinetic parameters were determined using seven different concentrations of the substrates and by nonlinear regression analysis using Kaleida Graph (Synergy Software).

around pH 7.5. The enzyme activity was discontinuously measured at 80°C, the optimal temperature for the growth of the organism. The assay mixture (250 μl) comprised 100 mM Tris-HCl (pH 7.5), 5 mM glucose, 2 mM ATP, 4 mM $MgCl_2$, and an appropriate amount of enzyme. The reaction was started by the addition of 10 μl of the enzyme solution. After incubation at 80°C for 30, 60, 90, and 120 s, the reaction was stopped by rapid cooling on ice. Then the coupled assay mixture (250 μl) comprising 100 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 0.4 mM $NADP^+$, and 1 Unit of G6PDH was added to the reaction mixture. After incubation at 25°C for 5 min, the absorbance at 340 nm was measured. The specific activity was 169 U mg^{-1} at 80°C. To examine the thermal stability of StoHK, 10 μg of the purified enzyme was incubated in 100 μl of 100 mM Tris-HCl (pH 7.5) at 80°C and 90°C for up to 120 min, and then the remaining activity was measured at 50°C. StoHK retained 86 and 20% of the initial activity even after incubation for 120 min at 80°C and 90°C, respectively, indicating that it is a highly thermostable enzyme. These results indicate that StoHK effectively functions under in vivo conditions.

Substrate specificity and kinetic parameters of StoHK. To determine the specificity and kinetic parameters for sugar substrates, the phosphorylation of sugar substrates was measured spectrophotometrically at 50°C by coupling the production of ADP to the oxidation of NADH using pyruvate kinase (PK) and lactate dehydrogenase (LDH) from rabbit muscle (Sigma) as auxiliary enzymes (PK/LDH-coupled assay). The assay mixture (500 μl) comprised 100 mM Tris-HCl (pH 7.5), 2 mM ATP, 4 mM $MgCl_2$, 0.15 mM NADH, 4 mM phosphoenolpyruvate, 10 mM KCl, 8 Units of PK, 10 Units of LDH, different concentrations of sugar substrates, and an appropriate amount of enzyme. The reaction was started by the addition of 10 μl of the enzyme solution and followed by monitoring the decrease in absorbance at 340 nm ($\varepsilon_{340\ nm}$ = 6.22 mM^{-1} cm^{-1}). Before the measurements, we confirmed that PK and LDH are not rate-limiting. The following substrate concentrations were used: glucose, 0.015 to 1.0 mM; mannose, 0.025 to 1.5 mM; glucosamine, 0.050 to 2.0 mM; GlcNAc, 0.10 to 3.0 mM; 2-deoxyglucose, 0.10 to 3.0 mM; and fructose, 2.5 to 150 mM.

As summarized in Table 2, StoHK catalyzed the ATP-dependent phosphorylation of not only glucose but also its C-2 epimer (mannose) and C-2 derivatives of it (2-deoxyglucose, glucosamine, and GlcNAc) following Michaelis-Menten kinetics. The specific activity determined by a PK/LDH-coupled assay (66 U mg^{-1} at 50°C) was slightly higher than that determined by a G6PDH-coupled assay, probably because StoHK is strongly inhibited by the reaction product ADP, as described below. Fructose was phosphorylated by StoHK, but it would not be a physiological substrate of the enzyme because the K_m value is 30 mM. Galactose was phosphorylated at rates of 0.8 and 0.08 U mg^{-1} at concentrations of 1 and 10 mM, respectively. Ribose, xylose, *N*-acetylmannosamine, glucose-1-phosphate, and fructose-6-phosphate (10 mM) were not phosphorylated by the enzyme.

StoHK is the first example of a thermostable enzyme that can phosphorylate both glucose and GlcNAc with similar catalytic efficiency. ATP-HK from *A. pernix* can slightly phosphorylate GlcNAc, but the V_{max} value is much lower than that for glucose. The GlcNAc kinase from *Candida albicans* (34) and RokA from the gram-negative bacterium *Bacteroides fragilis* (3), which have been reported to catalyze the phosphorylation of both glucose and GlcNAc, should not be thermostable. StoHK may be useful for the industrial synthesis of various hexose-6-phosphates because of its thermostability and broad substrate specificity for hexoses.

The substrate specificity of StoHK for phosphoryl donors was examined at 50°C using 5 mM glucose as a phosphoryl acceptor in the presence of 4 mM Mg^{2+}. StoHK could use various nucleoside triphosphates (2 mM each), ATP (set as 100%), CTP (81%), ITP (64%), GTP (52%), and UTP (37%) as phosphoryl donors. Because StoHK showed almost equal activity with CTP and with ATP, the K_m values for ATP and CTP were determined at 50°C using 5 mM glucose as a phosphoryl donor. As shown in Table 2, the K_m values for ATP and CTP were 0.12 and 0.52 mM, respectively, suggesting that ATP is a physiological phosphoryl donor. ADP, CDP, GDP, UDP, and PP_i (2 mM each) and polyphosphate (2 mg ml^{-1}) did not serve as phosphoryl donors.

The previously reported archaeal ATP-HKs show broad specificity for nucleoside triphosphates, but they cannot use CTP as a phosphoryl donor; the *A. pernix* enzyme (11) uses ATP (100%), ITP (40%), GTP (9%), and UTP (3%), and the *T. tenax* one (8) uses ATP (100%), ITP (40%), GTP (18%), and UTP (17%). Bacterial ATP-GKs also show broad specificity for nucleoside triphosphates, but they cannot use CTP

TABLE 3. The effects of metabolites on the activity of StoHK

Metabolite[a]	Concentration (mM)	Remaining activity (%)[b]
ADP	0.1	38
	1	8.1
AMP	0.1	76
	1	27
CDP	0.1	100
	1	67
PP_i	1	100
	10	46
Fructose-6-phosphate	1	100
	10	67

[a] The following metabolites did not affect the enzyme activity: phosphoenolpyruvate, P_i, glyceraldehyde, citrate, lactate, pyruvate, fructose-1,6-bisphosphate, and G6P (10 mM each), and NAD^+, NADH, $NADP^+$, and NADPH (1 mM each).

[b] The enzyme activity was measured at 50°C using half-saturating concentrations of glucose (0.050 mM) and ATP (0.12 mM) as substrate in the presence of 4 mM Mg^{2+}.

efficiently. Therefore, StoHK differs from other known glucose-phosphorylating enzymes in its ability to use both ATP and CTP as phosphoryl donors efficiently.

Effects of metabolites on the activity of StoHK. The effects of various metabolites on the activity of StoHK were examined using half-saturating concentrations of glucose and ATP.

StoHK was strongly inhibited by ADP, whereas it was not considerably affected by other metabolites tested (Table 3). Inhibition of the enzyme by ADP was examined with various concentrations of glucose (0.050 to 1.0 mM) and a fixed concentration of ATP (2 mM) as well as with various concentrations of ATP (0.1 to 1.0 mM) and a fixed concentration of glucose (5 mM). ADP competitively inhibited StoHK as to ATP, with the K_i value being 18 μM, whereas ADP noncompetitively inhibited StoHK as to glucose (data not shown). ADP-GK from *P. furiosus* has been reported to be strongly inhibited by AMP (K_i = 0.06 mM) (33), whereas known archaeal ATP-HKs have no regulatory properties. The regulatory property of StoHK as to ADP is a new feature not observed for known archaeal ATP-HKs. It seems reasonable to assume that, under low-energy charged conditions, glucose is used for energy production rather than for biosynthesis due to inhibition of StoHK by ADP.

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