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# Biochemical Characterization, Cloning, and Sequencing of ADP-Dependent (AMP-Forming) Glucokinase from Two Hyperthermophilic Archaea, *Pyrococcus furiosus* and *Thermococcus litoralis*[1]

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**The ADP-dependent (AMP-forming) glucokinases from the hyperthermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis* catalyze the phosphorylation of glucose using ADP as the essential phosphoryl group donor. Both enzymes were purified to homogeneity and characterized with regard to each other. The enzymes had similar enzymological properties as to substrate specificity, coenzyme specificity, optimum pH, and thermostability. However, a difference was observed in the subunit composition; while the *T. litoralis* enzyme is a monomer with a molecular mass of 52 kDa, the *P. furiosus* enzyme has a molecular mass of about 100 kDa and consists of two subunits with identical molecular masses of 47 kDa. The genes encoding these enzymes were cloned and sequenced. The gene for the *P. furiosus* enzyme contains an open reading frame for 455 amino acids with a molecular weight of 51,265, and that for the *T. litoralis* enzyme contains an open reading frame for 467 amino acids with a molecular weight of 53,621. About 59% similarity in amino acid sequence was observed between these two enzymes, whereas they did not show similarity with any ATP-dependent kinases that have been reported so far. In addition, two phosphate binding domains, and adenosine and glucose binding motifs commonly conserved in the eukaryotic hexokinase family were not observed.**

**Key words: archaea, glucokinase, hexokinase, hyperthermophiles, *Pyrococcus furiosus*, *Thermococcus litoralis*.**

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Hyperthermophiles are a group of microorganisms that exhibit optimum growth temperatures of above 80°C (*1*, *2*). Almost all of them are classified as archaea, the third domain of life (*3*), and recent studies have revealed that these hyperthermophilic archaea have novel sugar metabolic pathways, and some enzymes that were previously unknown in bacteria and eucarya (*4*, *5*).

Recently, a novel sugar kinase, ADP-dependent (AMP-forming) glucokinase (ADP-GK), was discovered in the hyperthermophilic archaeon *Pyrococcus furiosus* (*6*). This is the first description of an ADP-dependent (AMP-forming) kinase to date. The enzyme requires ADP as the phosphoryl group donor instead of ATP and is involved in a modified Embden-Meyerhof pathway in this organism (*4*, *5*).

In a phylogenetic hexokinase family tree constructed by comparing 60 sequences of sugar kinases, Bork *et al.* (*7*) observed that glucokinases appear in the following three clusters: (i) the mammalian glucokinases; (ii) the yeast glucokinases, which were grouped with yeast hexokinases; and (iii) the bacterial glucokinases. They have stated that a divergent evolutionary relationship between these glucokinases was unlikely and argued that evolutionary convergence to glucose specificity must have occurred independently in mammals, yeast, and bacteria (*7*). The hyperthermophilic archaea are relatively deeply branched archaea and are considered to be phylogenetically ancient organisms. Therefore, structural analysis of the ADP-dependent glucokinases from these organisms may provide abundant information for phylogenetic analysis of the glucokinases. Our interest in the evolutionary origin of glucokinases led us to determine the primary structures of the ADP-dependent glucokinases from different archaea. While this work was in progress, a putative glucokinase gene was identified in the *P. furiosus* genome (GenBank Accession Number AF127910) (*8*). However, information about the molecular properties of the ADP-dependent glucokinase is still lacking.

In the present study, we purified the ADP-dependent glucokinases from two hyperthermophilic archaea, *P. furiosus* and *Thermococcus litoralis*, and characterized the two en-

[1] The nucleotide sequences reported in this paper have been submitted to the GenBank nucleotide sequence databases, and are available under accession numbers E14588 and E14589.

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zymes in detail with regard to each other. We cloned and sequenced the genes encoding these two enzymes, and analyzed their molecular properties.

## MATERIALS AND METHODS

*Materials*—*Escherichia coli* strain JM109 was obtained from Toyobo (Osaka). *P. furiosus* DSM 3638 and *T. litoralis* DSM 5473 were from the German Collection of Microorganisms (Braunschweig, Germany). [$\gamma$-$^{32}$P] ATP and [$\alpha$-$^{32}$P]dCTP were from Daiichi Pure Chemicals (Tokyo). All other chemicals were of reagent grade.

*Growth of the Organisms*—*P. furiosus* was anaerobically grown in the absence of S° at 90°C for 17.5 h with maltose as the carbon source (*9*). *T. litoralis* was grown under the same conditions as those used for *P. furiosus* except for the cultivation temperature (at 88°C) (*10*).

*Enzyme assay*—ADP-GK activity was assayed spectrophotometrically by measuring the formation of NADPH in the presence of diapholase and nitroblue tetrazolium in a coupled assay with glucose-6-phosphate dehydrogenase. The reaction system contained, unless specified otherwise, 50 mM Tris/HCl, pH 7.5, 20 mM glucose, 2 mM ADP, 2 mM $MgCl_2 \cdot 6H_2O$, 5 units/ml glucose-6-phosphate dehydrogenase (Toyobo), 5 units/ml Diapholase (Toyobo), 0.025 % nitroblue tetrazolium, 1 mM NADP, and 1 % Triton X-100, in a total volume of 1.0 ml. The reaction was started by the addition of 20 μl of enzyme solution. After incubation for 10 min at 37°C , the reaction was terminated by the addition of 2 ml of 0.1 M HCl. The concentration of formazan formed was determined from the change in the absorbance at 550 nm ($\varepsilon$=0.795 $mM^{-1}$ $cm^{-1}$). One unit of enzyme activity was defined as the amount of enzyme required to convert 1 μmol of glucose per min at 37°C. Protein was determined by the method of Bradford (*11*). Bovine serum albumin was used as the standard.

*Purification of ADP-GK from P. furiosus*—All procedures were carried out at room temperature under open conditions. Cells (about 200 g wet weight) from a 200-liter culture were suspended in 1 liter of 10 mM Tris/HCl, pH 8.0, and then disrupted by sonication for 30 min on ice. The cell debris was removed by centrifugation at 15,000 ×*g* for 30 min, and the supernatant solution was dialyzed against the same buffer and used as the crude extract for the purification. The crude extract was applied to a column (5 × 25 cm) of DEAE-Sepharose fast flow (Pharmacia Biotech) equilibrated with 10 mM Tris/HCl, pH 8.0. Protein was eluted with a 4,000-ml linear gradient of 0–1.0 M NaCl in the same buffer. The active fractions were pooled, and then NaCl was added to 4 M. The enzyme solution was loaded on a Phenyl-Sepharose fast flow column (2.6 × 35 cm; Pharmacia) equilibrated with 10 mM Tris/HCl, pH 8.0, containing 4 M NaCl. The column was developed with a linear gradient of 4.0 to 0 M NaCl (1,600 ml). The active fractions were pooled and then dialyzed against 10 mM Tris/HCl, pH 8.0. The desalted enzyme was loaded on a Q-Sepharose fast-flow column (2.6 × 18 cm; Pharmacia) equilibrated with 10 mM Tris/HCl, pH 8.0. Protein was eluted with a 800-ml linear gradient of 0–0.7 M NaCl in the same buffer. The active fractions were pooled and then dialyzed against 5 mM potassium phosphate buffer, pH 7.0. The enzyme solution was applied to a hydroxyapatite column (2.6 × 10 cm) equilibrated with the same buffer. The column was washed with 3 volumes of the same buffer, and then the protein was eluted with a linear gradient (400 ml) of 5–200 mM potassium phosphate buffer, pH 7.0. The pooled active fractions were dialyzed against 50 mM Tris/HCl, pH 7.5, supplemented with 250 mM NaCl, concentrated by ultrafiltration, and then subjected to gel filtration on Superose 12PG (1.6 × 10 cm; Pharmacia) pre-equilibrated with the same buffer. The active fractions were pooled and dialyzed against 10 mM Tris/HCl, pH 7.5, and then $(NH_4)_2SO_4$ was added up to 2 M. The enzyme solution was loaded on a RESOURCE ISO column (0.64 × 3 cm; Pharmacia) equilibrated with 10 mM Tris/HCl, pH 7.5, containing 2 M $(NH_4)_2SO_4$. The column was developed with a linear gradient of 2.0 to 0 M $(NH_4)_2SO_4$ (30 ml). The fractions containing homogeneous ADP-GK, as judged on electrophoresis, were pooled, and then the solution was dialyzed against 10 mM Tris/HCl, pH 7.5.

*Purification of ADP-GK from T. litoralis*—Cells (about 40 g wet weight) from a 35 liter culture were suspended in 1 liter of 10 mM Tris/HCl, pH 8.5. Preparation of the crude extract and the first two chromatography steps were performed by the same procedures as those described for the *P. furiosus* enzyme except for the use of 10 mM Tris/HCl, pH 8.5, as the standard buffer. After Phenyl-Sepharose fast flow column chromatography, the pooled active fractions were dialyzed against 10 mM Tris/HCl, pH 8.5, containing 250 mM NaCl, concentrated by ultrafiltration, and then subjected to gel filtration on Sephacryl-S200HR (2.6 × 60 cm; Pharmacia) pre-equilibrated with the same buffer. The active fractions were pooled and then dialyzed against 10 mM Tris/HCl, pH 8.5. The desalted enzyme was loaded on a Mono Q HR 5/5 column (1 ml; Pharmacia) equilibrated with 10 mM Tris/HCl, pH 8.0. Elution was carried out with a 30-ml linear gradient of 0–1 M NaCl in the same buffer. The active fractions were pooled and then dialyzed against 10 mM Tris/HCl, pH 7.5.

*Polyacrylamide Gel Electrophoresis*—SDS–polyacrylamide gel electrophoresis (PAGE, 12% acrylamide slab gel, 1mm thick) was performed by the procedure of Laemmli (*12*). The protein band was stained with Coomassie Brilliant Blue R 250.

*Temperature and pH Optima*—The optimal temperature was determined by running the standard assay for ADP-GK activity at temperatures from 37°C to 100°C. The optimal pH of the enzyme was determined by running the standard assay at 37°C with $KH_2PO_4$-$K_2HPO_4$ buffer (0.1 M), Tris/HCl buffer (0.1 M), and glycine-NaOH buffer (0.1 M) for pH ranges 6.5–7.5, 7.0–8.5, and 8.0–10.0, respectively.

*Substrate specificity*—The reactivity of D-fructose, D-mannose, D-galactose, 2-deoxy-D-glucose, D-sorbitol, D-myoinocitol, D-mannitol, sucrose, and D-1,5-anhydroglucitol as substrates for ADP-GK was tested by measuring the formation of AMP from ADP by HPLC. The reaction mixture contained 50 mM Tris/HCl, pH 7.5, 20 mM each substrate, 2 mM ADP, 2 mM $MgCl_2 \cdot 6H_2O$, and 20 μl of enzyme preparation, in a total volume of 0.5 ml. After incubation for 10 min at 37°C, the reaction was stopped by cooling on ice. After 5 min, each solution was passed through a cellulose acetate filter (pore size, 0.2 μm; ADVANTEC, Tokyo). An aliquot (20 μl) of each filtrate was applied to a column (7.6 mm × 25 cm) of Asahipak GS320HQ (Asahi Chemical Industry, Shizuoka). $NaH_2PO_4$ (200 mM, pH 5.0) was used as the



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mobile phase at the flow rate of 1.0 ml/min. The effluent from the column was monitored with a UV detector at the wavelength of 260 nm. ATP, ADP and AMP were separated on a column at retention times of about 7.6, 8.6, and 11.6 min, respectively. The hexokinase from yeast (Oriental Yeast, Tokyo) and the glucokinase from *Bacillus stearothermophilus* (Sigma Chemical, St. Louis, MO) were used instead of ADP-GK in the reaction mixture for substrate specificity comparison. For detection of the specificity for the phosphoryl group donor, GDP, CDP, UDP, IDP, ATP, GTP, CTP, ITP, and AMP (each 2 mM) were used instead of ADP in the standard assay mixture described under "Enzyme assay." The divalent cation requirement was tested by the addition of 2 mM $MgCl_2$, $CoCl_2$, $NiCl_2$, $MnCl_2$, $ZnCl_2$, $PbCl_2$, $CaCl_2$, or $CuCl_2$ to the standard assay mixture.

*Molecular Mass Determination*—The molecular mass of ADP-GK was determined by gel filtration on a TSK gel column G3000SWXL (7.8 mm × 30 cm) (Tosoh) pre-equilibrated with the standard buffer containing 0.2 M NaCl. Thyroglobulin (670 kDa), gamma globulin (158 kDa), and ovalbumin (49 kDa) were used as molecular mass standards. The subunit molecular mass of the purified enzyme was determined by SDS-PAGE. The marker proteins (NEW ENGLAND BioLabs) used were as follows: bovine liver glutamate dehydrogenase (62 kDa), rabbit muscle aldolase (47.5 kDa), rabbit muscle triosephosphate isomerase (32.5 kDa), and bovine milk β-lactoglobulin A (25 kDa).

*N-Terminal Amino Acid Sequence Analysis of the P. furiosus ADP-GK and Design of Degenerate Probe*—N-Terminal sequence analysis of the purified *P. furiosus* ADP-GK was performed by automated Edman degradation with an Applied Biosystems 473A protein sequencer, which gave the following sequence: PTWEELYKNAIEKAIKSVPKVKGVLLGYNTNIDAIKYLD. The degenerate oligonucleotides, 5′-GG(A/T)TA(C/T)AACAC(A/T)AACAT(A/T)GA(C/T)GC(A/T)AT(A/T)AAGTA-3′, were designed as the DNA probe for the *P. furiosus* ADP-GK gene based on the amino acid sequence of GYNTNIDAIKY (the sequence underlined above). A high specific activity probe (probe A) was generated using 200 ng of this oligonucleotide mixture, T4 polynucleotide kinase (8.5 U) and 740 kBq of [γ-$^{32}$P]ATP.

*Cloning and Sequencing of the ADP-GK Genes*—To obtain a clone containing the ADP-GK gene, *P. furiosus* chromosomal DNA was prepared by the method of Ramakrishnan and Adams (*13*), digested with several restriction enzymes, and then separated by 0.8% agarose gel electrophoresis. The separated DNA fragments on the agarose gel were subjected to Southern blotting with probe A. An approximately 5 kb *Bgl*II fragment that gave a positive signal on Southern hybridization was extracted from the gel. The fragment was inserted into the *Bam*HI site of plasmid pUC118 and then *E. coli* JM109 cells were transformed. Transformants were selected on a Luria-Bertarni (LB) plate containing ampicillin (0.005%). The colonies were transferred and fixed on Biodyne-A nylon membranes (Pall Gelman Laboratory, Ann Arbor, MI). Pre-hybridization and hybridization with probe A were performed according to the manufacturer's instructions. Autoradiographs were obtained by exposure to X-ray film (Kodak X-OMAT AR) at –70°C for 24 h. After screening of recombinant plasmids by Southern hybridization, a positive plasmid containing the 5 kb *Bgl*II fragment, pPGK1, was isolated and used as a template for DNA sequencing.

For cloning of the *T. litoralis* ADP-GK gene, the 1.3 kb *Eco*RV fragment was cut out from pPGK1 and used as the DNA probe. A high specific activity probe (probe B) was generated by a random primer method using a *Bca*BEST Labeling Kit (TaKaRa Shuzo, Kyoto) and 740 kBq of [α-$^{32}$P]dCTP, according to the manufacturer's instructions. *T. litoralis* chromosomal DNA was prepared according to the method described above, digested with several restriction enzymes, and then separated by 0.8% agarose gel electrophoresis. The separated DNA fragments on the agarose gel were subjected to Southern blotting with probe B. An approximately 2.5 kb *Eco*T 221 fragment that gave a positive signal on Southern hybridization was extracted from the gel. The fragment was inserted into the *Pst*I site of plasmid pUC119 and then *E. coli* JM109 cells were transformed. Transformants were selected on an LB plate containing ampicillin (0.005%). Colony hybridization and autoradiography were performed by the same methods as those described for the *P. furiosus* ADP-GK gene. After screening of recombinant plasmids by Southern hybridization, a positive plasmid containing the 2.5 kb *Eco*T 221 fragment, pTGK1, was isolated and used as a template for DNA sequencing. The sequencing was performed by the dideoxynucleotide chain–termination method with an Applied Biosystems PRISM 310 DNA sequencer. Sequence data were analyzed using GENETYX-SV/RC9.0 software (Software Development).

## RESULTS

*Purification and Physical Characterization of ADP-GKs from P. furiosus and T. litoralis*—Table I is a summary of the purification of ADP-GK from the *P. furiosus* extract. The enzyme was purified about 1,480-fold with a 25% recovery. About 0.56 mg of the purified enzyme was obtained from 200 liters of *P. furiosus* culture. Table II shows the results of purification of the enzyme from the *T. litoralis* extract. The enzyme was purified about 630-fold with a 21% recovery. About 0.35 mg of the purified enzyme was obtained from 35 liters of *T. litoralis* culture. Both enzymes were found to be homogeneous on SDS-PAGE (Fig. 1).

The molecular mass of the *P. furiosus* ADP-GK was estimated to be about 100 kDa and that of the *T. litoralis* en-

TABLE I. **Purification of ADP-GK from *P. furiosus*.**

| Step | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Crude extract | 3,330 | 372 | 0.112 | 100 | 1 |
| DEAE- Sepharose FF | 608 | 344 | 0.566 | 93 | 5.05 |
| Phenyl- Sepharose FF | 45.3 | 286 | 6.31 | 77 | 56.3 |
| Q- Sepharose FF | 21.6 | 243 | 11.3 | 65 | 101 |
| Hydroxyapatite | 4.30 | 175 | 40.7 | 47 | 363 |
| Superose 12HR | 1.28 | 104 | 81.3 | 28 | 726 |
| RESOURCE ISO | 0.56 | 93.0 | 166 | 25 | 1,480 |



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zyme to be about 54 kDa by gel filtration. From the results of SDS-PAGE, the subunit molecular masses of the *T. litoralis* and *P. furiosus* enzymes were determined to be about 52 and 47 kDa, respectively (Fig. 1). These results show that the native enzyme from *P. furiosus* has a dimer structure composed of two identical subunits. On the other hand, the *T. litoralis* enzyme has a monomer structure.

The ADP-GKs from *P. furiosus* and *T. litoralis* showed similar properties as to optimum temperature and pH for the reaction, and thermostability. The activity of the enzymes increased with an increase in temperature from 37 to 100°C. The activity at 50°C was about twice that at 37°C. The highest activity was observed at 100°C and was about 8 times that at 37°C. The optimum temperature may be above 100°C. We were not able to assay at temperatures above 100°C because of the instability of the auxiliary enzymes under the assay conditions. The optimum pHs of the two enzymes were around pH 7.5. The two enzymes were stable up to 95°C (at pH 7.5); both enzymes retained the full activity upon heating at 90°C for 10 min and more than 95% of the full activity at 100°C for 10 min.

*Substrate Specificity and Kinetic Constants*—The purified ADP-GKs from *P. furiosus* and *T. litoralis* showed activity only in the forward direction. The two enzymes showed comparable activity with ADP and CDP as the phosphoryl group donor. GDP, IDP, UDP, ATP, GTP, CTP, and ITP were inert (Table III). The enzymes required divalent cations for their activity. $MgCl_2$ was most effective and it was able to replace $MnCl_2$ or $CoCl_2$ to some extent (Table III). The ability of the enzymes to catalyze the phosphorylation of various sugars was examined. The enzymes catalyze the phosphorylation of D-mannose, D-galactose, D-glucosamine and 2-deoxy-D-glucose to a limited extent, besides D-glucose (Table IV). In addition, both enzymes exhibited high reactivity for D-1,5-anhydroglucitol. The enzyme from *T. litoralis* showed the highest activity with D-1,5-anhydroglucitol as a phosphoryl group acceptor (about 1.7-times that with

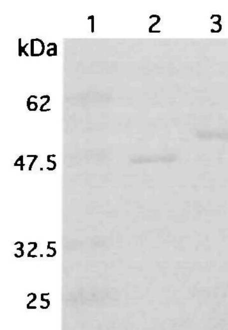


Fig. 1. **SDS-PAGE of the purified ADP-GKs from *P. furiosus* and *T. litoralis*.** Lanes: 1, molecular mass standards; 2, *P. furiosus* ADP-GK; 3, *T. litoralis* ADP-GK.

TABLE II. **Purification of ADP-GK from *T. litoralis*.**

| Step | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Crude extract | 1,050 | 122 | 0.116 | 100 | 1 |
| DEAE-Sepharose FF | 20.0 | 90.6 | 4.53 | 74 | 39.1 |
| Phenyl-Sepharose FF | 5.0 | 54.2 | 10.8 | 44 | 93.1 |
| Sephacryl S-200 | 1.24 | 32.5 | 26.2 | 27 | 226 |
| Mono Q | 0.35 | 25.6 | 73.1 | 21 | 630 |

TABLE III. **Specificity for phosphoryl group donors and cation dependence of the two ADP-GKs.**

| Phospholyl group donor | Relative activity | | Divalent cation | Relative activity | |
|---|---|---|---|---|---|
| | *P. furiosus* ADP-GK (%) | *T. litoralis* ADP-GK (%) | | *P. furiosus* ADP-GK (%) | *T. litoralis* ADP-GK (%) |
| ADP | 100 | 100 | $Mg^{2+}$ | 100 | 100 |
| GDP | 3 | 2 | $Co^{2+}$ | 93 | 22 |
| CDP | 111 | 122 | $Ni^{2+}$ | 11 | 7 |
| UDP | 2 | 3 | $Mn^{2+}$ | 47 | 6 |
| IDP | 2 | 2 | $Zn^{2+}$ | 4 | 0 |
| ATP | 0 | 0 | $Pb^{2+}$ | 0 | 0 |
| GTP | 0 | 0 | $Ca^{2+}$ | 0 | 0 |
| CTP | 0 | 0 | $Cu^{2+}$ | 0 | 0 |
| ITP | 0 | 0 | | | |

TABLE IV. **Comparison of the specificities for phosphoryl group acceptors.**

| Phospholyl group acceptor | Relative activity | | | |
|---|---|---|---|---|
| | *P. furiosus* ADP-GK (%) | *T. litoralis* ADP-GK (%) | *B. stearothermophilus*ATP-GK (%) | YeastATP-HK (%) |
| D-Glucose | 100 | 100 | 100 | 100 |
| D-Mannose | 13 | 13 | 5 | 83 |
| D-Galactose | 7 | 9 | 2 | 24 |
| D-Fructose | 0 | 2 | 0 | 260 |
| 2-Deoxy-D-glucose | 3 | 4 | 0 | 135 |
| D-Glucosamine | 72 | 67 | 0 | 76 |
| D-1,5-Anhydroglucitol | 68 | 166 | 2 | 2 |
| D-Sorbitol | 0 | 0 | 0 | 0 |
| D-Myoinocitol | 0 | 0 | 0 | 0 |
| D-Mannitol | 0 | 0 | 0 | 0 |
| Sucrose | 0 | 0 | 0 | 0 |



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D-glucose). The hexokinase (ATP-HK) from yeast and the glucokinase (ATP-GK) from *B. stearothermophilus* did not act on this compound (Table IV).

Both enzymes showed typical Michaelis-Menten kinetics. The $K_m$ values for D-glucose, D-1,5-anhydroglucitol, D-glucosamine, ADP, CDP, $Mg^{2+}$, and $Co^{2+}$ at 37°C were calculated to be 0.4 ($V_{max}/K_m$=0.18 × $10^3$), 2.0 (0.037 × $10^3$), 1.9 (0.038 × $10^3$), 0.057 (1.3 × $10^3$), 0.56 (0.13 × $10^3$), 0.037 (2.0 × $10^3$), and 0.058 mM (1.3 × $10^3$) for the *T. litoralis* enzyme, respectively, and those for the *P. furiosus* enzyme were 0.64 ($V_{max}/K_m$=0.26 × $10^3$), 8.3 (0.020 × $10^3$), 0.54 (0.31 × $10^3$), 0.07 (2.4 × $10^3$), 0.83 (0.20 × $10^3$), 0.041 (4.1 × $10^3$), and 0.071 mM (2.3 × $10^3$), respectively.

*Nucleotide Sequences of the ADP-GK Genes*—For sequencing of the *P. furiosus* ADP-GK gene, a recombinant plasmid, pPGK1, was used as a template as described under "MATERIALS AND METHODS." Sequence analysis revealed an open reading frame whose deduced amino acid sequence corresponded to the determined N-terminal protein sequence. The complete nucleotide sequence of the *P. furiosus* ADP-GK gene comprises 1,365 bp coding for 455 amino acids with a calculated molecular weight of 51,265 (GenBank Accession Number E14588), which corresponds to the subunit molecular mass of about 50 kDa determined on SDS-PAGE. For sequencing of the *T. litoralis* ADP-GK gene, pTGK1 was used as a template. The complete nucleotide sequence of the *T. litoralis* ADP-GK gene comprises 1,401 bp coding for 467 amino acids with a calculated molecular weight of 53,621 (GenBank Accession Number E14589), which corresponds to the molecular mass of 54 kDa determined on gel filtration. *E. coli* JM109, carrying pPGK1 or pTGK2, exhibited hyperthermostable ADP-GK activity, which was not lost on incubation at 80°C for 10 min, confirming that both genes encoded ADP-GK (data not shown).

*Amino Acid Sequence Alignment*—On amino acid sequence alignment, the identity between the ADP-GKs from *P. furiosus* and *T. litoralis* was observed to be 58.9% (Fig. 2). Both enzymes exhibited very low identity with other ATP-dependent glucokinases and hexokinases. In the N-terminal region, the *T. litoralis* enzyme was 8 amino acids longer than the *P. furiosus* enzyme. Multiple sequence alignment showed the presence of several conserved regions throughout these proteins. The conserved regions did not show similarity with any ATP-dependent kinases that have been reported so far.

## DISCUSSION

In this study, we purified ADP-GKs from the two anaerobic hyperthermophilic archaea, *P. furiosus* and *T. litoralis*, and characterized them. The genes encoding the enzymes were cloned into *E. coli* and sequenced. This is the first report of the purification, characterization and determination of the primary structure of the *T. litoralis* enzyme. The purification and basic properties of the *P. furiosus* enzyme have already been reported by Kengen *et al.* (*14*). We here developed a more efficient purification procedure, and investigated more detailed properties of the enzyme and the primary structure.

The specific activity of the purified *P. furiosus* enzyme is about 2.2 times that in the case of *T. litoralis*. The specific activity of the *P. furiosus* enzyme has been reported to be 307 μmol/min/mg at 50°C (*14*). This is compatible with the specific activity (160 units/mg) determined at 37°C in this study, because the activity at 50°C is about twice that at 37°C. The ADP-GKs from *P. furiosus* and *T. litoralis* showed similar properties as to kinetic constants, optimum temperature, and optimum pH of the reaction, and thermostability. A remarkable difference between the two enzymes is in the subunit composition. The enzyme from *P. furiosus* has a native molecular mass of 100 kDa and a subunit size of 47

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P.furiosus       1 --------MPTWEELYKNAIEKAIKSVPKVKGVLLGYNTNIDAIKYLDSKDLEERIIKAG   52
T.litoralis      1 MKESLKDRIRLWKRLYVNAFENALNAIPNVKGVLLAYNTNIDAIKYLDKDDLEKRVTEIG   60
                              *  ** ** * *    * ****** ************  *** *    *

P.furiosus      53 KEEVIKYSEELPDKINTVSQLLGSILWSIRRGKAAELFVESCPVRFYMKRWGWNELRMGG  112
T.litoralis     61 KEKVFEIIENPPEKISSIEELLGGILRSIKLGKAMEWFVESEEVRRYLREWGWDELRIGG  120
                   ** *    *  * **     *** ** **  *** * ****  ** *   *** *** **

P.furiosus     113 QAGIMANLLGGVYGVPVIVHVPQLSRLQANLFLDGPIYVPTLENGEVKLIHPKEFSGDEE  172
T.litoralis    121 QAGIMANLLGGVYRIPTIVHVPQNPKLQAELFVDGPIYVPVFEGNKLKLVHPKDAIAEEE  180
                   *************  * ******   *** ** *******  *    ** ***     **

P.furiosus     173 NCIHYIYEFPRGFRVFEFEAPRENRFIGSADDYNTTLFIREEFRESFSEVIKNVQLAILS  232
T.litoralis    181 ELIHYIYEFPRGFQVFDVQAPRENRFIANADDYNARVYMRREFREGFEEITRNVELAIIS  240
                     *********** **   ********  *****     * **** * *   ** *** *

P.furiosus     233 GLQALTK---E--NYKEPFEIVKSNLEVLNEREIPVHLEFAFTPDEKVREEILNVLGMFY  287
T.litoralis    241 GLQVLKEYYPDGTTYRDVLDRVESHLNILNRYNVKSHFEFAYTANRRVREALVELLPKFT  300
                   *** *         *      * * *  **      * *** *    ***     *  *

P.furiosus     288 SVGLNEVELASIMEILGEKKLAKELLAHDPVDPIAVTEAMLKLAKKTGVKRIHFHTYGYY  347
T.litoralis    301 SVGLNEVELASIMEIIGDEELAKEVL-EGHI--FSVIDAMNVLMDETGIERIHFHTYGYY  357
                   *************** *   **** *         *  **  *   **  **********

P.furiosus     348 LALTEYKGEHVRDALLFAALAAAAKAMKGNITSLEEIREATSVPVNEKATQVEEKLRAEY  407
T.litoralis    358 LALTQYRGEEVRDALLFASLAAAAKAMKGNLERIEQIRDALSVPTNERAIVLEEELEKEF  417
                   **** * ** ******** ***********    * ** * *** ** *   ** *  *

P.furiosus     408 -GIKEGIGEVEGYQIAFIPTKIVAKPKSTVGIGDTISSSAFIGEFSFTL-            455
T.litoralis    418 TEFENGLIDMVDRQLAFVPTKIVASPKSTVGIGDTISSSAFVSEFGMRKR            467
                        *       * ** ****** ****************  **
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Fig. 2. **Alignment of the amino acid sequences of the ADP-GKs from *P. furiosus* and *T. litoralis*.** Asterisks represent conserved residues in the two enzymes.



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kDa. On the other hand, the *T. litoralis* enzyme has a native molecular mass of 54 kDa and a subunit size of 52 kDa. These data indicate that the *P. furiosus* ADP-GK has a dimer structure composed of two identical subunits, whereas the *T. litoralis* enzyme has a monomer structure. On the amino acid alignment, it was revealed that the *T. litoralis* enzyme is 8 amino acids longer than the *P. furiosus* enzyme in the N-terminal region. The difference in the structures of the N-terminal regions of the two enzymes might be responsible for the difference in subunit composition between the enzymes.

ATP-dependent glucokinases from bacteria are usually homodimers with a subunit size of 24–30 kDa (*15, 16*). Eukaryotic hexokinases have a dimeric structure with subunits of 50 kDa (*17, 18*), whereas eukaryotic glucokinases have a monomeric structure (50 kDa) (*17, 19*). In this regard, the *P. furiosus* enzyme is similar to the eukaryotic hexokinase but the *T. litoralis* enzyme is similar to the eukaryotic glucokinase. In general, the substrate specificity of eukaryotic glucokinases or hexokinases for sugars is relatively low. For example, yeast hexokinase utilizes D-mannose, D-fructose, and 2-deoxy-D-glucose as phosphoryl group acceptors, as well as D-glucose (Table IV). The enzymes from *P. furiosus* and *T. litoralis* exhibit limited activity or do not act on these sugars. On the other hand, the glucokinases from bacteria exhibit high specificity for D-glucose, as shown in the case of the *B. stearothermophilus* enzyme (Table IV). In this respect, both enzymes are similar to the bacterial glucokinases. However, glucokinases or hexokinases that are able to utilize D-1,5-anhydroglucitol as a phosphoryl group acceptor have not been described so far. The enzymes from yeast and *B. stearothermophilus* do not act on D-1,5-anhydroglucitol (Table IV). Therefore, the high activity for this compound is one of the remarkable characteristics of the two enzymes.

In a phylogenetic hexokinase family tree constructed by comparing 60 sequences of sugar kinases, Bork *et al.* (*7*) observed that glucokinases appear in the following three clusters: (i) the mammalian glucokinases, (ii) the yeast glucokinases, and (iii) the bacterial glucokinases. On the basis of the amino acid sequence, the ADP-GKs from *P. furiosus* and *T. litoralis* did not show similarity with any sugar kinases that have been reported so far. This indicates that the enzymes represent a novel family of sugar kinases. In addition, Bork *et al.* (*20*) found that the three-dimensional structures of the actin, hexokinase, and Hsp70 protein families contained common motifs interacting with the ATP molecule, which are the "Phosphate-1" and "Phosphate-2" motifs in contact with the β- and γ-phosphate of ATP, and the "Connect-1" and "Connect-2" motifs at the interface between the subdomains. They also suggested the presence of sugar binding motifs in several sugar kinases (*7*). These binding motifs that interact with adenosine or sugar were not apparent in the ADP-GKs from *P. furiosus* and *T. litoralis* on amino acid sequence alignment (not shown). The two enzymes showed about 59% similarity in amino acid sequence and shared several conserved regions (Fig. 4). This suggests that the functionally important residues for the binding of D-glucose and ADP may be present in these regions. To clarify the residues that contribute to the substrate binding, three-dimensional structure analysis is under investigation.

Recently, we reported the presence of a kinase that utilizes AMP as the essential phosphate acceptor in *P. furiosus* (*21*). The enzyme utilized phosphoenolpyruvate as the phosphoryl group donor and formed ATP in the presence of $P_i$. Thus, we have proposed a novel type of energy metabolism in the modified Embden-Meyerhof pathway of *P. furiosus*; the AMP-dependent kinase may be responsible for the production of ATP from AMP formed through the ADP-GK and ADP-dependent phosphofructokinase reactions (*21*). The activity of the AMP-dependent kinase dramatically increases during glycolysis (unpublished data), suggesting that a novel regulatory mechanism is present in the glycolytic pathway of *P. furiosus*. Our next aim is to shed light on the regulation of energy metabolism in the modified Embden-Meyerhof pathway of *P. furiosus* and *T. litoralis* including ADP-GK, ADP-dependent phosphofructokinase, and the AMP-dependent kinase. That may reveal the significance of the novel type of energy metabolism in evolution.

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