**Purification and Characterization of a Novel ADP-dependent Glucokinase from the Hyperthermophilic Archaeon Pyrococcus furiosus**

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Pyrococcus furiosus uses a modified Embden-Meyerhof pathway during growth on poly- or disaccharides. Instead of the usual ATP-dependent glucokinase, this pathway involves a novel ADP-dependent (AMP-forming) glucokinase. The level of this enzyme and some other glycolytic enzymes appeared to be closely regulated by the substrate. Growth on cellulose resulted in a high specific activity of 0.96 units mg⁻¹, whereas on pyruvate a 10-fold lower activity was found. The ADP-dependent glucokinase was purified 1350-fold to homogeneity. The oxygen-stable enzyme had a molecular mass of 93 kDa and was composed of two identical subunits (47 kDa). The glucokinase was highly specific for ADP, which could not be replaced by ATP, phosphoenolpyruvate, GDP, PP₃, or polyphosphate. D-Glucose could be replaced only by 2-deoxy-D-glucose, albeit with a low efficiency. The Km values for D-glucose and ADP were 0.73 and 0.033 mM, respectively. An optimum temperature of 105 °C and a half-life of 220 min at 100 °C are in agreement with the requirements of this hyperthermophilic organism. The properties of the glucokinase are compared to those of less thermoactive glucokinases.

EXPERIMENTAL PROCEDURES

Growth of Organisms—AdP (monopotassium salt, less than 0.2% ATP), ATP (disodium salt), glucose-6-phosphate dehydrogenase (D-glucose-6-phospho-NADP oxidoreductase, EC 1.1.1.49; yeast), phosphoglucoisomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9; yeast), and phosphomannose isomerase (D-mannose-6-phosphate ketol-isomerase, EC 5.3.1.8; yeast) were obtained from Boehringer GmbH (Mannheim, Germany). Fructose-6-phosphate, D-galactose, 2-deoxy-D-glucose, sodium phosphate glass (type 35), and adenosine-5'-diphosphate-agaroses were from Sigma Chemie (Bornem, Belgium). D-Glucose, D-fructose, and D-mannose were from Merck (Darmstadt, Germany). All other chemicals were of analytical grade. Phenyl-Sepharose CL-4B, Mono-Q HR 5/5, and phenyl-Superose HR 5/5 were purchased from Pharmacia LKB Biotechnology (Woerden, The Netherlands). Hydroxyapatite Bio-Gel HT and the Prep-Gel system was from Bio-Rad (Veenendaal, The Netherlands). Gasses were supplied by Hoek-Loos (Schiedam, The Netherlands). P. furiosus (DSM 3638) was obtained from the German Collection of Microorganisms (Braunschweig, Germany).

Growth of Organism—P. furiosus was routinely grown at 90 °C on an artificial seawater medium, supplemented with tungsten (10 μM Na₂WO₄), yeast extract (1 g/titer), and vitamins, as described before (6). Routine culturing was performed in stoppered 120-ml serum bottles, containing 50 ml of medium and pressurized with 150 kPa N₂/CO₂ (80:20). Starch (5 g/titer), maltose (10 mM), cellulose (5 mM), pyruvate (40 mM), or peptone (5 g/titer) were used as substrates. For the preparation of cell extracts, cultures were subcultured at least five times (1% inoculum) on the substrate of interest prior to extraction.

Mass culturing (200 liters) was performed on the same medium except that Na₂S was omitted, the fermenter was sparged with N₂, and potato starch was used as substrate (5 g/titer).

Preparation of Cell-free Extracts—To determine the effect of the substrate on enzyme levels, cell extracts were prepared aerobically from

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the 50-ml cultures. The contents of each bottle was centrifuged for 20 min at 22,800 × g. The supernatant was discarded, and the cell pellet was resuspended in 1 ml of distilled water. The cell suspension was sonicated three times for 30 s. Cell debris were removed by centrifugation, and the supernatant was used as cell-free extract.

For use in enzyme purifications, cells and cell extracts were handled aerobically. Cells were harvested at mid-log phase by centrifugation at 5,800 × g for 10 min at 100,000 × g. The cell extract, containing 35–45 mg protein/ml, was stored at −20 °C until use.

Protein was determined with a Coomasie Brilliant Blue G250 protein assay kit (Bio-Rad). The absorbance of the sample was measured at 595 nm.

Determination of Enzyme Activity—The enzyme assays were performed aerobically in stopped 1-ml quartz cuvettes as described before (9). Specific enzyme activities were calculated from initial rates and expressed in units mg−1 protein. 1 unit was defined as that amount of enzyme required to convert 1 μmol of glucose per min.

ADP-dependent glucokinase was determined by measuring the formation of NADPH in a coupled assay with yeast glucose-6-phosphate dehydrogenase. The sample was assayed at 50 °C. At this temperature, the yeast enzyme remained active, and the Pyrococcus enzyme was sufficiently active to measure its activity to the limit of the assay mixture. The assay was performed using 100 mM TrisCl, pH 7.8, 10 mM MgCl2, 0.5 mM NADP, 15 mM D-glucose, 2 mM ADP, 0.35 units of D-glucose-6-phosphate dehydrogenase, and 5–50 μl of enzyme preparation. The absorbance of NADPH was followed at 334 nm (ε = 6.18 mM−1 cm−1). Care was taken that the activity of the auxiliary enzyme was always in excess of the glucokinase activity.

Phosphoglucose isomerase (EC 5.3.1.9) and ADP-dependent phosphofructokinase were determined at 50 °C using auxiliary enzymes as described before (9).

Substrate Specificity—The substrate specificity was tested using purified glucokinase. The use of 2-deoxy-D-glucose and D-galactose as possible substrates for the glucokinase was tested using the standard assay mixture. The yeast enzyme remained active, and the glucokinase activity was calculated from initial rates and expressed in units mg−1 protein.

The concentrations of ADP and D-glucose were varied from 0.5 to 4.0 mM and 5 to 20 mM, respectively.

Temperature Effect and Thermostability—The temperature effect on enzyme activity was determined by incubating an appropriate amount of purified enzyme in 1 ml of 1% glycerol and heating the sample for 10 min at 30 °C, 50 °C, 60 °C, 70 °C, 80 °C, and 90 °C. The enzyme activity was measured using the standard assay mixture. The temperature optimum was determined at 50 °C in 200 mM Tris/maleate buffer over the pH range 5.5–9.0.

The thermostability of the glucokinase was determined by incubating purified glucokinase in 200 mM Tris/maleate buffer containing 30% glycerol at 50 °C for 5 min. The enzyme was then assayed using the standard assay mixture.

Purity of the enzyme was confirmed by SDS-PAGE analysis with the following molecular mass standards: lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), chicken egg albumin (45 kDa), bovine serum albumin monomer and dimer (66 and 132 kDa), and urease hexamer (545 kDa).

The N-terminal sequence of the glucokinase was determined by performing PAGE at various acrylamide percentages (5, 6, 7, 8, 9, and 10%), as described by Hedrick and Smith (14). The following molecular mass standards were used: lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), chicken egg albumin (45 kDa), bovine serum albumin monomer and dimer (66 and 132 kDa), and urease hexamer (545 kDa).

The enzyme was desalted by ultrafiltration. The enzyme pool was applied to a Mono-Q HR 5/5 column equilibrated in 50 mM Pipes/Cl−(pH 6.2). Active fractions were pooled, concentrated to 1 ml, and loaded on a phenyl-Sepharose HR 5/5 column equilibrated in 50 mM Pipes/Cl−(pH 6.2). The glucokinase was eluted during a linear 40-ml gradient (0–1 M NaCl) at 0.2 M NaCl. The enzyme pool was desalted and concentrated with Macrosep (30 kDa) concentrators (Filtron). Complete purification was accomplished by continuous elution electrophoresis on a Prep Cell apparatus (Bio-Rad). A 1-ml sample from the concentrated enzyme pool was loaded on the gel (8% acrylamide, 0.1% ampholytes, and 0.1% acrylamide in 300 mM Pipes/Cl−(pH 6.2)). The enzyme was eluted from the gel in Tris (25 mM), glycine (192 mM) buffer, pH 8.3. Only those fractions that gave single bands on a native gel were combined.

Purity of the enzyme was checked by native and denaturing SDS-PAGE. For determination of the subunit composition by SDS-PAGE, protein samples were diluted in sample buffer, containing 2% SDS (w/v) and 5% 2-mercaptoethanol, and subsequently heated at 100 °C. In some cases, 2-mercaptoethanol was omitted from the sample buffer, and the sample was not heated. Silver staining was performed using the reagent kit from E. Merck (Darmstadt, Germany). Activity staining was performed on native PAGE gels by coupling the glucokinase activity to the generation of nitro blue tetrazolium. Therefore, the gels were incubated at 37 °C in the dark for 30 min in a staining mix with the following composition: 100 mM TrisCl, pH 7.8, 0.001% phenazin methosulfate, 0.035% nitro blue tetrazolium, 15 mM MgCl2, 0.5 mM NAPD, 3 mM ADP, 15 mM D-glucose, 185 mM NaCl, and D-glucose-6-phosphate dehydrogenase (1.75 units).

Molecular Mass Determination—The molecular mass of the native glucokinase was determined by performing PAGE at various acrylamide percentages (5, 6, 7, 8, 9, and 10%), as described by Hedrick and Smith (14). The following molecular mass standards were used: lactalbumin (14.2 kDa), carbonic anhydrase (29 kDa), chicken egg albumin (45 kDa), bovine serum albumin monomer and dimer (66 and 132 kDa), and urease hexamer (545 kDa).

For use in enzyme purifications, cells and cell extracts were handled aerobically. The cell extract, containing 2% SDS (w/v) and 5% 2-mercaptoethanol, and subsequently heated at 100 °C. In some cases, 2-mercaptoethanol was omitted from the sample buffer, and the sample was not heated. Silver staining was performed using the reagent kit from E. Merck (Darmstadt, Germany). Activity staining was performed on native PAGE gels by coupling the glucokinase activity to the generation of nitro blue tetrazolium. Therefore, the gels were incubated at 37 °C in the dark for 30 min in a staining mix with the following composition: 100 mM TrisCl, pH 7.8, 0.001% phenazin methosulfate, 0.035% nitro blue tetrazolium, 15 mM MgCl2, 0.5 mM NAPD, 3 mM ADP, 15 mM D-glucose, 185 mM NaCl, and D-glucose-6-phosphate dehydrogenase (1.75 units).

Temperature Effect and Thermostability—The temperature effect on enzyme activity was determined by incubating an appropriate amount of purified enzyme in 1 ml of 1% glycerol and heating the sample for 10 min at 30 °C, 50 °C, 60 °C, 70 °C, 80 °C, and 90 °C. The enzyme reaction was started by injecting 10 μl of 100 mM ADP. After 15–30 min, the reaction was stopped by putting the vials on ice, and the temperature of the sample was measured. The assay mixture was then assayed using the standard assay mixture.

Thermostability of the glucokinase was determined by incubating purified glucokinase in 200 mM Tris/maleate buffer containing 30% glycerol at 50 °C for 5 min. The enzyme was subsequently assayed using the standard assay mixture.

RESULTS

Glucokinase Levels on Different Carbon Sources—To discern the inducible or constitutive nature of the ADP-dependent glucokinase, cells of P. furiosus were grown on various carbon sources (Fig. 1). The level of the ADP-dependent glucokinase was found to vary from almost zero (0.003 units mg−1) during growth on peptone to 0.96 units mg−1 during growth on cellobiose. The non-glycolytic substrate pyruvate showed a relatively low glucokinase activity (0.074 units mg−1). Starch and
maltose gave intermediate values of 0.43 and 0.49 units mg⁻¹, respectively. The activity of the subsequent enzymes in the glycolysis, viz. phosphoglucone isomerase and the ADP-dependent phosphofructokinase, were also determined. The level of both enzymes appeared to vary in a similar way as the glucokinase, i.e., the highest activity on cellulose and lower activities on starch, maltose, and pyruvate.

Purification of the Glucokinase—The glucokinase was purified aerobically because no enzyme activity was lost upon storage of cell-free extracts at 4°C under air. After fractionation of the broken cell suspension at 100,000 × g, most of the total amount of activity (92%) was recovered in the supernatant, indicating that the enzyme is located in the cytoplasm. During initial purification attempts, PAGE showed that the enzyme copurified with several other proteins, suggesting that the enzyme adhered to these proteins. Therefore, the zwitterionic detergent CHAPS was added to the buffers, which did not affect the activity negatively.

The use of affinity chromatographic techniques, like ADP-agarose (either ribose-linked or N6-linked) or various dye-ligand-agaroses (Dynamex screening kit, Amicon), was unsuccessful because the enzyme did not bind to any of the ligands, even in the presence of 10 mM MgCl₂. Therefore, a series of seven sequential purification steps were required to obtain a homogeneous preparation as judged by a silver-stained PAGE gel (Table I). The colorless enzyme was 1346-fold purified with 2.1% recovery and showed a specific activity of 307 units mg⁻¹ at 50°C. The identity of the band was confirmed by activity staining.

Physical Properties—The molecular mass of the native enzyme as determined by PAGE at various acrylamide concentrations was 93 kDa (not shown). SDS-PAGE of the 980-fold purified protein gave a single band of 47 kDa, irrespective of the time of heating in sample buffer or the presence of 2-mercaptoethanol (Fig. 2). Apparently, the 93-kDa native enzyme easily disintegrates into two identical 47-kDa subunits. This result is in accordance with the immediate and complete inhibition of glucokinase activity that was found upon addition of 5 mM SDS (data not shown).

Catalytic Properties—The ADP-dependent glucokinase exhibited a high activity (>65% of maximum) between pH 6 and 9, with an optimum at pH 7.5. As all other kinases, the enzyme required divalent cations for activity (Table II). MgCl₂ was most effective, followed by MnCl₂, which resulted in 77% of the activity found with MgCl₂. No activity was found in the absence of divalent cations in the presence of EDTA. With respect to the phosphoryl group donor, the glucokinase was highly specific for ADP. ATP, GDP, phosphoenolpyruvate, PP₇, or polyphosphate were unable to replace ADP (Table II). The glucokinase was also rather specific for the type of sugar. D-Fructose, D-mannose, and D-galactose could not be phosphorylated, and only 2-deoxy-D-glucose was able to replace glucose to a limited (9.2%) extent (Table II).

Kinetic Parameters—Michaelis-Menten constants were determined according to Lineweaver-Burk. A Kₘ value of 0.73 ± 0.06 and 0.033 ± 0.003 mM was found for glucose and ADP, respectively. Apparent Vₘ₉ values were 249 ± 18 and 194 ± 15 units mg⁻¹ for glucose and ADP, respectively.

Thermostability and Temperature Optimum—The thermostability of the purified glucokinase was determined at 100°C and 110°C. At 110°C, all activity was lost after 30 min of incubation. Addition of MgADP, glucose, or both did not affect the stability. Therefore, no attempts were made to determine the half-life of the enzyme at this temperature. At 100°C, however, the glucokinase was remarkably stable. Inactivation followed first-order kinetics with a half-life value of 220 min (not shown).

The temperature dependence of the activity is shown in Fig. 3. The optimum temperature was found at 105°C (15-min incubation period). Because of a rapid denaturation above this temperature, this optimum value may increase or decrease depending on the time of incubation (shorter or longer incuba-
tion time, respectively). An Arrhenius plot of the data (Fig. 3, inset) showed a breakpoint at 60 °C, resulting in activation energy values of 54.3 kJ mol\(^{-1}\) between 30 and 60 °C and 37.4 kJ mol\(^{-1}\) between 60 and 105 °C.

N-terminal Amino Acid Sequence Analysis—Two independent attempts to determine the N-terminal sequence did not give an unambiguous and ungapped sequence, indicating that the N terminus may be blocked. Those amino acids that were identified as identical by both analyses gave the following sequence (first 10 residues, X = ambiguous residue): NH₂MTXELYKN(I/A). This sequence did not show similarity with any sequence given in the SWISSPROT data base.

**DISCUSSION**

P. furiosus has recently been shown to utilize a modified Embden-Meyerhof pathway, which involves a glucokinase and a phosphofructokinase that are both ADP-dependent (9). Here, the ADP-dependent glucokinase was purified and characterized. Cell-free extracts of P. furiosus contained high levels of this enzyme, especially when the organism was grown on cellobiose. This high level of the glucokinase and also of the phosphoglucone isomerase and the phosphofructokinase in cellobiose-grown cells as compared to maltose-grown cells clearly shows that at least the first steps of the glycolysis are closely regulated in this hyperthermophilic Archaeon. This also follows from the low activity of these enzymes on pyruvate- and peptone-grown cells.

The cytoplasmic and oxygen-stable glucokinase was purified more than 1000-fold to homogeneity. Thus, the glucokinase constitutes less than 0.1% of the total cellular protein. This seems a rather low value for such a key enzyme. However, using the experimentally determined relationship between activity and temperature, the specific activity at 100 °C amounts to 2,233 units mg\(^{-1}\) (\(k_{\text{cat}} = 3,500 \text{ s}^{-1}\)), which is the highest reported (Table III). Moreover, it has been calculated before that the specific activity in cell-free extracts is more than satisfactory to sustain the glucose flux (9).

The ADP-dependent glucokinase had a native molecular mass of approximately 93 kDa and consisted of two identical subunits of approximately 47 kDa. This \(\alpha_2\) composition is observed also for bacterial glucokinases and eukaryotic hexokinases, but it differs from the eukaryotic glucokinases (bakers' yeast, rat liver), which show a monomeric structure (Table III). Furthermore, the P. furiosus glucokinase differs from most bacterial enzymes by its native molecular mass, which is about twice the usual size of about 50 kDa. In this respect, it resem-

![Fig. 2. SDS-polyacrylamide gel electrophoresis of the glucokinase from P. furiosus.](image)

Lane 1 shows a set of marker proteins with their molecular mass indicated. Lanes 2-5 contained the 980-fold purified protein (0.45 mg protein/lane). Lanes 2 and 3 contained glucokinase diluted in sample buffer without and with 2-mercaptoethanol, respectively, and which were not boiled. Lanes 4 and 5 contained glucokinase that was boiled in sample buffer for 2 and 45 min, respectively. Proteins were stained with Coomassie Brilliant Blue R250.

![Fig. 3. Dependence of glucokinase activity on temperature.](image)

Activity was determined by measuring the amount of glucose-6-phosphate formed after incubation for an appropriate period of time at the desired temperature. Inset, Arrhenius plot of the data from 30 to 105 °C.

| Sugar          | Relative activity | Phosphoryl group donor | Relative activity | Divalent cation | Relative activity |
|----------------|-------------------|------------------------|-------------------|----------------|------------------|
| d-Glucose      | 100               | ADP                    | 100               | Mg\(^{2+}\)    | 100              |
| 2-Deoxy-d-glucose | 9.2              | ATP                    | ND                | Mn\(^{2+}\)    | 77               |
| d-Fructose     | ND\(^a\)          | GDP                    | ND                | Ca\(^{2+}\)    | 17               |
| o-Mannose      | ND                | PEP                    | ND                | Zn\(^{2+}\)    | 5                |
| o-Galactose    | ND                | Poly-P                 | ND                | Co\(^{2+}\)    | 1                |

\(^a\) ND, not detectable, i.e. the activity was less than 0.3% of the activity under optimal conditions.

![Table II Substrate specificity and cation dependence of the glucokinase from P. furiosus](image)
Aspergillus niger
Hxk 30 100 50 ND 32 18
Propionibacterium shermanii
Gluc 30 58 58 57 38 19

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