Molecular and Biochemical Characterization of the ADP-dependent Phosphofructokinase from the Hyperthermophilic Archaeon Pyrococcus furiosus*

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Pyrococcus furiosus uses a modified Embden-Meyerhof pathway involving two ADP-dependent kinases. Using the N-terminal amino acid sequence of the previously purified ADP-dependent glucokinase, the corresponding gene as well as a related open reading frame were detected in the genome of P. furiosus. Both genes were successfully cloned and expressed in Escherichia coli, yielding highly thermostable ADP-dependent glucokinase and phosphofructokinase. The deduced amino acid sequences of both kinases were 21.1% identical but did not reveal significant homology with those of other known sugar kinases. The ADP-dependent phosphofructokinase was purified and characterized. The oxygen-stable protein had a native molecular mass of approximately 180 kDa and was composed of four identical 52-kDa subunits. It had a specific activity of 88 units/mg at 50 °C and a pH optimum of 6.5. As phosphoryl group donor, ADP could be replaced by GDP, ATP, and GTP to a limited extent. The \( K_m \) values for fructose 6-phosphate and ADP were 2.3 and 0.11 mM, respectively. The phosphofructokinase did not catalyze the reverse reaction, nor was it regulated by any of the known allosteric modulators of ATP-dependent phosphofructokinases. ATP and AMP were identified as competitive inhibitors of the phosphofructokinase, raising the \( K_m \) for ADP to 0.34 and 0.41 mM, respectively.

During growth on poly- or disaccharides, the hyperthermophilic archaeon Pyrococcus furiosus uses a novel type of glycolytic pathway that is deviant from the classical Embden-Meyerhof pathway in several respects (1, 2). First, instead of the classical ATP-dependent hexokinase, the pathway involves a novel ADP-dependent glucokinase (3, 4). Second, a novel ADP-dependent phosphofructokinase replaces the more common ATP-dependent phosphofructokinase (3). Third, the pathway is modified in the degradation of glyceraldehyde 3-phosphate, which involves glyceraldehyde-3-phosphate dehydrogenase instead of the conventional couple glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase (5, 6). Modifications of the classical Embden-Meyerhof pathway at one or more of these three steps have also been observed in members of the hyperthermophilic archaeal genera Thermococcus, Desulfurococcus, and Thermoproteus (2, 7). The presence of these modifications in P. furiosus and other hyperthermophilic microorganisms suggests that these are adaptations to elevated temperatures as a result of an altered biochemistry or a decreased stability of biomolecules.

Although ATP is regarded as the universal energy carrier and the most common phosphoryl group donor for kinases, several gluco- and phosphofructokinases with a different cosubstrate specificity have been described. Beside ADP-dependent gluco- and phosphofructokinases that have been demonstrated in Pyrococcus and Thermococcus spp. (3, 4, 7), polyphosphate-dependent glucokinases have been found in several other microorganisms. In addition, the glucokinase of Propionibacterium can use both ATP and polyphosphate as phosphoryl group donor (8). Furthermore, PP-dependent phosphofructokinases have been described in several eukaryas and bacteria and the hyperthermophilic archaeon Thermoproteus tenax (9). Phylogenetic analyses of phosphofructokinases grouped these enzymes into three clusters. In a multiple alignment of representatives of each cluster, functionally important residues were identified that were highly conserved between all phosphofructokinases (9). ADP-dependent phosphofructokinases were not included in this study, because primary sequences of these enzymes were not yet available.

In this paper, we describe the cloning, expression, purification, and characterization of the ADP-dependent phosphofructokinase from P. furiosus. It is the first molecular and biochemical characterization of an ADP-dependent phosphofructokinase to date.

EXPERIMENTAL PROCEDURES

Materials—Acetyl phosphate (potassium-lithium salt, crystallized), ADP (disodium salt), AMP (disodium salt, crystallized), aldolase (\( \text{n-fructose-1,6-bisphosphate} \ \text{n-glyceraldehyde-3-phosphate-lyase}, \ EC\ 4.1.2.13; \text{rabbit muscle})\), ATP (disodium salt), fructose 1,6-bisphosphate (trisodium salt, crystallized), GDP (dilithium salt), glucose 6-phosphate (disodium salt), glucose-6-phosphate dehydrogenase (\( \text{n-glyceraldehyde-3-phosphate-dehydrogenase} \ (\text{EC}\ 1.2.1.12; \text{yeast})\), glycerol-3-phosphate dehydrogenase (\( \text{sn-glycerol-3-phosphate} \ (\text{NADP}^+ \ \text{oxidoreductase})\), EC 1.1.1.49; yeast), glycerol-3-phosphate dehydrogenase (\( \text{sn-glycerol-3-phosphate} \ (\text{NADP}^+ \ \text{2-oxidoreductase})\), EC 1.1.1.8; \text{rabbit muscle}), NADH (disodium salt), phosphoenolpyruvate (tricelohexylamilmonium salt), phophoglucone isomerase (\( \text{glucose-6-phosphate ketol-isomerase} \), EC 5.3.1.1; \text{yeast}), and triosephosphate isomerase (\( \text{n-glyceraldehyde-3-phosphate-ketol-isomerase} \), EC 5.3.1.1; \text{rabbit muscle}) were obtained from Roche Molecular Biochemicals. \( \text{N-Fructose-1-phosphate} \ (\text{barium salt}), \text{n-fructose 2,6-bisphosphate} \ (\text{sodium salt}), \text{n-fructose 6-phosphate} \ (\text{disodium salt}), \beta-NADP^+ \ (\text{sodium salt}), \text{sea salts}, \text{sodium phosphate glass type 35}, \text{tetrapotassium pyrophosphate, triphosphate pentasodium}, \) and trisodium trimetaphos-
P. furiosus ADP-dependent Phosphofructokinase

The previously obtained N-terminal amino acid sequence of the ADP-dependent glucokinase from P. furiosus, partially purified from cell-free extract of P. furiosus. All purification steps were done without protection against oxygen. To prevent microbial contamination, all buffers contained 0.02% sodium azide. Phosphofructokinase activity was recovered from cell-free extract following precipitation between 40 and 60% ammonium sulfate saturation. The subsequent purification included chromatography on phenyl-Superose HR 5/5, Q-Sepharose fast flow, hydroxyapatite Bio-Gel HT, mono Q HR 5/5, and Superdex 200 prep gel filtration.

Preparation of Cell-free Extract from P. furiosus—P. furiosus cells from a 200-liter culture were harvested by continuous centrifugation (2200 g) and resuspended in 10 ml of 20 mM Tris/HCl buffer, pH 8.5. The suspension was passed twice through a 0.45-μm filter and loaded onto a Q-Sepharose column that was equilibrated with 20 mM Tris/HCl buffer, pH 8.5. Bound proteins were eluted by a linear gradient of NaCl (0 to 1 M in Tris/HCl buffer).

Active fractions were pooled and desalted with 20 mM Tris/HCl buffer, pH 8.5, using a Centricon filter with a 30-kDa cutoff.

Protein Concentration and Purity—Protein concentrations were determined with Coomassie Brilliant Blue G250 as described before (13) using bovine serum albumin as a standard. The purity of the enzyme was checked by SDS-PAGE as described before (10).

Determination of Enzyme Activity—ADP-dependent phosphofructokinase activity was measured aerobically in stopped 1-ml quartz cuvettes at 50 °C as described before (3).

The subunit molecular mass of the purified recombinant protein was determined on a Superdex 200 gel filtration column using 100 mM Tris/HCl buffer, pH 7.8, with 150 mM NaCl. The column was calibrated using the following standard proteins: ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldolase (158 kDa), and catalase (232 kDa).

Molecular mass determination of the partially purified phosphofructokinase from P. furiosus cell-free extract was determined on a Superdex 200 gel filtration column using 100 mM Tris/HCl buffer, pH 7.8, with 150 mM NaCl. The column was calibrated using the following standard proteins: ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldolase (158 kDa), and catalase (232 kDa).

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taken that the auxiliary enzymes were not limiting at the various pH values.

Substrate Specificity—As possible phosphoryl group donors, ATP, GTP, pyrophosphate, phosphoenolpyruvate, acetylphosphate, tri- and polyphosphate, trimetaphosphate (each 2.5 mM), and polyphosphate (sodium phosphate glass type 35, 0.25 mg/ml) were used in the activity assay instead of ADP. The divalent cation requirement was tested by adding 10 mM MnCl₂, CaCl₂, CoCl₂ or ZnCl₂ instead of MgCl₂.

Kinetic Parameters—Kinetic parameters were determined at 50 °C by varying the concentration of ADP (0.0125–5 mM) or fructose 6-phosphate (0.1–10 mM) in the assay mixture in the presence of 10 mM fructose 6-phosphate or 2.5 mM ADP, respectively. Data were analyzed by computer-aided direct fit to the Michaelis-Menten curve. Furthermore, the data were used to construct Hill plots (log (V/Vₘₐₓ) - V) versus log S.

Allosteric Effectors—Regulation of phosphofructokinase activity by possible allosteric modulators was investigated by adding adenine nucleotides (ATP, ADP, or AMP; 2, 5, and 10 mM), metabolites (glucose, pyruvate, phosphoenolpyruvate, or citrate; 5 mM) or fructose 6-bisphosphate (0.1 and 1 mM) to the assay mixture. Furthermore, the effect of KCl and NaCl (30, 150 and 500 mM) on the enzyme activity was tested.

RESULTS

Purification of the Phosphofructokinase from P. furiosus Cell-free Extract—Cell-free extracts of P. furiosus showed a phosphofructokinase activity of 0.038 units/mg. However, despite the use of various chromatographic techniques, we were unable to obtain a highly purified enzyme, because it tended to stick to other proteins, resulting in similar band patterns upon PAGE after each purification step. When applied to a hydrophobic interaction column, phosphofructokinase activity was completely lost. Moreover, the use of dye affinity chromatography was not successful; although the phosphofructokinase did bind to a number of the tested dye ligands, it could not be eluted specifically with ADP. Aspecific elution with NaCl did not result in loss of contaminating proteins. Consequently, following chromatography on five different columns, the enzyme was purified 80-fold to a specific activity of 3 units/mg but still contained several contaminating proteins (Fig. 1).

Cloning of the Phosphofructokinase Gene—Using the previ-
ously obtained N-terminal amino acid sequence of the ADP-dependent glucokinase (4), a putative glucokinase gene was identified in the P. furiosus genome sequence. Expression of the gene in E. coli resulted in an ADP-dependent glucokinase activity of 20 units/mg in cell-free extracts at 50 °C, confirming that the gene indeed encoded the glucokinase3. When the glucokinase gene, designated *gkA*, was used to search the P. furiosus genome, highest homology (25.7% nucleotide identity) was found with a 1365-base pair open reading frame predicted to encode a 455-amino acid protein. It was considered that this open reading frame might encode the ADP-dependent phosphofructokinase, and therefore the open reading frame was amplified by polymerase chain reaction and cloned into pET9d, resulting in plasmid pLUW572. DNA sequence analysis of pLUW572 confirmed the successful and faultless cloning of the open reading frame into pET9d (not shown).

Overexpression of the Phosphofructokinase Gene in E. coli—SDS-PAGE analysis of a cell-free extract of *E. coli* BL21(DE3) harboring pLUW572 revealed an additional band of approximately 50 kDa, which corresponded with the calculated molecular mass (52.3 kDa) of the gene product. This band was absent in extracts of *E. coli* BL21(DE3) carrying the pET9d plasmid without insert. In a cell-free extract of *E. coli* BL21(DE3) harboring pLUW572, an ADP-dependent phosphofructokinase activity of 3.48 units/mg was measured at 50 °C, confirming that indeed the *P. furiosus* phosphofructokinase gene, designated *pfkA*, had been cloned and expressed. The enzyme could be produced for up to 5% of the total E. coli cell protein without inducing gene expression by adding isopropyl-1-thio-β-D-galactopyranoside. Therefore, no attempts were made to optimize the overexpression.

Primary Sequence Comparison—On an amino acid level, the identity between the glucokinase and phosphofructokinase from *P. furiosus* was 21.1%. Comparison of the deduced amino acid sequence of the phosphofructokinase with those of proteins present in the GenBank data base showed high similarity with two hypothetical proteins from *Pyrococcus horikoshii* (PH1645, 75.2% identity; PH0589, 23.1% identity). Cloning and expression of the corresponding genes demonstrated that the proteins are an ADP-dependent phosphofructokinase and an ADP-dependent glucokinase, respectively (data not shown). Furthermore, 48.6% identity was found with a hypothetical protein from *Methanococcus jannaschii* (MJ1604), which turned out to be an ADP-dependent phosphofructokinase3. Multiple sequence alignment showed several conserved regions through the five proteins (Fig. 2). Comparison of the conserved regions with sequences present in the GenBank data base did not reveal additional similarities.

Purification and Physical Characterization of the Recombinant Phosphofructokinase—The recombinant phosphofructokinase was easily purified by a heat incubation and anion exchange chromatography to at least 95% homogeneity as judged by SDS-PAGE (Fig. 1). The specific activity of the purified protein was 88 units/mg at 50 °C. On SDS-PAGE, the purified recombinant protein did not appear at the same height as the most abundant band in the partially purified *P. furiosus* fraction. However, because the phosphofructokinase activity of the partially purified *P. furiosus* cell-free extract is 3 units/mg, the enzyme represents only 3% of the total protein in the extract and can therefore not be the most dominant band in lane 2 of the SDS-PAGE gel.

SDS-PAGE of the purified recombinant phosphofructokinase gave a single band at 52 kDa (Fig. 1). The native molecular mass of the partially purified phosphofructokinase from *P. furiosus* cell-free extract, as determined by gel filtration chromatography, was approximately 180 kDa. This is in good agreement with the molecular mass determination of the purified recombinant phosphofructokinase. A native molecular mass of the phosphofructokinase of 179 kDa was calculated from the calibration curve (Fig. 3), suggesting that the phosphofructokinase is a homotetramer. The phosphofructokinase showed activity between pH 5.5 and 7.0, with an optimum at pH 6.5 (data not shown).

Substrate Specificity of the Recombinant Phosphofructokinase—The purified phosphofructokinase only showed activity in the forward direction. The enzyme showed highest activity with ADP as a phosphoryl group donor, which could be replaced by GDP, ATP, and GTP to a limited extent (Table I). Divalent cations were required for activity of the enzyme, as shown by complete lack of activity in the presence of EDTA. Phosphofructokinase activity was highest in the presence of MgCl2, followed by CoCl2 (Table I). The partially purified enzyme from *P. furiosus* cell-free extract showed the same substrate specificity pattern (data not shown).

Kinetic Parameters of the Recombinant Phosphofructokinase—The purified phosphofructokinase showed Michaelis-Menten kinetics at 50 °C, with the following constants that were determined according to direct fit: *Km* values of 2.3 ± 0.3 and 0.11 ± 0.01 mM for fructose 6-phosphate and ADP, respectively, and *Vmax* values of 194 ± 13 and 150 ± 5 units/mg for fructose 6-phosphate and ADP, respectively. *Km* values determined for the partially purified enzyme from *P. furiosus* cell-free extracts were in the same order of magnitude. Further-

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3 C. H. Verhees, J. E. Tuininga, S. W. M. Kengen, J. van der Oost, A. J. M. Stams, and W. M. de Vos, manuscript in preparation.
more, Hill coefficients of 1.1 (fructose 6-phosphate) and 0.95 (ADP) were determined, indicative of noncooperative binding of the substrates to each subunit of the tetrameric enzyme.

**Allosteric Effectors of the Recombinant Phosphofructokinase**—The addition of glucose, pyruvate, phosphoenolpyruvate, citrate, or fructose 2,6-bisphosphate did not show any effect on the phosphofructokinase activity. Both NaCl and KCl had a negative effect on the phosphofructokinase activity (42 and 43% activity in 300 mM NaCl and KCl, respectively).

Furthermore, the phosphofructokinase activity was negatively affected by the addition of ATP or AMP to the assay mixture. Because subsequent addition of MgCl₂ did not restore activity, the negative effect was not because of binding of Mg²⁺ to the ATP or AMP, resulting in lower availability of the ions for the substrate ADP. The addition of 5 mM ATP or AMP resulted in an increase in $K_m$ values for ADP from 0.11 to 0.34 ± 0.02 or 0.41 ± 0.03 mM, respectively, whereas the $V_{max}$ did not change (Fig. 4). This indicates competitive inhibition of the phosphofructokinase by ATP and AMP. Apparently, the phosphofructokinase is not allosterically regulated by ATP, AMP, or any of the other tested compounds.

**DISCUSSION**

*P. furiosus* uses a modified Embden-Meyerhof pathway involving two novel-type kinases, i.e. an ADP-dependent glucokinase, which has previously been purified and characterized (4), and an ADP-dependent phosphofructokinase. In cell-free extracts of mass-cultured *P. furiosus* cells grown on starch, a phosphofructokinase activity of 0.038 units/mg was measured. Purification of the ADP-dependent phosphofructokinase from cell-free extracts of *P. furiosus* was hampered, because the enzyme tended to stick to other proteins, and both dye affinity and hydrophobic interaction chromatography could not be used in the purification. However, an alternative approach became available following the identification of the *P. furiosus* pfkA gene encoding the phosphofructokinase, which was successfully overexpressed in *E. coli*.

The recombinant phosphofructokinase was purified from *E. coli* to 95% homogeneity in a two-step purification. The specific activity of the purified protein was 88 units/mg at 50 °C, which is approximately 2300-fold higher than the activity in crude cell-free extract of *P. furiosus* (0.038 units/mg). This suggests that the phosphofructokinase represents a very small fraction (0.043%) of the total *P. furiosus* cell protein, which is unexpected for a catalytic enzyme present in an important metabolic pathway. However, using the experimentally determined relationship between activity and temperature ($Q_10 = 2$ (15)), it can be calculated that the specific activity at 100 °C would be 2816 units/mg. Furthermore, it has been calculated before that the specific activity of phosphofructokinase in cell-free extracts of *P. furiosus* is sufficiently high to sustain the glucose flux (3).

The ADP-dependent phosphofructokinase had a native molecular mass of 180 kDa and a subunit size of 52 kDa, in agreement with the deduced molecular mass of 52.3 kDa from the amino acid sequence. These data suggest that the phosphofructokinase has a tetrameric structure, which is most common for phosphofructokinases. ATP-dependent phosphofructokinases from bacteria and mammals are usually homotetramers with a subunit size of 33 and 85 kDa, respectively. Yeast phosphofructokinases show $\alpha_4\beta_4$ octameric structures with subunits of 112 and 118 kDa, whereas PP₇-dependent phosphofructokinases have been described to be monomers (110 kDa), homodimers (subunits of 48–55 kDa), homotetramers (subunits of 45 kDa), or heterotetramers (subunits of 60 and 65 kDa) (16).

The reaction catalyzed by the phosphofructokinase was found to be irreversible. Therefore, *P. furiosus* needs a separate fructose-1,6-bisphosphate phosphatase to catalyze the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate during gluconeogenesis. Indeed, this enzyme has been detected in cell-free extract with a specific activity of 0.026 units/mg at 75 °C (17). The irreversibility of the phosphofructokinase reac-

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**TABLE 1**

Substrate specificity and cation dependence of the ADP-dependent phosphofructokinase from *P. furiosus*

| Phosphoryl group donor | Relative activity (%) | Divalent cation Relative activity |
|------------------------|----------------------|---------------------------------|
| ADP                    | 100                  | Mg²⁺ 100                         |
| GDP                    | 28                   | Co²⁺ 81                          |
| ATP                    | <10                  | Mn²⁺ 43                          |
| GTP                    | <6                   | Ca²⁺ 8                           |
| Phosphoenolpyruvate    | ND                   | Zn²⁺ ND                          |
| Pyrophosphate          | ND                   |                                  |
| Triphosphate           | ND                   |                                  |
| Acetylphosphate        | ND                   |                                  |
| Trimetaphosphate       | ND                   |                                  |
| Polyphosphate          | ND                   |                                  |

*ND, not detectable.
tion has also been described for ATP-dependent phosphofructokinases, although PP\textsubscript{i}-dependent phosphofructokinases catalyze reversible reactions (16).

Apparent $K_m$ values of 2.3 and 0.11 mM were found for fructose 6-phosphate and ADP, respectively. These values were determined at 50 °C, which is much lower than the optimal growth temperature of $P$. furiosus. Because temperature can have a dramatic effect on $K_m$ values, we have realized that $K_m$ values at the optimum growth temperature of 100 °C could differ considerably from the data obtained in this study. Apparent $K_m$ values at 55 °C of the ADP-dependent phosphofructokinases from cell-free extracts of _Thermococcus celer_ and _T. litoralis_ were 2.5 and 4 mM, respectively, for fructose 6-phosphate and 0.2 and 0.4 mM, respectively, for ADP (7). However, the possible temperature effect makes it difficult to compare kinetic values of microorganisms with different optimal growth temperatures (100 °C for _P. furiosus_ and 85 °C for both _Thermococcus_ strains). For the purified PP\textsubscript{i}-dependent phosphofructokinase from _T. tenax_ (optimal growth temperature 85 °C), much lower $K_m$ values were found: 0.053 mM for fructose-6-phosphate and 0.023 mM for PP\textsubscript{i} (9).

The ADP-dependent phosphofructokinase also showed activity with ATP, GTP, and GDP as phosphoryl group donors. In the case of ATP or GTP, however, the reaction product (ADP or GDP, respectively) is again an efficient phosphoryl group donor. Therefore, the relative activities with these compounds are probably overestimated. Furthermore, because of this fact, we were not able to determine kinetic values for ATP.

The phosphofructokinase was found to be inhibited by ATP and AMP through a competitive mechanism. In the case of ATP, this is not surprising, because ATP itself is a substrate and must therefore be able to bind to the catalytic site. In view of the role of phosphofructokinases in regulating the glycolytic pathway, it is surprising to see that ATP and AMP have the same (negative) effect on the activity of the phosphofructokinase. Allosterically regulated phosphofructokinases are usually inhibited by ATP but stimulated by AMP. ATP-dependent phosphofructokinases from _E. coli_ and _Bacillus stearothermophilus_ are allosterically activated by ADP and GDP and inhibited by phosphoenolpyruvate. Both yeast and mammalian phosphofructokinases are regulated by a large variety of effectors. Beside allosteric regulation by ATP and AMP, the enzymes are inhibited by citrate and activated by phosphate. Only mammalian enzymes are allosterically activated by fructose 1,6-bisphosphate. A very potent allosteric stimulator of eukaryotic phosphofructokinases is fructose 2,6-bisphosphate, which acts synergistically with AMP. This compound has been detected in most eukaryotes but never in prokaryotes (16). Apparently, the ADP-dependent phosphofructokinase from _P. furiosus_ is not allosterically regulated at all, and therefore it can not act as the major control point of the glycolytic pathway. Alternatively, the glyceraldehyde-3-phosphate ferredoxin oxidoreductase could be an important enzyme in control of the glycolysis of _P. furiosus_ (6). The PP\textsubscript{i}-dependent phosphofructokinase from _T. tenax_ is not allosterically controlled either, nor does it function as the major control point of the glycolytic pathway of this organism (9).

Hill plot analysis indicated that the phosphofructokinase did not cooperatively bind either of the substrates ADP and fructose 6-phosphate, in contrast to the ATP-dependent phosphofructokinases from _E. coli_ and _B. stearothermophilus_, which were found to show cooperative binding to fructose 6-phosphate but not to ATP (16).

The assumption that the open reading frame related to the glkA, found in the _P. furiosus_ genome, might encode the ADP-dependent phosphofructokinase was based on the observation that the N-terminal amino acid sequence of the glucokinase did not show any homology to known sugar kinases (4). Furthermore, in the _P. furiosus_ genome data base, no sequence could be found that showed significant homology to either gluc- or phosphofructokinases. Because both enzymes are ADP-dependent kinases, they could have identical ADP and sugar binding sites and might therefore be homologous to each other.

This hypothesis was confirmed when the expressed open reading frame indeed turned out to encode the ADP-dependent phosphofructokinase. Primary sequence analysis of the deduced amino acid sequence of the glucokinase and the phosphofructokinase showed that the proteins are significantly homologous and share several conserved regions. The functionally important residues for substrate binding that have been described for ATP- and PP\textsubscript{i}-dependent phosphofructokinases (9) did, however, not seem to be present in any of the sequences of the ADP-dependent kinases, suggesting they represent a novel group of kinases. Altogether, these findings suggest that the glucokinase and the phosphofructokinase from _P. furiosus_ are phylogenetically related. Further research is focused on scientific evidence for this suggestion.

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