ADP-dependent Glucokinase/Phosphofructokinase, a Novel Bifunctional Enzyme from the Hyperthermophilic Archaeon * Methanococcus jannaschii*

Received for publication, January 29, 2002, and in revised form, February 18, 2002

Published, JBC Papers in Press, February 20, 2002, DOI 10.1074/jbc.C200059200

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A gene encoding an ADP-dependent phosphofructokinase homologue has been identified in the hyperthermophilic archaeon *Methanococcus jannaschii* via genome sequencing. The gene encoded a protein of 462 amino acids with a molecular weight of 53,361. The deduced amino acid sequence of the gene showed 52 and 29% identities to the ADP-dependent phosphofructokinase and glucokinase from *Pyrococcus furiosus*, respectively. The gene was overexpressed in *Escherichia coli*, and the produced enzyme was purified and characterized. To our surprise, the enzyme showed high ADP-dependent activities for both glucokinase and phosphofructokinase. A native molecular mass was estimated to be 55 kDa, and this indicates the enzyme is monomeric. The reaction rate for the phosphorylation of d-glucose was almost 3 times that for d-fructose 6-phosphate. The *Km* values for d-fructose 6-phosphate and d-glucose were calculated to be 0.010 and 1.6 mM, respectively. The *Km* values for ADP were 0.032 and 0.63 mM when d-glucose and d-fructose 6-phosphate were used as a phosphoryl group acceptor, respectively. The gene encoding the enzyme is proposed to be an ancestral gene of an ADP-dependent phosphofructokinase and glucokinase. A gene duplication event might lead to the two enzymatic activities.

In general, ATP is regarded as the universal energy carrier and the most common phosphoryl group donor for kinases. However, several gluco- and phosphofructokinases have been reported to have different phosphoryl group donor specificity. The glucokinase from *Mycobacterium tuberculosis* can utilize both ATP and polyphosphate as the phosphoryl group donor (1). PP, phosphofructokinases in several archaea and bacteria and in the hyperthermophilic archaean *Thermoproteus tenax* (2–4). Recently novel sugar kinases, ADP-dependent (AMP-forming) glucokinase (ADP-GK) and phosphofructokinase (ADP-PFK), were discovered in the hyperthermophilic archaeon *Pyrococcus furiosus* (5). Those enzymes require ADP as the phosphoryl group donor instead of ATP and are involved in a modified Embden-Meyerhof pathway in this organism. The hyperthermophilic archaea are relatively deeply branched archaea and are considered to be phylogenetically ancient organisms. Therefore, structural analysis of the kinases from these organisms may provide abundant information for phylogenetic analysis of the sugar kinases. We cloned and sequenced the gene encoding the ADP-GK from *P. furiosus* and *Thermococcus litoralis* (6). About 59% identity in amino acid sequence was observed between these two enzymes, although they did not show similarity with any ATP-dependent kinases that have been reported so far. In addition, the amino acid sequence of the *P. furiosus* ADP-GK showed high identity (26%) with that reported for the *P. furiosus* ADP-PFK (7). This suggests that those kinases belong to a novel kinase family and might have evolved from a common origin.

Recently a gene encoding the ADP-PFK homologue has been identified from genome information in the hyperthermophilic archaeon *Methanococcus jannaschii* (8). Verhees et al. (9) have expressed the gene in *Escherichia coli* and revealed that the produced enzyme has a unique activity. They performed characterization of the enzyme with regard to the ADP-PFK activity. They also analyzed the same gene of *M. jannaschii* and found that the identity (29%) in amino acid sequence between the *M. jannaschii* ADP-PFK homologue and *P. furiosus* ADP-GK is somewhat higher than that (26%) observed between *P. furiosus* ADP-PFK and -GK. We expressed the gene in *E. coli* and examined characteristics of the product. As a result, we found that the produced enzyme has high ADP-dependent activity for both glucokinase and phosphofructokinase. We show here that the enzyme is a novel type of enzyme, a bifunctional ADP-dependent glucokinase/phosphofructokinase (ADP-GK/PFK). The enzyme was proposed to be a common origin of the ADP-GK and -PFK from genome analysis. This is the first example of a kinase that catalyzes phosphorylation of both d-glucose and d-fructose 6-phosphate.

**EXPERIMENTAL PROCEDURES**

Overexpression and Purification of Recombinant Protein—The gene (MJ1604) encoding the ADP-PFK homologue, which shows high similarity to that of the *P. furiosus* ADP-PFK, has been identified in the *M. jannaschii* genome (8). The plasmid DNA pET15b, which carries an ADP-PFK, has been identified in the *M. jannaschii* genome (8). The plasmid DNA pET15b, which carries an ADP-PFK, has been identified in the *M. jannaschii* genome (8). The plasmid DNA pET15b, which carries an ADP-PFK, has been identified in the *M. jannaschii* genome (8). The plasmid DNA pET15b, which carries an ADP-PFK, has been identified in the *M. jannaschii* genome (8). The plasmid DNA pET15b, which carries an ADP-PFK, has been identified in the *M. jannaschii* genome (8). The plasmid DNA pET15b, which carries an ADP-PFK, has been identified in the *M. jannaschii* genome (8). The plasmid DNA pET15b, which carries an ADP-PFK, has been identified in the *M. jannaschii* genome (8). The plasmid DNA pET15b, which carries an ADP-PFK, has been identified in the *M. jannaschii* genome (8).
Bifunctional ADP-dependent GK/PPK from M. jannaschii

Properties of ADP-GKs and PFKs from hyperthermophilic archaea

| Parameter | T. litoraisa ADP-GK | P. furiosusaADP-GK | P. furiosusa6 ADP-PPK | M. jannaschii ADP-GK/PPK |
|-----------|---------------------|--------------------|-----------------------|--------------------------|
| Native molecular mass (kDa) | 50 | 100 | 180 | 55 |
| Subunit molecular mass (kDa) | 46 | 51 | 52 | 53 |
| Kₘ value (mM) | | | | |
| d-Glucose | 0.4 | 0.64 | 1.6 | |
| ADP | 0.057 | 0.07 | 0.032 | |
| d-Fructose 6-phosphate | | 2.3 | 0.010 | |
| ADP | | 0.11 | 0.63 | |

Phosphoryl group acceptor (%)

| | d-Glucose | d-Fructose | d-Glucosamine | d-Manneose | d-Galactose | 2-Deoxy-d-glucose | d-Fructose 6-phosphate | d-Fructose 1-phosphate | d-Glucose 6-phosphate |
| | 100 | 100 | ND | ND | 100 | ND | ND | ND | ND |

Phosphoryl group donor (%)

| | ADP | GDP | CDP | | | | | | |
| | 100 | 100 | 100 | 100 | 100 | ND | ND | ND | ND |

Divalent cation (%)

| | Mg²⁺ | Co²⁺ | Ni²⁺ | Mn²⁺ | Zn²⁺ | Pb²⁺ | Ca²⁺ | | |
| | 100 | 22 | 7 | 6 | ND | ND | 8 | | |

Table I

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ADP-GK was determined by measuring the formation of NAD.PH from ADP and glucose. The concentrations of glucose were: 2.0 mM, 2.3 mM, 2.6 mM, 2.9 mM, and 3.2 mM. Dixon plots of v versus fructose 6-phosphate at several concentrations of glucose. The concentrations of glucose were: 2.0 mM, 1.0 mM, and 0.67 mM.

![Figure 1](image)

**FIG. 1. Inhibition analysis.** A, double-reciprocal plots of v versus glucose concentrations at several fixed concentrations of fructose 6-phosphate. The concentrations of fructose 6-phosphate used were: 0 mM (1), 0.002 mM (2), 0.003 mM (3), 0.005 mM (4), and 0.007 mM (5). B, Dixon plots of v versus fructose 6-phosphate at several concentrations of glucose. The concentrations of glucose were: 2.0 mM (1), 1.0 mM (2), and 0.67 mM (3).
fructose 1-phosphate, and d-glucose 6-phosphate as substrates was tested by measuring the formation of AMP from ADP by high performance liquid chromatography (HPLC) basically as described by Koga et al. (6). The reaction mixture contained 50 mM BisTris Buffer (pH 6.5), a 20 mM concentration of each substrate, 2 mM ADP, 2 mM MgCl₂·6H₂O, 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, Japan). NaH₂PO₄ (200 mM, pH 5.0) was used instead of ADP in the standard assay mixture. The divalent cation requirement was tested by the addition of 2 mM MgCl₂, CoCl₂, NiCl₂, MnCl₂, ZnCl₂, PbCl₂, or CaCl₂ to the standard assay mixture. The molecular mass of the enzyme was determined by gel filtration on a TSK gel column G3000SW (7.8 mm × 30 cm) (Tosoh, Tokyo, Japan), and the subunit molecular mass of the purified enzyme was determined by SDS-PAGE as described previously (6).

**RESULTS AND DISCUSSION**

**Expression of the Gene and Purification of the Recombinant Enzyme—**The E. coli strain BL21(DE3) codon plus RIL transformed with the expression vector pMJGK/PPF exhibited high activities for both ADP-dependent glucokinase and phosphofructokinase, which were not lost by incubation at 80 °C for 10 min. Hereafter we refer to the enzyme as the M. jannaschii ADP-GK/PPK. The enzyme was purified to homogeneity from the extract of E. coli cells. About 10 mg of the purified enzyme was obtained from 2 liters of the E. coli culture. The specific activity of the purified M. jannaschii ADP-GK/PPK was estimated to be 7.7 μmol/min/mg at 50 °C for phosphofructokinase activity at the optimum pH of 6.5. The specific activity for the ADP-PPFK reaction of the M. jannaschii enzyme has been reported to be 8.2 μmol/min/mg at 50 °C by Verhees et al. (9), and this is compatible with that measured in this study. On the other hand, the specific activity for the ADP-GK reaction of the enzyme was about 3 times that for the ADP-PPFK reaction and was estimated to be 21.5 μmol/min/mg at 50 °C. Upon heating at 80 °C for 10 min, the enzyme retained its full activity but lost 20% of the activity at 90 °C after a 10-min incubation. The purified ADP-GK/PPFK showed activity only in the forward direction.

**Characteristics of the M. jannaschii ADP-GK/PPK—**The biochemical characteristics of the purified enzyme were determined and compared with those of the ADP-GKs from P. furiosus and T. litoralis (6) and ADP-PPFK from P. furiosus (7). The deduced amino acid sequence of the M. jannaschii ADP-GK/PPK gene showed 52, 29, and 33% identities to the P. furiosus ADP-GK/PFK, P. furiosus ADP-GK, and T. litoralis ADGK, respectively. SDS-PAGE of the purified enzyme gave only one band; the subunit molecular mass was determined to be about 53 kDa, and was consistent with the molecular weight (55,524) calculated from the amino acid sequence including the His tag sequence. The native molecular mass of the enzyme determined by HPLC was about 55 kDa; this indicates the enzyme is monomeric. Although the P. furiosus ADP-PPFK shows high homology with the M. jannaschii ADP-GK/PPK, it has a tetramer structure composed of four identical subunits (7), which is most common for phosphofructokinases. The Thermococcus zilligii ADP-PPFK has been reported to have the same structure (8). In this regard, the M. jannaschii ADP-GK/PPK is similar to the T. litoralis ADGK (Table I).

ADP could be replaced by GDP to some extent for both the ADP-GK and -PPFK reactions of the M. jannaschii enzyme (Table I). When d-glucose was used as a phosphoryl group acceptor, ADP could be replaced by CDP to a limited extent. ATP,
GTP, pyrophosphate, triplyphosphate, trimetaphosphate, polyphosphate, and phosphoenolpyruvate were inert. The ADP-GKs from *P. furiosus* and *T. litoralis* utilize GDP to a very limited extent as the phosphoryl group donor, although the two enzymes showed comparable activity for ADP and GDP. On the other hand, the *P. furiosus* ADP-PFK has some reactivity for GDP. In this respect, the *M. jannaschii* enzyme is similar to the *P. furiosus* ADP-PFK (Table I). The enzymes required divalent cations for both activities. MgCl₂ and CaCl₂ were comparatively effective, and they were able to be replaced by NiCl₂, MnCl₂, PbCl₂, or CoCl₂ to some extent (Table I). However, the ADP-GKs from *P. furiosus* and *T. litoralis* do not utilize CaCl₂, and the *P. furiosus* ADP-PFK shows very low activity for CaCl₂. Therefore, the reactivity for CaCl₂ is one of the remarkable characteristics of the enzymes. The ability of the enzyme to catalyze the phosphorylation of various sugars was examined. The enzyme catalyzed the phosphorylation of d-fructose and 2-deoxy-d-glucose to a limited extent in addition to d-glucose and d-fructose 6-phosphate (Table I). d-Glucosamine, d-mannose, d-galactose, d-fructose 1-phosphate, and d-glucose 6-phosphate were inert. Typical Michaelis-Menten kinetics were observed for both the phosphorylation of d-glucose and d-fructose 6-phosphate at 50 °C. The apparent *Kₘ* values for fructose 6-phosphate and d-glucose were calculated to be 0.010 and 1.6 mM, respectively. The *Kₘ* values for ADP were 0.032 and 0.63 mM when d-glucose and d-fructose 6-phosphate were used as a phosphoryl group acceptor, respectively. To date, the ADP-PFK that utilizes d-glucose as a phosphoryl group acceptor has not been found. The ADP-GKs do not utilize d-fructose 6-phosphate (Table I). To the best of our knowledge, the ADP-GK/PFK of *M. jannaschii* is the only enzyme having significantly high levels of both activities. We performed inhibition studies for the glucokinase activity of the enzyme with fructose-6-phosphate as an inhibitor. The double-reciprocal plots of *v* versus *t* of glucose concentrations at several fixed concentrations of fructose 6-phosphate showed a typical competitive inhibition pattern (Fig. 1A). *Kₘ* for fructose 6-phosphate was calculated to be 0.0047 mM from Dixon plots (Fig. 1B). This indicates that the substrate recognition site of the enzyme for glucose and fructose 6-phosphate is identical.

**Genome Analysis**—Fig. 2 shows an amino acid alignment of ADP-GK and -PFK homologues from *M. jannaschii*, *P. furiosus*, *Pyrococcus horikoshii* OT-3, and *Pyrococcus abyssi*. A phylogenetic tree produced using the alignment of Fig. 2 by the neighbor-joining method is shown in Fig. 3. The genome information of these organisms is available at the Kyoto Encyclopedia of Genes and Genomes (genome.ad.jp/kegg/) and the Utah Genome Center (www.genome.utah.edu/). The data bases were screened for homologues of ADP-GKs and -PFKs using blastP. As shown in Fig. 3, the ADP-GKs clustered together and are separated from another cluster of the ADP-PFKs. Interestingly both the ADP-GK and -PFK homologues are present in *P. furiosus*, *P. horikoshii* OT-3, and *P. abyssi* but not in *M. jannaschii*, except for the ADP-GK/PFK. This suggests that the gene encoding the enzyme might be an ancestral gene of the ADP-GK and -PFK, and a gene duplication event has lead to the two enzymatic activities. Recently the crystal structure of the *T. litoralis* ADP-GK has been solved (11). Coocrystallization with ADP elucidated the substrate recognition site of the enzyme for glucose and fructose 6-phosphate in those kinases. As shown in Fig. 2, several residues of the *M. jannaschii* ADP-GK/PFK are conserved in all the ADP-GKs but not in the ADP-PFKs. In particular, Lys 31, Tyr 32, Asp 37, Ser 44, Glu 81, Leu 113, Glu 130, Asp 141, Ala 192, Lys 382, Asn 387, Lys 435, and Ser 446 of the *M. jannaschii* enzyme are all conserved in the ADP-GKs including the *T. litoralis* ADP-GK (not shown). Some or one of those residues might be responsible for the reactivity of the ADP-dependent kinases for d-glucose. To clarify the amino acid residues that are responsible for the binding of d-glucose and d-fructose 6-phosphate, x-ray reflection analysis of the *M. jannaschii* ADP-GK/PFK is under investigation.

The presence of a glycolytic pathway in methanogens has been proposed on the basis of enzyme analyses of several mesophilic and thermophilic methanogens (9, 12) and the genome sequence of *M. jannaschii*, which revealed the presence of several glycolytic enzyme homologues (13). A number of methanogens have been known to synthesize glycogen intracellularly and accumulate it as a reserve polysaccharide (9, 12). Under starvation, the degradation of the glycogen storage has been observed in the mesophilic archaeon *Methanococcus maripaludis* (12). The recent characterization of the amino acid sequence of the ADP-GKs from *P. furiosus* and *T. litoralis* (6) and that of the ADP-PFKs from *P. furiosus* (7) and *T. zilligii* (8) resulted in the identification of a homologue in the genome of *M. jannaschii* (8). These observations suggest that a modified Embden-Meyerhof pathway, present in *P. furiosus*, might also be operational in methanogens. It is therefore interesting to examine whether the production of methane from glycogen or d-glucose by methanogen itself is permitted. The observations described above and our results suggest that such a probability is not completely ruled out.

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