Arch Microbiol (2002) 177 : 401–409
DOI 10.1007/s00203-002-0405-7

ORIGINAL PAPER

Thomas Hansen · Meike Musfeldt · Peter Schönheit

ATP-dependent 6-phosphofructokinase from the hyperthermophilic bacterium *Thermotoga maritima*: characterization of an extremely thermophilic, allosterically regulated enzyme

Received: 2 November 2001 / Revised: 22 January 2002 / Accepted: 25 January 2002 / Published online: 5 March 2002

Abstract The ATP-dependent 6-phosphofructokinase (ATP-PFK) of the hyperthermophilic bacterium *Thermotoga maritima* was purified 730-fold to homogeneity. The enzyme is a 140-kDa homotetramer composed of 34 kDa subunits. Kinetic constants were determined for all substrates in both reaction directions at pH 7 and at 75 °C. Rate dependence (forward reaction) on fructose 6-phosphate (F-6-P) showed sigmoidal kinetics with a half-maximal saturation constant ($S_{0.5}$) of 0.7 mM and a Hill coefficient of 2.2. The apparent K_m for ATP was 0.2 mM and the apparent V_{max} value was about 360 U/mg. The enzyme also catalyzed in vitro the reverse reaction with an apparent K_m for fructose 1,6-bisphosphate and ADP of 7.6 mM and 1.4 mM, respectively, and an apparent V_{max} of about 13 U/mg. Divalent cations were required for maximal activity; Mg^{2+}, which was most effective, could partially be replaced by Mn^{2+} and Fe^{2+}. Enzyme activity was allosterically regulated by classical effectors of ATP-PFKs of Eukarya and Bacteria; it was activated by ADP and inhibited by PEP. The enzyme had a temperature optimum of 93 °C and showed a significant thermostability up to 100 °C. Using the N-terminal amino acid sequence of the subunit, the *pfk* gene coding for ATP-PFK was identified and functionally overexpressed in *Escherichia coli*. The purified recombinant ATP-PFK had identical kinetic and allosteric properties as the native enzyme purified from *T. maritima*. The deduced amino acid sequence showed high sequence similarity to members of the PFK-A family. In accordance with its allosteric properties, ATP-PFK of *T. maritima* contained the conserved allosteric effector-binding sites for ADP and PEP.

Keywords *Thermotoga maritma* · Hyperthermophilic bacteria · ATP-dependent 6-phosphofructokinase · PFK-A family · Allosteric regulation · Thermostability · Embden-Meyerhof pathway · Evolution

T. Hansen · M. Musfeldt · P. Schönheit (✉)
Institut für Allgemeine Mikrobiologie,
Christian-Albrechts-Universität Kiel,
Am Botanischen Garten 1–9, 24118 Kiel, Germany
e-mail: peter.schoenheit@ifam.uni-kiel.de,
Tel.: +49-431-8804328, Fax: +49-431-8802194

Introduction

ATP-dependent 6-phosphofructokinase (ATP-PFK, EC 2.7.1.11) catalyzes the phosphorylation of fructose 6-phosphate (F-6-P) to fructose 1,6-bisphosphate (F-1, 6-BP) with ATP as phosphoryl donor. The enzyme is present in all domains of life – Bacteria, Archaea and Eukarya. A common property of most ATP-PFKs from the domains of Bacteria and Eukarya is their homotetrameric stucture and the allosteric regulation of activity by compounds of intermediary metabolism. The bacterial enzymes are usually composed of 34-kDa subunits and are allosterically activated by ADP and inhibited by phosphoenolpyruvate (PEP) (Blangy et al. 1968; Evans and Hudson 1979; Uyeda 1979). Thus, these enzymes represent key regulatory sites of sugar degradation via the Embden-Meyerhof pathway.

Recently, we have characterized two ATP-PFKs from the domain of Archaea, from the hyperthermophilic Crenarchaeota *Desulfurococcus amylolyticus* (Hansen and Schönheit 2000) and *Aeropyrum pernix* (Hansen and Schönheit 2001). The enzymes of both organisms were characterized as extremely thermophilic, homotetrameric enzymes consisting of 34-kDa subunits. Unlike most ATP-PFKs of bacteria and eukarya, the two archaeal enzymes were not allosterically regulated. To comparatively analyze ATP-PFKs from hyperthermophiles, in particular with respect to their allosteric behaviour, we characterized the ATP-PFK from the hyperthermophile *T. maritma*. In the 16S-rRNA-based phylogenetic tree, *T. maritima* (Huber et al. 1986) belongs to the deepest branches in the domain of Bacteria (Woese 1987). In previous studies, we have shown that *T. maritima* contains high activities of ATP-PFK as part of the classical Embden-Meyerhof pathway involved in glucose degradation (Schröder et al. 1994; Selig et al. 1997). The analysis of the allosteric properties of ATP-PFK in *T. maritima* is important to understand the regulation of the Emden-Meyerhoff pathway in a phylogenetic ancestral bacterium.

During completion of this work. a paper by Ding et al. (2001) appeared reporting heterologous expression of putative *pfk* gene from *T. maritima*. The recombinant His-tagged protein represents a homotretrameric ATP-dependent PFK, which was partially characterized with respect to its molecular composition and some kinetic properties. The temperature dependence of enzyme activity was not given. The authors reported a hyperbolic rate dependence of ATP-PFK activity for F-6-P and did not detect significant response to the common allosteric effectors ADP and PEP. Thus, Ding et al. concluded that ATP-PFK in *T. maritima* is not a site of classical allosteric regulation by ADP and PEP in glucose degradation via the Emden-Meyerhof pathway. However, it has to be considered that all kinetic determinations including rate dependence on F-6-P and the effects of ADP and PEP were determined at 50 °C, i.e. far below the physiological temperature of about 80 °C, the optimal temperature for growth. Furthermore, the His-tag present in the recombinant protein might have affected the kinetic and allosteric properties of the enzyme. Thus, to study the kinetic and regulatory properties of ATP-PFK from *T. maritima* under physiological relevant conditions, it is necessary to analyze these parameters with the native enzyme, purified from *Thermotoga*, at temperatures near 80 °C.

In this communication we report the purification and characterization of ATP-PFK from *T. maritima* grown on starch as carbon and energy source. We show that ATP-PFK represents an extremely thermophilic, homotetrameric protein with the classical allosteric behaviour towards the common effectors ADP and PEP when assayed at the 75 °C, near the optimal growth temperature. The gene coding for ATP-PFK was identified using the N-terminal amino acid sequence of the subunit and was functionally overexpressed in *Escherichia coli*. The purified recombinant enzyme showed almost identical molecular, kinetic and regulatory properties as the native enzyme purified from *T. maritima*. Most of the data of this paper have been presented on the second NorFA Workshop on "Biology of Thermophiles" at the University of Iceland (June 2–6, 2000).

Materials and methods

Growth of the organism and preparation of cell free extracts

Thermotoga maritima MSB 8 (DSM 3109) (Huber et al. 1986) was grown anaerobically at 80 °C in a 100-l Biostat fermenter on a medium containing starch (5 g/l) and yeast extract (5 g/l) as carbon and energy source. Cells were harvested at the late-exponential growth phase. The following steps were carried out under anoxic conditions: Cell extracts were prepared from 32 g of frozen cells (wet mass) that were suspended in 100 ml 50 mM Tris/HCl, pH 7.0, containing 2 mM dithiothreithol (buffer A). Upon thawing and passage three times through a French pressure cell at 8 MPa, the cells were almost completely disrupted. Cell debris and unbroken cells were removed by ultracentrifugation for 90 min at 100,000×*g* at 4 °C.

Purification of ATP-dependent 6-phosphofructokinase

All chromatographic steps were carried out at 4 °C. The 100,000×*g* supernatant was applied to a HiLoad Q-Sepharose column (10 cm×2.6 cm) equilibrated with buffer A. Protein was eluted at a flow rate of 3 ml/min with 90 ml 50 mM piperazine, pH 6.4 (25 °C), containing 2 mM dithiothreithol (buffer B) as well as a combination of fixed NaCl concentrations and linear gradients from 0 M NaCl to 2 M NaCl in buffer B: 0–0.8 M NaCl (300 ml), 0.8–1.2 M NaCl (120 ml), 1.2–2 M NaCl (120 ml). Fractions containing the highest PFK activity (84 ml, 1.0–1.3 M NaCl) were pooled, diluted to a final NaCl concentration of 60 mM with buffer A including 2.5 mM $MgCl_2$ (buffer A′), and applied to a ATP-Agarose column (7 cm×1 cm) at a flow rate of 1.3 ml/min equilibrated with buffer A′. After washing the column with 15 ml buffer A′, protein was desorbed at a flow rate of 0.5 ml/min with fixed concentration as well as linear gradients from 0 to 50 mM of both F-6-P and ADP in buffer A′: 1 mM (6 ml), 2.5 mM (5 ml), 2.5 mM–15 mM (10 ml), 15 mM (5 ml), 15 mM–50 mM (5 ml), and 50 mM (2 ml). Fractions with the highest PFK activity (4.8 ml, 10–35 mM F-6-P+10–35 mM ADP) were pooled, adjusted to pH 5 with 1 M acetic acid, and loaded on a Uno S 1 column (1 ml) previously equilibrated with 50 mM MES, pH 5.3 (25 °C), containing 1 mM dithiothreithol (buffer C). Protein was eluted at a flow rate of 1 ml/min combining fixed NaCl concentrations with a linear NaCl gradient from 0 to 2 M (7 ml) in buffer C. PFK-activity-containing fractions of 0.5 ml were recovered from 0.7–1.6 M NaCl, those obtained from 0.9–1.6 M (2.5 ml) were essentially pure. The eluate was stored at –20 °C. Under these conditions activity remained about constant.

Analytical assays

The purity of the preparations was checked by SDS-PAGE in 14% polyacrylamide gels followed by staining with Coomassie brilliant blue R 250 according to standard procedures (Laemmli 1970). Protein concentrations were determined by the method of Bradford (Bradford 1976) with bovine serum albumin as standard. Gel filtration chromatography was carried out at ambient temperature on a Superdex 200 (50 mM Tris/HCl, 150 mM NaCl, pH 7.0, 1 ml/min).

Determination of N-terminal amino acid sequence

The purified protein was run on a 13% polyacrylamide gel in the presence of 6 M urea following the procedure of Schaegger and von Jagow (1987). Blotting onto a poly(vinylidene diflouride) membrane and N-terminal microsequencing on a model 473A sequencer (Applied Biosystems) was carried out as described in Meyer et al. (1996).

Enzyme assays and determination of kinetic parameters

Since the enzyme activity was not sensitive to oxygen, all assays were carried out under oxic conditions. The ATP-dependent PFK activity ($F-6-P + ATP \rightleftharpoons F-1,6-BP + ADP$) was determined in both directions. The forward reaction was measured by coupling the ATP-dependent formation of F-1, 6-BP to the oxidation of NADH via F-1, 6-BP aldolase, triosephosphate isomerase and glycerol-3-phosphate-dehydrogenase, whereas the reverse reaction was investigated by coupling the ADP-dependent formation of F-6-P to the reduction of $NADP^+$ via glucose-6-phosphate isomerase (GPI) and glucose 6-phosphate dehydrogenase. ATP-PFK activity was measured at 50 °C in a continuous and above 50 °C in a discontinuous assay as previously described (Hansen and Schönheit 2000), except for the standard assay mixtures containing: (1) 100 mM triethanolamine (pH 7.0 at the respective temperature) and 5 mM F-6-P, 2 mM ATP, and $MgCl_2$:ATP at an optimized ratio of 5:1 (forward reaction), or (2) 50 mM F-1,6-P, 10 mM ADP, and $MgCl_2$:ADP at an optimized ratio of 2.5:1 (reverse reaction). Initial velocities were investigated in at least six parallel assays stopped at different time intervals. One unit (U) of ATP-PFK activity is defined as either the conversion of either 1 μmol F-6-P to F-1, 6-BP (forward reaction) or the formation of 1 μmol F-6-P from F-1, 6-BP (reverse reaction). The coupling enzymes in all assays were routinely tested to ensure that they were not rate-limiting.

pH dependence, substrate specificity, cation specificity, and effectors

The pH dependence of the enzyme was measured between 4.0 and 9.0 at 75 °C and 50 °C using either piperazine, phosphate, MES, Tris/HCl, triethanolamine, or ethanolamine at a concentration of 100 mM each. The cation and nucleotide specificities were examined using the standard discontinuous test system at 75 °C by exchanging either Mg^{2+} (5 mM) or ATP (1 mM) for alternative divalent cations (Ni^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}, and Fe^{2+}) or alternative phosphoryl donors (ITP, GTP, UTP, CTP, UDP, GDP, ADP, CDP, acetyl phosphate, and PP_i) at equimolar concentrations. For the test of substrate specificity for sugars, F-6-P was exchanged for fructose 1-phosphate, glucose 6-phosphate, fructose and glucose. The following classical effectors of ATP-PFKs from bacterial and eukaryotic sources were tested using the following concentrations: PEP (1 mM, 10 mM), ADP (0.5 mM, 2.5 mM), F-2,6-BP (1 mM), AMP (1, 10 mM), and citrate (1, 10 mM). In order to prevent nonenzymatic hydrolysis of ATP, which impaired the demonstration of allosteric effects, Mg·ATP solutions were prepared in the respective buffer right before use.

Temperature dependence and thermal stability

The temperature dependence of the enzyme activity was measured between 25 °C and 95 °C in 50 mM potassium phosphate buffer, pH 7.0. The activity was measured in the direction of F-1, 6-P formation using standard concentrations of F-6-P (5 mM), ATP (2 mM) and $MgCl_2$ (10 mM), which ensured specific activities close to V_{max}. The thermostability of the purified enzyme (5 µg in 40 µl phosphate buffer, pH 7.0) as well as the effects of potential stabilizing additives [1 M NaCl, 1 M KCl, 1 M $(NH_4)_2SO_4$)] were tested in sealed vials which were incubated at temperatures between 70 °C and 100 °C for 2–120 min. The vials were then cooled on ice for 10 min and remaining enzyme activity was tested at 50 °C and compared to the controls (unheated sample).

Identification and cloning of the gene encoding ATP-PFK from *Thermotoga maritima*

Using the N-terminal amino acid sequence of the 34-kDa subunit, one ORF (TM0209) was identified by a Blast search (Altschul et al. 1990) in the complete sequenced genome of *Thermotoga maritima* (Nelson et al. 1999). This ORF has previously been annotated as putative (*pfkA*) gene in the *Thermotoga* genome. To prove its coding function, the ORF was cloned and functionally overexpressed in *E. coli* as follows: The coding region of the ATP-PFK was amplified by PCR with *Pwo* polymerase from genomic DNA of *Thermotoga maritima* as a template. Using the primers 5′AGGCTGATC*CATATG*AAGAAGATAGCAGT3′ (forward) and 5′CGATT*GGATCC*CGTT CATGAAAGCATGTG′ (reverse) restriction sites (italicized) for *Nde*I and *Bam*HI, respectively, were introduced. Following amplification and *Nde*I/*Bam*HI double-digestion the PCR product was inserted into a pET-17b expression vector linearized by *Nde*I/*Bam*HI double-digestion. The resulting plasmid pET-17b-*atp-pfk* was introduced into *E. coli* JM109 and BL21-CodonPlus(DE3)-RIL via transformation. The inserted gene sequence was confirmed on each strand by the method of Sanger et al. (1977).

Functional overexpression of the *atp-pfk*-gene in *E. coli* and purification of recombinant *T. maritima* ATP-PFK

Transformed *E. coli* BL21-CodonPlus(DE3)-RIL cells were grown in 400 ml of Luria-Bertani medium at 37 °C to an optical density at 600 nm of 0.8, and ATP-PFK expression was initiated by induction following the addition of 1 mM IPTG. After 4 h of further growth, the cells were harvested by centrifugation at 4 °C and washed in 50 mM Tris/HCl, pH 7.0, containing 50 mM NaCl. The pellet was frozen at –20 °C. Cell extracts were prepared by French-press treatment of cell suspensions in buffer E [50 mM NaCl, 50 mM Tris/HCl (pH 7.0, 70 °C)]. After ultracentrifugation (100 000×*g* for 60 min) the solution was heat-precipitated at 70 °C for 30 min and centrifuged again. Homogeneous enzyme preparation was achieved by extensive dialysis against buffer A and chromatography on HiLoad Q-Sepharose and UnoQ1 as described above for the purification of native enzyme.

Source of material

All commercially available chemicals used were of reagent grade and were obtained from Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland), or Sigma (Deisenhofen, Germany). Yeast extract and peptone were from Difco (Stuttgart, Germany). Enzymes and coenzymes, restriction enzymes, T4 DNA ligase and other chemicals were from Roche Diagnostics (Mannheim, Germany), New England Biolabs (Beverly, Massachusetts, USA), Sigma (Deisenhofen, Germany), and PEQLAB (Erlangen, Germany). ATP, ADP, fructose 6-phosphate, and PEP, each at highest available purity, were purchased from Roche Diagnostics (Mannheim, Germany). Gases were from Linde (Hamburg, Germany). *Thermotoga maritima* MSB 8 (DSM 3109) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). *E. coli* BL21-CodonPus(DE3)-RIL expression host strains and pET-17b protein expression vector were purchased from Stratagene (La Jolla, California, USA), and Novagen (Madison, Wis., USA) respectively. Plasmids were prepared and gel-purified using kits from Qiagen (Hilden, Germany) and PEQLAB. All FPLC material (HiLoad Q-Sepharose; ATP-Agarose Sigma A 9264; Uno S) and columns used were from Pharmacia (Freiburg, Germany), Sigma and Biorad (Munich, Germany).

Results

Purification of ATP-PFK from *Thermotoga maritima*

ATP-PFK was purified aerobically from cell extracts of *T. maritma* by only three purification steps involving anion-exchange chromatography on Q-Sepharose, affinity chromatography on ATP-agarose, and cation exchange chromatography on Uno S1. The most efficient purification step was affinity chromatography, resulting in a 420-fold purification. Using this entire procedure the enzyme was purified about 730-fold to a specific activity of 62 U/mg (at 50 °C) with a yield of 12%. (Table 1). The purified protein was electrophoretically homogeneous as judged by

Table 1 Purification of ATP-PFK from *Thermotoga maritima*. Enzyme activity was measured at 50 °C in the direction of fructose 1, 6-bisphosphate (F-1, 6-BP) formation in a continuous assay using aldolase, triosephosphate isomerase, and α-glycerolphosphate dehydrogenase, as described in Materials and methods. 1 U=2 µmol NADH oxidized/min

Purification step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Cell-free extract	800	68	0.085	100	1
Q-Sepharose	73.2	29	0.4	43	5
ATP-agarose	0.37	13	36.13	20	425
Uno S1	0.13	8	62	12	729

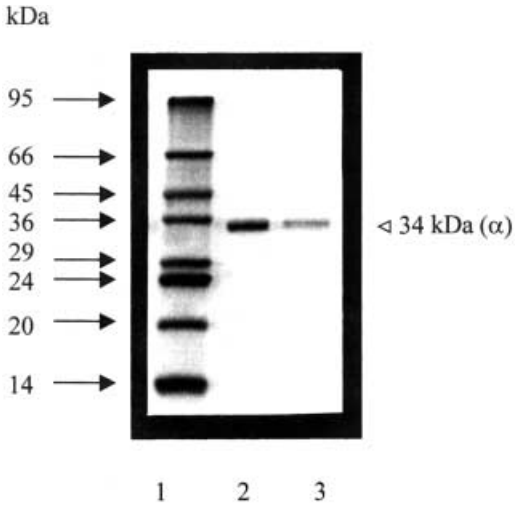


Fig. 1 Purified ATP-dependent phosphofructokinase (ATP-PFK) from *Thermotoga maritima* and recombinant PFK from transformed *Escherichia coli* as analyzed by SDS-PAGE (Laemmli 1970). Protein was denatured in SDS and separated in 14% slab gels (8 cm×7 cm), which were stained with Coomassie brilliant blue R 250. *Lane 1* Molecular mass standards in kDa, *lane 2* native enzyme purified from *T. maritima*, *lane 3* recombinant enzyme purified from *E. coli* (see Materials and methods)

denaturing SDS-PAGE (Fig. 1). Thus, ATP-PFK represents about 0.14% of the cellular protein of starch-grown *T. maritima.*

Molecular and catalytic properties

The apparent molecular mass of native ATP-PFK was determined by gel filtration on Superdex 200 and was approximately 140 kDa. SDS-PAGE revealed only one subunit with an apparent molecular mass of 34 kDa (Fig. 1), indicating a homotetrameric (α_4) structure of the native enzyme.

Kinetic constants of purified ATP-PFK were determined for both reaction directions (ATP + fructose 6 − phosphate ⟶ fructose 1, 6 − bisphosphate + ADP). The forward direction was measured both at 75 °C and 50 °C. The rate dependence at 75 °C on the F-6-P concentration followed sigmoidal kinetics (see Fig. 4) indicating a positive homotropic cooperativity of this substrate. The half maximal saturation constant ($S_{0.5}$) was about 0.7 mM, and a Hill coefficient of about 2.2 was calculated. The $S_{0.5}$ values increased at higher temperature (1 mM at 80 °C) and decreased at lower temperature (0.4 mM at 50 °C). Thus, at 50 °C the sigmoidal rate dependence reflecting allosteric cooperativity (Hill coefficient 1.6) was less pronounced. Rate dependence on ATP both at 75 and 50 °C followed Michaelis-Menten kinetics with an apparent K_m of about 0.2 mM for both temperatures. The corresponding apparent V_{max} values of the forward direction were about 360 U/mg at 75 °C and 70 U/mg at 50 °C, respectively.

The enzyme catalyzed in vitro the reverse reaction, i.e. ADP-dependent F-1, 6-BP conversion to F-6-P and ATP. This was demonstrated with the purified recombinant enzyme after heterologous expression in *E. coli* (see below). Rate dependence at 75 °C was hyperbolic for both substrates with the apparent K_m for F-1,6 -BP (7.6 mM) and for ADP (1.4 mM), respectively. The corresponding apparent V_{max} values were 13 U/mg. The high K_m for F-1, 6-BP as compared to F-6-P and the low V_{max} of F-6-P formation over F-1, 6-BP formation indicate that the enzyme catalyzes in vivo the phosphorylation of F-6-P to F-1, 6-BP during glucose catabolism rather than the reverse direction.

The pH optimum of ATP-PFK measured at 75 °C in the forward direction was at pH 6.7; 50% of activity was found at pH 5.7 and 80% at pH 7.7.

Substrate specificities

Various phosphoryl donors (other than ATP), sugars and cations were tested as substrates for ATP-PFK activity measured in the direction of F-1, 6-BP formation. ATP could not be replaced by PP_i or ADP. Besides ATP (100%, 70 U/mg at 50 °C), GTP (64%) and ITP (38%) served as effective phosphoryl donors for F-6-P phosphorylation. UTP (14%) and CTP (10%) were less effective, and ADP, UDP, GDP, pyrophosphate and acetyl phosphate were not used (<1%). The enzyme was specific for F-6-P as phosphoryl acceptor, while other sugars (glucose, glucose 6-phosphate, fructose, fructose 6-phosphate) were not phosphorylated. The enzyme required divalent cations for activity; Mg^{2+} (100%, 360 U/mg at 75 °C), which was most effective, could partially be replaced by Mn^{2+} (70%), Fe^{2+} (30%), and, less efficiently, by Co^{2+} (6%) and Ni^{2+} (4%).

Temperature optimum and stability

The temperature dependence of ATP-PFK is shown in Fig. 2A, B. At 40 °C the enzyme showed little activity, which, however, increased exponentially showing an optimum of 93 °C. Thus, ATP-PFK of the hyperthermophile *T. maritima* represents the most thermoactive of all ATP-PFKs described so far. From the linear part of the Arrhenius plot between 20 °C and 90 °C, an activation energy of 54 kJ/mol was calculated. The temperature stability of ATP-PFK was tested between 70 °C and 100 °C in 50 mM potassium phosphate buffer by incubating the enzyme up to 120 min, followed by measuring residual activity at 50 °C (Fig. 3). After 120 min incubation, the enzyme did not lose significant activity at 70 °C or 80 °C (<20%); at 95 and 100 °C, an almost complete loss of activity was observed after 60 min and 20 min, respectively. Addition of $(NH_4)_2SO_4$ (1 M) significantly stabilized ATP-PFK against heat inactivation at 100 °C; 60% residual activity was found after 120 min incubation time. NaCl or KCl, each 1 M, were without effect.

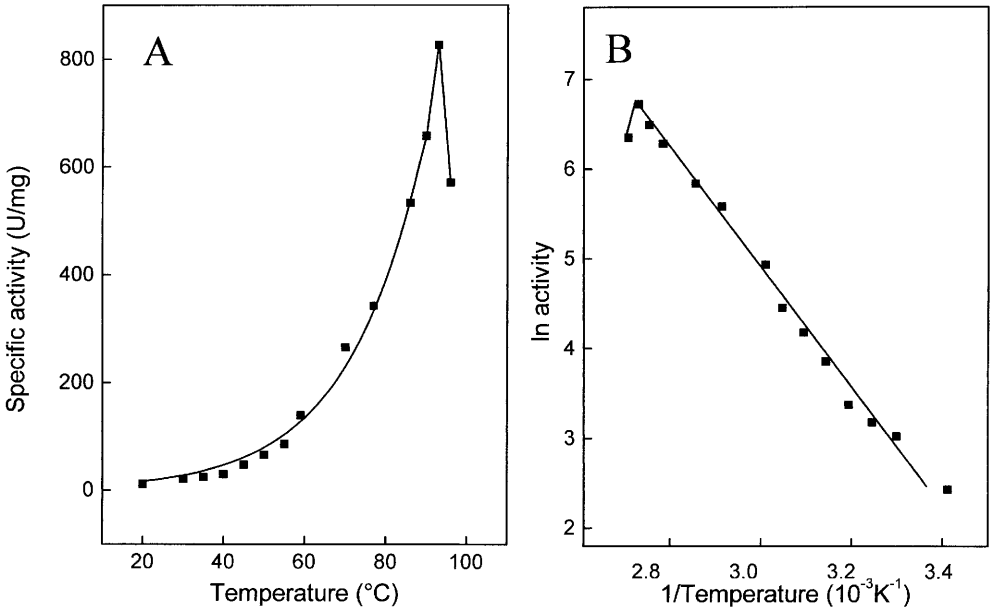


Fig. 2A, B Effect of temperature on the specific activity of the ATP-PFK from *T. maritima*. **A** Temperature dependence of the specific activity. **B** Arrhenius plot of the same data. The assay mixture contained 0.5 µg of enzyme, 100 mM triethanolamine, pH 7.0 (at the indicated temperature), 2 mM ATP, 10 mM $MgCl_2$ and 5 mM fructose-6-phosphate

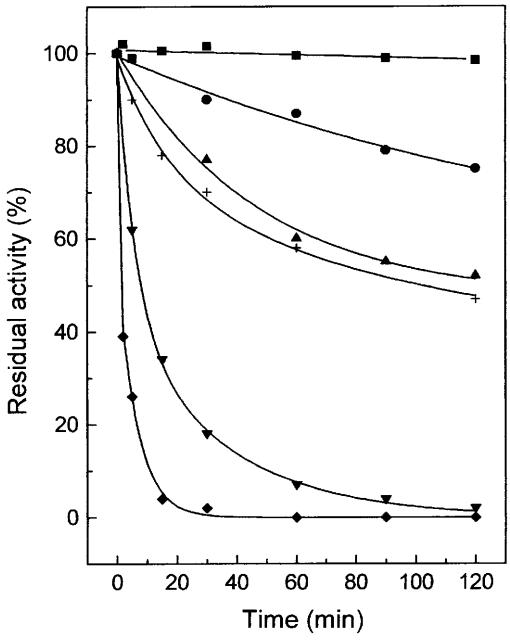


Fig. 3 Thermostability of ATP- PFK from *Thermotoga maritima*. Five µg of enzyme were incubated in 40 µl 50 mM potassium phosphate buffer, pH 7.0, between 70 °C and 100 °C: ■ 70 °C, l 80 °C, ▲ 90 °C, ▼ 95 °C, ◆ 100 °C; + 1 M ammonium sulfate added, 100 °C. At the times indicated, samples were cooled on ice for 10 min and assayed for remaining activity at 50 °C in the direction of fructose 1, 6-bisphosphate formation. 100% activity corresponded to specific activity of 70 U/mg

Effect of allosteric effectors on ATP-PFK activity

The effect of classical regulators for bacterial and eukaryal ATP-PFKs, such as PEP and ADP, was tested on ATP-PFK from *T. maritima.* Activity was assayed at 75 °C in the direction of F-6-P phosphorylation. The rate dependence of enzyme activity on increasing F-6-P concentrations in the presence of the effectors ADP and PEP is shown in Fig. 4A, B. ADP activates the enzyme; the addition of increasing concentrations of ADP (0.5 mM , 2.5 mM) resulted in a gradual change from sigmoidal to hyperbolic rate dependence paralleled by the decrease in $S_{0.5}$ for F-6-P from 0.7 to 0.35 mM. Thus, e.g. at a F-6-P concentration of 0.1 mM, enzyme activity increased up to three-fold in the presence 2.5 mM ADP. The V_{max} values were about 120% at 0.5 mM ADP; at 2.5 mM ADP, V_{max} was reduced to about 70%, probably due to product inhibition of the enzyme (Fig. 4B). Conversely, the addition of PEP (0.1 and 10 mM) resulted in an allosteric inhibition of enzyme activity (Fig. 4A, B) by increasing $S_{0.5}$ from 0.7 mM to 1.1 mM. V_{max} was reduced to 70% at 1 mM PEP and to 45% at 10 mM PEP. Inhibition by PEP was almost completely reversed by the addition of 2.5 mM ADP (not shown). Thus, both ADP and PEP exerted their classical effects towards hyperthermophilic ATP-PFK of *T. maritima.*

Cloning of the *pfk* gene encoding ATP-PFK from *T. maritima* and functional overexpression in *E. coli*

Based on the N-terminal amino acid sequence determined for the 34-kDa subunit, MRKIAVLTSGG DAGPMAAVRAVVRYGVRQGLEVIGVRR, a single ORF, TM0209, was identified in the genome of *T. maritma* which exactly matches the 38 N-terminal amino acid residues. The ORF, which was previously annotated as putative *atp-pfk* gene, encoding ATP-PFK (Nelson et al. 1999), contains 957 bp coding for a polypeptide of 319 amino acids with a calculated molecular mass of 34,487 Da. The coding function of putative *atp-pfk* gene was proved by its functional overexpression in *E. coli*. The gene was amplified by PCR, cloned into vector pET-17b and transformed into *E. coli* BL21-CodonPlus(DE3-RIL). After induction with IPTG, a polypeptide of 34 kDa was overexpressed show-

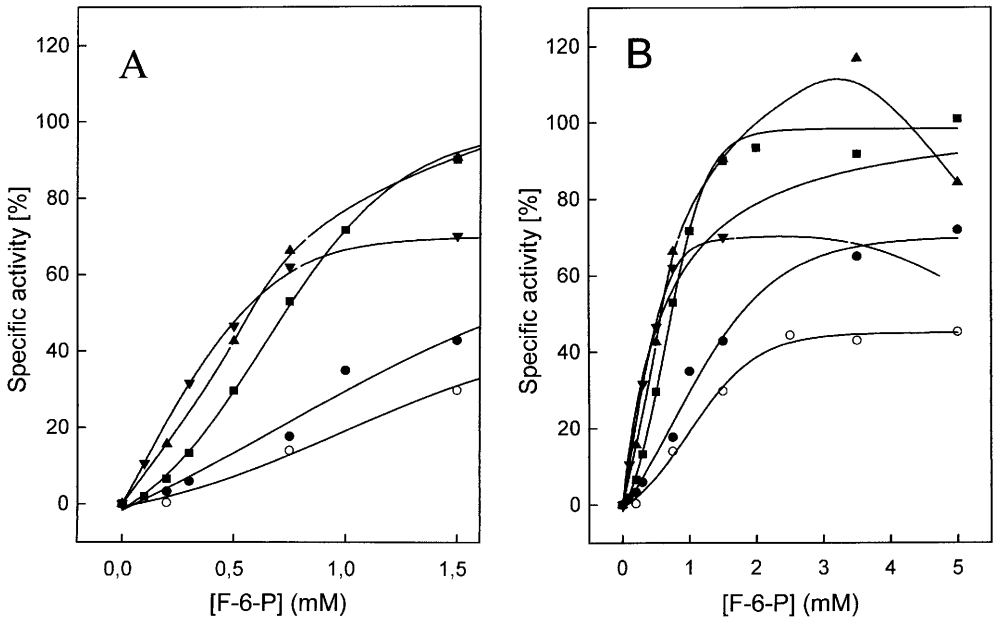


Fig. 4A, B Rate dependence of ATP-PFK from *T. maritima* on the fructose-6-phosphate (F-6-P) concentration at 75 °C in the presence and absence of effectors. Additives: ■ none, ▲ 0.5 mM ADP, ▼ 2.5 mM ADP, ● 1 mM PEP, ❍ 10 mM PEP. **A** 0–1.5 mM F-6-P, **B** 0–5 mM F-6-P. Enzyme activity was measured in a discontinuous assay system (see Material and methods). The assay mixture contained 0.15 µg enzyme, 100 mM triethanolamine, pH 7.0 at 75 °C, 2 mM ATP, 10 mM $MgCl_2$ and 0.1–5 mM F-6-P. 100% activity corresponded to specific activity of 360 U/mg

Table 2 Biochemical and kinetic properties of ATP-PFK from *T. maritima*. The molecular mass of native enzyme was determined by gel filtration, of subunits by SDS-PAGE. The apparent V_{max} values were determined at 75 °C (discontinuous assay) and 50 °C (continuous assay) in the direction of F-1, 6-BP formation [5 mM fructose 6-phosphate (F-6-P) and F-6-P formation [50 mM fructose 1, 6 bisphosphate (F-1, 6-BP)]. *nd* Not determined

Parameter	Substrate	Enzyme isolated from	
		Thermotoga maritima	*Escherichia coli* (recombinant)
Apparent molecular mass of native enzyme (kDa)		140	140
Subunit (kDa)		34	34
Calculated (kDa)		34.487	–
Oligomeric structure		α_4	α_4
pH optimum (75 °C)		6.7	6.7
Temperature optimum (°C)		93	93
Arrhenius activation energy (kJ mol^{-1}, 20 °C–90 °C)		54 kJ mol^{-1}	nd
Apparent $S_{0.5}$ (mM)	F-6-P (50 °C)	nd	0.4
	(75 °C)	0.72	0.7
	(80 °C)	1.0	nd
n (Hill coefficient)	F-6-P (75 °C)	2.2	2.0–2.2
Apparent K_m (mM)	ATP (75 °C)	0.2	0.24
	ADP (75 °C)	nd	1.4
	F-1, 6-BP (75 °C)	nd	7.6
V_{max} (U/mg, direction of F-6-BP formation) (75 °C)		nd	133
(50 °C)		nd	3
V_{max} (U/mg, direction of F-1,6-BP formation) (75 °C)		360	410
(50 °C)		65	80
Phosphoryl donor specificity[a] (%, 50 °C)	ATP>GTP, ITP>	100; 64; 38	100
	UTP>CTP	14; 10	nd
	ADP, GDP, UDP	0; 0; 0	0
	Acetyl phosphate,	0	0
	PP_i	0	0
Cation specificity[a] (%, 75 °C)	Mg^{2+}>Mn^{2+}>Fe^{2+}> Co^{2+}>Ni^{2+}	100; 70; 30 6; 4	nd

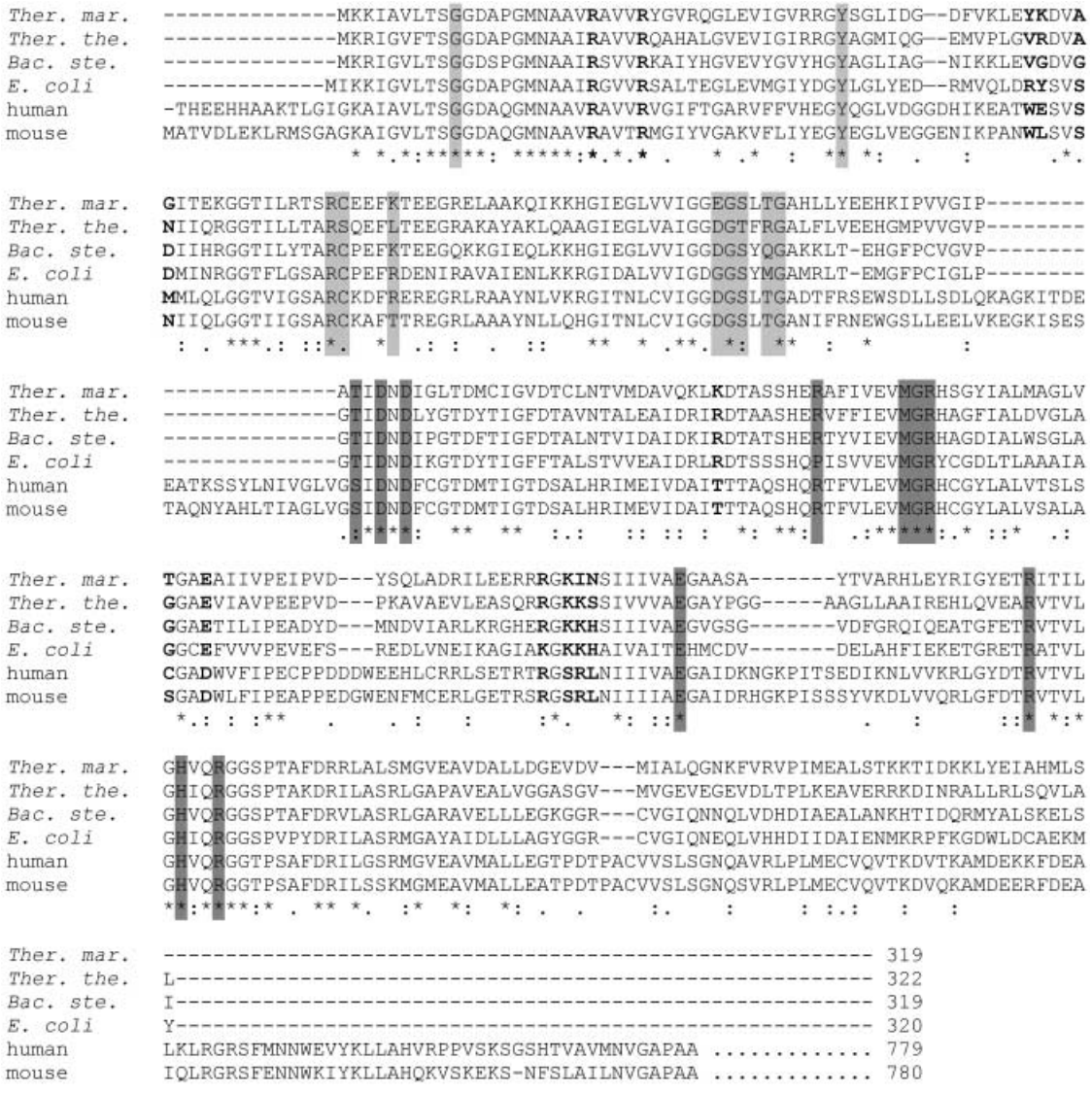
[a]Direction of F-1, 6-BP formation, % of V_{max}

ing thermoactive ATP-PFK activity. The recombinant ATP-PFK was purified from transformed *E. coli* about 20-fold by heat treatment, and chromatography on HiLoad Q sepharose and Uno Q1. About 50 mg purified protein were obtained from 25 g transformed *E. coli* cells (wet weight). As shown in Fig. 1B and Table 2, ATP-PFK purified from both *T. maritima* and transformed *E. coli* showed almost identical properties; which include, e.g. temperature optimum (93 °C) and thermostability, the kinetic constants for F-6-P, ADP, F-1, 6-BP and ATP and the allosteric regulation by ADP and PEP.

Discussion

In this communication, we describe the purification, characterization and heterologous overexpression of the encoding gene, of the ATP-dependent phosphofructokinase (ATP-PFK) from the hyperthermophilic bacterium *T. maritima*. The enzyme was characterized as an homotetramer of 34 kDa subunits, which is a common property of bacterial ATP-PFKs (Hofmann et al. 1999) and the archaeal ATP-PFK from *D. amylolyticus* (Hansen and Schönheit 2000) and *Aeropyrum pernix* (Hansen and Schönheit 2001). Most eukaryotic ATP PFKs are also homoteramers but contain larger subunits (see Fig. 5) (Poorman et al. 1984). With a temperature optimum at 93 °C, the *Thermotoga* ATP-PFK represents the most thermoactive of all

Fig. 5 Multiple sequence alignment of selected ATP-dependent phosphofructokinases of Bacteria and Eukarya (PFK-A family). Deduced amino acid sequences of *Thermotoga maritima (Ther. mar.)* (Nelson et al. 1999), *Thermus thermophilus (Ther. the.)* (Xu et al. 1991), *Bacillus stearothermophilus (Bac. ste.)* (French et al. 1987), *Escherichia coli (E. coli)* (Hellinga and Evans 1985), human (Sharma et al. 1989), mouse (Nakajima et al. 1994) were aligned. The sequences of the eukaryotic enzymes were truncated. Amino acids that form putative substrate-binding sites as well as effector-binding sites in accordance with the crystal structures of *B. stearothermophilus* and *E. coli* ATP-PFKs (Evans and Hudson 1979; Schirmer and Evans 1990; Shirakihara and Evans 1988) are shaded *gray* (substrate-binding for MgATP), shaded *dark gray* (substrate-binding for F-6-P), and marked *bold* (effector-binding site for ADP/PEP)



ATP-PFKs described so far, including those of the bacterial thermophilic *Thermus* species (Cass and Stellwagen 1975; Yoshida 1972) and of the archaea *D. amylolyticus* (Hansen and Schönheit 2000) and *A. pernix* (Hansen and Schönheit 2001).

The gene encoding ATP-PFK was functionally overexpressed in *E. coli*. The purified recombinant enzyme showed almost identical molecular, kinetic and regulatory properties as the native enzyme purified from *T. maritima*. The deduced amino acid sequence of *T. maritima* ATP-PFK exhibited a high degree of identity to most ATP-PFKs from bacterial and eukaryal species, which form the conserved PFK-A protein family (Hofmann et al. 1999; Poorman et al. 1984). A multiple sequence alignment of selected ATP-PFKs of the PFK-A family, from the bacteria *Thermus thermophilus*, *Bacilllus stearothermophilus*, and *E. coli* as well as from the eukaryotes human and mouse is given in Fig. 5. For the eukaryotic enzymes, which contain larger subunits, sequences are shown that have been truncated at the C-terminal extensions. Conserved amino acids, which represent substrate-bindings sites for F-6-P and MgADP and effector sites for PEP and ADP, as concluded from crystal structures of the *B. stearothermophilus* and *E. coli* ATP-PFK (Evans and Hudson 1979; Schirmer and Evans 1990; Shirakihara and Evans 1988), are marked (see legend to Fig. 5). Those amino acids representing binding sites in bacterial and eukaryal ATP-PFKs are also conserved in the *Thermotoga* enzyme. In particular, the glutamic acid E187 is conserved; this amino acid plays a crucial role in allosteric control by PEP binding in the ATP-PFKs from *E. coli* and *B. stearothermophilus* (Auzat et al. 1994; Evans and Hudson 1979; Schirmer and Evans 1990).

The presence of conserved substrate- and effector-binding sites in the *T. maritima* enzyme is in accordance with the observed classical allosteric nature of this hyperthermophilic enzyme. Rate dependence of the enzyme on F-6-P concentrations followed sigmoidal kinetics tested over a temperature range between 50 °C and 80 °C. The sigmoidal kinetics could best be demonstrated at higher temperatures due to higher $S_{0.5}$ values for F-6-P. Allosteric regulation has also been reported for thermophilic ATP-PFKs form *Thermus* species (*Thermus* X-1 (Cass and Stellwagen 1975) and *Thermus thermophilus* (strain HB8) (Yoshida 1972; Yoshida et al. 1971). In contrast to the *Thermotoga* enzyme, the ATP-PFK from *Thermus* X-1 showed higher $S_{0.5}$ values for F-6-P at lower temperatures (25 °C) as compared to 75 °C.

In summary, the extremely thermophilic bacterial ATP-PFK from *T. maritima* shows the classical allosteric behavior with ADP as positive or PEP as negative effectors, indicating that the allosteric properties of an ATP-PFK are not impaired by temperatures up to 100 °C. Therefore we conclude that ATP-PFK in the phylogenetic ancestral bacterium *Thermotoga* fulfills the same key regulatory function in the Embden-Meyerhof pathway as in other bacteria and in eukarya. In contrast, the extremely thermophilic archaeal ATP-PFK from *D. amylolyticus and A. pernix* are non-allosteric enzymes, suggesting that the modified Embden-Meyerhof pathways (see e.g. Selig et al. 1997) in these archaea are regulated at different sites.

During completion of this work, a paper by Ding et al. (2001) reported heterologous expression of putative *pfk* gene coding for ATP-PFK of *T. maritima*. The recombinant His-tagged protein was characterized and showed similar results with respect to molecular composition, and specificity for substrates, phosphoryl donors and cations as reported in this paper. In contrast to our data, these authors did not detect the classical allosteric behavior of ATP-PFK: they reported a hyperbolic rather than a sigmoidal rate dependence on fructose 6-phosphate and did not show significant response to the common allosteric regulators ADP and PEP. Since all kinetic measurements were made at 50 °C, the regulatory effects by ADP and PEP might have been overlooked, since the allosteric properties of the *Thermotoga* enzyme – according to our data – are less pronounced at this low, physiologically not relevant temperature.

Ding et al. (2001) also described the expression of a gene coding for a second, pyrophosphate (PP)-dependent, PFK in *T. maritima*. Both ATP-PFK and PP-PFK were reported to be expressed in vivo; however, specific activities of both enzymes in cell extracts were not given. In starch-grown cells of *T. maritima* we measured (at 50 °C, pH 6.7) up to 50-fold higher activities of ATP-PFK (150 mU) over PP-PFK (2 mU/mg), indicating that ATP-PFK is the relevant PFK in glucose degradation in *T. maritima*. In accordance, ATP-PFK activity was significantly higher (>50-fold) in starch-grown cells than in peptone- or gluconate-grown-cells of *T. maritma*, indicating sugar-specific induction of the enzyme (data not shown).

Acknowledgements The authors thank Dr. R. Schmid (Mikrobiologie, Universität Osnabrück) for N-terminal amino acid sequencing and H. Preidel for mass culturing *T. maritima*. The expert technical assistance of K. Lutter-Mohr and A. Brandenburger is also gratefully acknowledged. Some preliminary experiments on the purification and expression of ATP-PFK of *T. maritima* were carried by B. Reichstein and T. Maretzki during an advanced student course. This work was supported by grants of the Fonds der Chemischen Industrie.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Auzat I, Le Bras G, Branny P, De La Torre F, Theunissen B, Garel JR (1994) The role of Glu187 in the regulation of phosphofructokinase by phosphoenolpyruvate. J Mol Biol 235:68–72

Blangy D, Buc H, Monod J (1968) Kinetics of the allosteric interactions of phosphofructokinase from *Escherichia coli*. J Mol Biol 31:13–35

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Cass KH, Stellwagen E (1975) A thermostable phosphofructokinase from the extreme thermophile *Thermus* X-1. Arch Biochem Biophys 171:682–694

Ding YH, Ronimus RS, Morgan HW (2001) *Thermotoga maritima* phosphofructokinases: expression and characterization of two unique enzymes. J Bacteriol 183:791–794

Evans PR, Hudson PJ (1979) Structure and control of phosphofructokinase from *Bacillus stearothermophilus*. Nature 279:500–504

Faguy DM, Doolittle WF (1999) Lessons from the *Aeropyrum pernix* genome. Curr Biol 9:R883–R886

French BA, Valdez BC, Younathan ES, Chang SH (1987) High-level expression of *Bacillus stearothermophilus* 6-phosphofructo-1-kinase in *Escherichia co*li. Gene 59:279–283

Hansen T, Schönheit P (2000) Purification and properties of the first-identified, archaeal, ATP-dependent 6-phosphofructokinase, an extremely thermophilic non-allosteric enzyme, from the hyperthermophile *Desulfurococcus amylolyticus*. Arch Microbiol 173:103–109

Hansen T, Schönheit P (2001) Sequence, expression and characterization of the first archaeal ATP- dependent 6-phosphofructokinase, a non-allosteric enzyme related to the PFK-B sugar kinase family, from the hyperthermophilic crenarchaeote *Aeropyrum pernix*. Arch Microbiol 177:62–69

Hellinga HW, Evans PR (1985) Nucleotide sequence and high-level expression of the major *Escherichia coli* phosphofructokinase. Eur J Biochem 149:363–373

Hofmann K, Bucher P, Falquet L, Bairoch A (1999) The PROSITE database, its status in 1999. Nucleic Acids Res 27:215–219

Huber R, Langworthy TA, König H, Thomm M, Woese CR, Sleytr UB, Stetter KO (1986) *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90 °C. Arch Microbiol 144:324–333

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Meyer C, Schmid R, Scriba PC, Wehling M (1996) Purification and partial sequencing of high-affinity progesterone- binding site(s) from porcine liver membranes. Eur J Biochem 239:726–731

Nakajima H, Noguchi T, Hamaguchi T, Tomita K, Hanafusa T, Kono N, Tanaka T, Kuwajima M, Matsuzawa Y (1994) Expression of mouse phosphofructokinase-M gene alternative transcripts: evidence for the conserved two-promoter system. Biochem J 303:449–453

Nelson KE, Clayton RA, Gill SR, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Nelson WC, Ketchum KA, McDonald L, Utterback TR, Malek JA, Linher KD, Garrett MM, Stewart AM, Cotton MD, Pratt MS, Phillips CA, Richardson D, Heidelberg J, Sutton GG, Fleischmann RD, Eisen JA, Fraser CM (1999) Evidence for lateral gene transfer between Archaea and bacteria from genome sequence of *Thermotoga maritima*. Nature 399:323–329

Poorman RA, Randolph A, Kemp RG, Heinrikson RL (1984) Evolution of phosphofructokinase–gene duplication and creation of new effector sites. Nature 309:467–469

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Schaegger H, von Jagow G (1987) Tricine-sodium-dodecylsulfate-polyacrylamide gel electrophoresis for the seperations of proteins in the range from 1–100 kDa. Anal Biochem 166:368–379

Schirmer T, Evans PR (1990) Structural basis of the allosteric behaviour of phosphofructokinase. Nature 343:140–145

Schröder C, Selig M, Schönheit P (1994) Glucose fermentation to acetate, CO_2 and H_2 in the hyperthermophilic eubacterium *Thermotoga maritima*: involvement of the Embden-Meyerhof pathway. Arch Microbiol 161:460–470

Selig M, Xavier KB, Santos H, Schönheit P (1997) Comparative analysis of Embden-Meyerhof and Entner-Doudoroff glycolytic pathways in hyperthermophilic archaea and the bacterium *Thermotoga*. Arch Microbiol 167:217–232

Sharma PM, Reddy GR, Vora S, Babior BM, McLachlan A (1989) Cloning and expression of a human muscle phosphofructokinase cDNA. Gene 77:177–183

Shirakihara Y, Evans PR (1988) Crystal structure of the complex of phosphofructokinase from *Escherichia coli* with its reaction products. J Mol Biol 204:973–994

Uyeda K (1979) Phosphofructokinases. Adv Enzymol 48:193–244

Woese CR (1987) Bacterial evolution. Bacteriol Rev 51:221–271

Xu J, Seki M, Denda K, Yoshida M (1991) Molecular cloning of phosphofructokinase 1 gene from a thermophilic bacterium, *Thermus thermophilus*. Biochem Biophys Res Commun 176: 1313–1318

Yoshida M (1972) Allosteric nature of thermostable phosphofructokinase from an extreme thermophilic bacterium. Biochemistry 11:1087–1093

Yoshida M, Oshima T, Imahori K (1971) The thermostable allosteric enzyme: phosphofructokinase from an extreme thermophile. Biochem Biophys Res Commun 43:36–39