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ORIGINAL PAPER

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Sequence, expression, and characterization of the first archaeal ATP-dependent 6-phosphofructokinase, a non-allosteric enzyme related to the phosphofructokinase-B sugar kinase family, from the hyperthermophilic crenarchaeote *Aeropyrum pernix*

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Abstract The gene (ORF APF0012) encoding the ATP-dependent 6-phosphofructokinase (ATP-PFK) of the hyperthermophilic archaeon *Aeropyrum pernix* was identified, cloned, and functionally expressed in *Escherichia coli*. The deduced amino acid sequence showed similarity (25–40%) to members of PFK-B sugar kinases. The purified recombinant enzyme is a heterotetramer of 115 kDa, composed of 34-kDa subunits. Rate dependence (at 85 °C) on both fructose 6-phosphate (F-6-P) and ATP followed Michaelis-Menten kinetics with apparent K_m values of 0.25 mM and 0.68 mM, respectively; apparent V_{max} values were about 5 U/mg. The enzyme was specific for ATP as phosphoryl donor, but showed a broader spectrum of phosphoryl acceptors: in addition to F-6-P, glucose 6-phosphate, adenosine, fructose, ribose 5-phosphate, and ribose were accepted. Enzyme activity required divalent cations; Mg^{2+}, which was most effective, could partially be replaced by Co^{2+}, Ni^{2+}, or Mn^{2+}. The enzyme had a temperature optimum of 90 °C and showed a significant thermostability up to 100 °C. ATP-PFK activity was not allosterically regulated by classical effectors of ATP-PFKs of eukarya and bacteria, such as ADP and phosphoenolpyruvate. In accordance, this archaeal ATP-PFK did not contain the typical conserved binding sites for these effectors. This is the first report of a sequence of an archaeal ATP-PFK related to the PFK-B sugar kinase family.

Keywords Hyperthermophilic archaea · *Aeropyrum pernix* · ATP-dependent 6-phosphofructokinase · Non-allosteric · Sugar kinase · PFK-B family · PFK-A family

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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. In bacteria and eukarya, ATP-PFKs represent key regulatory enzymes of sugar degradation via the classical Embden-Meyerhof pathway. ATP-PFKs have been characterized from various bacterial and eukaryal species. A general feature 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, allosterically activated by ADP, and inhibited by phosphoenolpyruvate (Blangy et al. 1968). ATP-PFKs from eukaryotes contain larger subunits and are allosterically regulated by a larger number of effectors including citrate and fructose 2,6-bisphosphate (F-2,6-BP) [for a review see Uyeda (1979)]. In accordance with their allosteric behaviour, conserved allosteric effector binding sites have been identified in the deduced amino acid sequences of bacterial and eukaryal ATP-PFKs.

Recently, we purified and characterized the first ATP-PFK from the domain of Archaea, from the hyperthermophilic crenarchaeote *Desulfurococcus amylolyticus* (Hansen and Schönheit 2000). The extremely thermophilic enzyme consists of a homotetramer composed of 34-kDa subunits. In contrast to most ATP-PFKs from the domains of Bacteria and Eukarya, the archaeal enzyme was not allosterically regulated, either by classical effectors of ATP-PFKs or by a variety of other compounds of intermediary metabolism. Furthermore, the N-terminal amino acid sequence (29 amino acids) did not show significant similarity to known PFKs from bacteria and eukarya (Hansen and Schönheit 2000). However, when the complete genome of the crenarchaeote *Aeropyrum pernix* was released (Kawarabayasi et al. 1999), significant sequence similarity to the ORF APE0012 was found. This ORF was annotated as the putative sugar kinase of the PFK-B family

(Hofmann et al. 1999) which includes the minor PFK (PFK-B or PFK2) of *Escherichia coli* (Daldal 1983). *A. pernix* is an aerobic hyperthermophilic creanarchaeote (temperature optimum 90 °C) that is phylogenetically closely related to the anaerobic *D. amylolyticus* and, more distantly, to the aerobic *Sulfolobus* sp. (Sako et al. 1996). Recently, we have shown that *A. pernix* cells grown on starch and peptone contained ATP-PFK activity as part of a sugar degradation pathway (Reichstein and Schönheit, unpublished results).

In this communication we report the cloning and heterologous expression of ORF APE0012 from *A. pernix*. The ORF was shown to code for an extremely thermophilic ATP-PFK, which was not allosterically regulated by ADP and phosphoenolpyruvate and showed substrate specificities similar to PFK-B sugar kinases. This is the first report on the sequence of an archaeal ATP-PFK showing similarity to the PFK-B sugar kinase family.

Materials and methods

Identification of hypothetical ORFs encoding ATP PFKs in archaea

BLASTP searches were carried out using the N-terminal amino acid sequence of the ATP-PFK from *Desulfurococcus amylolyticus* (Hansen and Schönheit 2000). Following the identification and verification of APE0012 as an ATP-PFK, the deduced amino acid sequences of APE0012 were used for BLASTP, PSI-BLAST, Gapped BLAST, and TBLASTN (Altschul et al. 1990, 1997) searches to identify additional putative archaeal members of the PFK-B family. Additional sequences of the PFK-B family were retrieved from the PROSITE database (Hofmann et al. 1999) and the Pfam database (Bateman et al. 2000). Sequence alignments were constructed using the neighbour-joining method of Clustal W (Thompson et al. 1994).

Cloning and expression of ORF APE0012 encoding ATP-PFK activity

The ORF APE0012 was cloned and functionally overexpressed in *Escherichia coli* as follows: The coding regions of the genes were amplified from genomic DNA from *A. pernix* K1 by PCR with *Pwo* polymerase. The following primers were used: 5′GATTAGGTACATATGCTAGAGCATCTGGT3′ (forward APE0012), 5′ CTGACGGTAAAGCTTACTAAAATCTCCA-C3′ (reverse APE0012). For the addition of 5′ T overhangs, the PCR product designated *atppfkap* was incubated with *Taq* polymerase for 5 min at 72 °C and then cloned into pBAD via a linearized vector activated with topoisomerase I. The vector pBAD-*atppfkap* was introduced into *E. coli* LMG 194 cells via transformation. The inserted gene sequence as well as orientation was confirmed by sequencing each strand following the method of Sanger et al. (1977). Transformed *E. coli* cells were grown in 400 ml RM medium containing 0.2% glucose at 30 °C to an optical density at 600 nm of 1.0. *A. pernix* ATP-PFK expression was initiated by the addition of 0.1% L-arabinose.

Purification of recombinant *A. pernix* ATP-PFK

Cells were harvested by centrifugation at 4 °C at the late exponential growth phase (OD_{600}~2.8). Cell extracts were prepared by French press treatment (130 MPa) of the cell suspension in buffer A (50 mM NaCl, 50 mM sodium phosphate, pH 7.0). *A. pernix* ATP-PFK was purified from recombinant *E. coli* as follows: After ultracentrifugation of cell extracts (100,000×*g* for 60 min) the solution was heat precipitated at 75 °C for 45 min and centrifuged again. The following chromatographic steps were performed at 4 °C. The supernatant was diluted four-fold with buffer B (50 mM Tris/HCl, pH 7.5) and applied to a Bio-Scale Q5 column (5 ml) previously equilibrated with buffer B. Protein was eluted at a flow rate of 1 ml/min with linear NaCl gradients from 0 M NaCl to 2 M NaCl in buffer B: 0–0.5 M NaCl (50 ml), 0.5–2 M NaCl (25 ml). Fractions containing the highest PFK activity (15 ml, 0.1–0.25 M) were pooled, heat precipitated at 85 °C for 30 min, and centrifuged (5,000×*g* for 15 min). Following the addition of DNase I (0.1 mg) and $MgCl_2$ (final concentration 10 mM) the supernatant was concentrated by ultrafiltration (YM 30 membrane, Millipore, Eschborn). NaCl was added to a final of concentration of 1 M before heat precipitation at 95 °C for 15 min. After centrifugation (5,000×*g* for 15 min), protein was applied to a Superdex 200 HiLoad gel filtration column equilibrated with 150 mM NaCl in buffer B. Protein was eluted at a flow rate of 1 ml/min. The eluate was stored at 4 °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 (1976) with bovine serum albumin (BSA) as standard. Gel-filtration chromatography was carried out at ambient temperature on a Superdex 200 column (50 mM Tris/HCl, 150 mM NaCl, pH 7.0, 1 ml/min).

Enzyme assays and determination of kinetic parameters

Since the enzyme activity was not sensitive to oxygen, all assays were performed under oxic conditions. The ATP-dependent PFK activity (F-6-P+ATP→F-1,6-BP+ADP) was determined in the direction of F-1,6-BP formation. The 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 (TIM), and glycerol-3-phosphate dehydrogenase. ATP-PFK activity was measured at 50 °C in a continuous assay and above 50 °C in a discontinuous assay as previously described (Hansen and Schönheit 2000), except that the standard assay mixtures contained 50 mM sodium phosphate (pH 6.7 at the respective temperature) and 2 mM F-6-P, 2 mM ATP, and $MgCl_2$:ATP at an optimized ratio of 4:1. Initial velocities were investigated in parallel assays stopped at different time intervals. One unit of ATP-PFK activity is defined as the conversion of 1 µmol F-6-P to F-1,6-BP. 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 85 °C using either piperazine, phosphate, MES, Tris/HCl, triethanolamine, or ethanolamine at a concentration of 50 mM each. The cation and nucleotide specificities were examined using the standard discontinuous test system at 85 °C by exchanging either Mg^{2+} (4 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 adenosine, fructose, fructose 1-phosphate, glucose 6-phosphate, ribose, ribose 5-phosphate. The following classical effectors of ATP-PFKs from bacterial and eukaryal sources were tested using the following concentrations: phosphoenolpyruvate (1 mM, 10 mM), ADP (0.5 mM, 10 mM), AMP (1, 10 mM), and citrate (1, 10 mM).

Temperature dependence and thermal stability

The temperature dependence of the enzyme activity was measured between 40 °C and 100 °C in 50 mM sodium phosphate buffer, pH 6.7. The activity was measured in the direction of F-1,6-P formation using standard concentrations of F-6-P (2 mM), ATP (2 mM) and $MgCl_2$ (8 mM), which ensured specific activities close to the V_{max}. The thermostability of the purified enzyme (15 µg in 100 µl phosphate buffer, pH 7.0) as well as potential stabilizing additives (1 M NaCl, 1 M KCl and 1 M ammonium sulfate) were tested in sealed vials that were incubated at temperatures between 80 °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 85 °C and compared to the controls (unheated sample).

Source of material

All commercially available chemicals used were of reagent grade and obtained from Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland), or Sigma (Deisenhofen, Germany). Yeast extract and peptone were from Difco (Stuttgart, Germany); enzymes, T4 DNA ligase and coenzymes were from Roche Diagnostics (Mannheim, Germany), New England Biolabs (Beverly, Mass., USA), and PEQLAB (Erlangen, Germany). Substrates were from Roche Diagnostics (ATP, ADP, F-6-P), Merck (fructose), and Sigma (F-1-P, ribose, ribose 5-phosphate, adenosine). Gases were from Linde (Hamburg, Germany). *A. pernix* K1 (DSM 11879) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Linearized vector activated with topoisomerase I, pET-17b protein expression vector, and *E. coli* LMG194 expression host strain were purchased from Invitrogen (Groningen, Netherlands). Genomic DNA, plasmid DNA, and DNA in gels were purified using kits from Qiagen (Hilden, Germany) and PEQLAB. All FPLC material and columns (Bio-Scale Q5 Superdex 200 HiLoad gel filtration column) used were from Pharmacia (Freiburg, Germany), Sigma and Biorad (Munich, Germany).

Results

Identification, cloning and functional overexpression of *A. pernix* ATP-PFK

Based on the N-terminal amino acid sequence determined for the 34-kDa subunit of the *D. amylolyticus* ATP-PFK, (MNKSIDVVTVGHALVDIRIVVNEFP) (Hansen and Schönheit 2000), the ORF APE0012 (42% identity based on the N-termini) was identified by a Blast search (Altschul et al. 1990) in the completely sequenced genome of *A. pernix* (Kawarabayasi et al. 1999). This ORF, which might encode an ATP-PFK, has previously been annotated as a putative sugar kinase of the PFK-B family of carbohydrate kinases. The ORF (AEP0012) starts with ATG, stops with TAG and contains 956 bp coding for a polypeptide of 310 amino acids with a calculated molecular mass of 33,392 Da. AFP0012 was characterized as gene *atppfk*, encoding ATP-PFK *from A. pernix*, by its functional overexpression in *E. coli* as follows: ORF AFP0012 was amplified by PCR and cloned into vector pBAD. The resulting plasmid, designated pBAD-*atppfkap*, was introduced into *E. coli* LMG 194 via transformation. After induction with 0.1% L-arabinose, a polypeptide of 34 kDa was overexpressed that showed thermoactive ATP-PFK activity. The recombinant ATP-PFK was purified about 200-fold from *E. coli* transformants by heat treatments and chromatography on Bio-Scale Q5 and gel filtration.

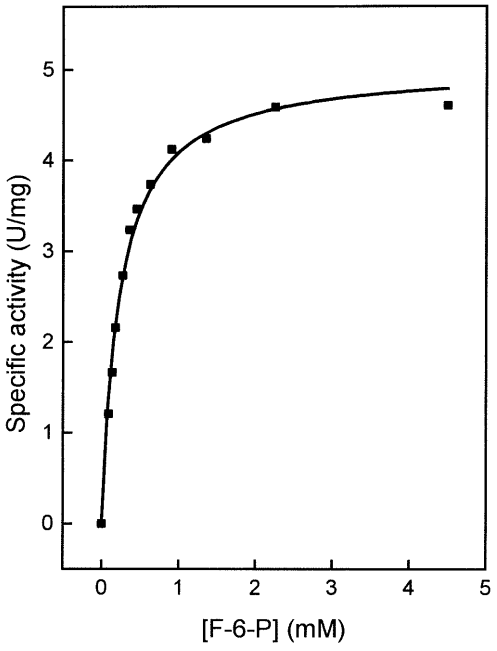

Fig. 1 Rate dependence of ATP-dependent phosphofructokinase (PFK) from *Aeropyrum pernix* on the fructose 6-phosphate (F-6-P) concentration at 85 °C. *Insert* Double reciprocal plot of the rates vs the corresponding substrate concentrations. Enzyme activity was measured in a discontinuous assay system (see Materials and methods). The assay mixture contained 10 µg of enzyme, 50 mM sodium phosphate, pH 6.7 at 85 °C, 2 mM ATP, 8 mM $MgCl_2$ and 0.2–5 mM fructose 6-phosphate

Molecular and catalytic properties of recombinant *A. pernix* ATP-PFK

The purified recombinant ATP-PFK was electrophoretically homogeneous as judged by denaturing SDS-PAGE, showing one subunit with an apparent molecular mass of 34 kDa. The apparent molecular mass of native ATP-PFK, determined by gel filtration, was approximately 115 kDa, indicating a homotetrameric (α_4) structure.

Rate dependence (at 85 °C) on both F-6-P (Fig. 1) and ATP followed Michaelis-Menten kinetics, with apparent K_m values of 0.25 mM and 0.68 mM, respectively; apparent V_{max} values were about 5 U/mg. ATP could not be replaced by ADP or PP_i, defining this archaeal PFK as an ATP-dependent enzyme (Table 1). Besides ATP (100%, 5 U/mg at 85 °C), GTP (76%) and ITP (76%) served as effective phosphoryl donors. The enzyme accepted several phosphoryl acceptors in addition to F-6-P, such as glucose 6-phosphate, adenosine, fructose, ribose 5-phosphate, and ribose; apparent K_m and V_{max} values are given in Table 1. Enzyme activity required divalent cations; Mg^{2+} (100%, 5 U/mg at 85 °C), which was the most effective, could partially be replaced by Co^{2+} (46%), Ni^{2+} (15%), or Mn^{2+}

Table 1 Biochemical and kinetic properties of ATP-dependent 6-phosphofructokinase from *Aeropyrum pernix*. Molecular mass of native enzyme was determined by gel filtration, molecular mass of subunits by SDS-PAGE. The apparent V_{max} values and K_m values were determined at 85 °C (discontinuous assay) in the direction of fructose 1,6-bisphosphate formation (5 mM fructose 6-phosphate)

Parameter		Value
Apparent molecular mass (kDa)	Native enzyme	115
	Subunit	34
	Calculated	33.392
Oligomeric structure		α_4
pH optimum		6.7
T_{Opt} (°C)		90
Arrhenius activation energy (kJ/mol, 40–90 °C)		57
Apparent K_m (mM)	F-6-P	0.25
	ATP	0.68
Apparent V_{max} (U/mg)		5
Phosphoryl donor specificity (% of V_{max})	ATP>GTP>ITP	100, 76; 76
	ADP, PP_i, acetyl phosphate	0; 0; 0
Cation specificity (% of V_{max})	Mg^{2+}>Co^{2+}>Ni^{2+}>	100; 46; 15
	Mn^{2+}>Ca^{2+}>Cu^{2+}	9; 5; 4
Substrate specificity (85 °C)		
Apparent K_m (mM)	Glucose 6-phosphate	0.6
	Adenosine	1
	Fructose	1
	Ribose 5-phosphate	3
	Ribose	5
Apparent V_{max} (U/mg)	Glucose 6-phosphate	1.0
	Adenosine	1.1
	Fructose	1.0
	Ribose 5-phosphate	2.5
	Ribose	1.4
K_i (M)	$(NH_4)_2SO_4$	0.05
	KCl	0.1
	NaCl	1

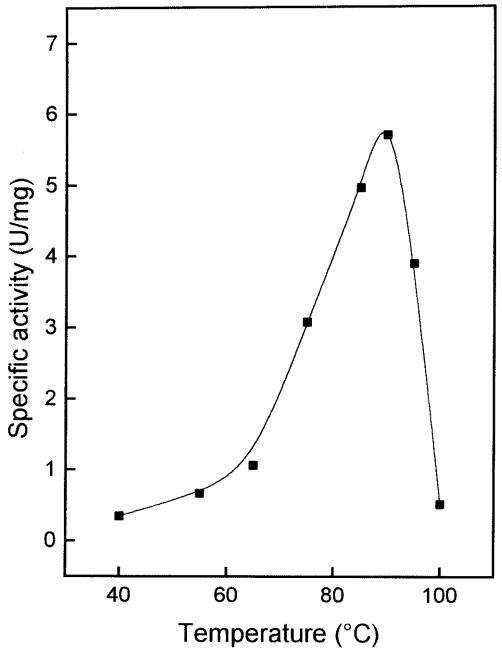

Fig. 2 Temperature dependence of the specific activity of the ATP-dependent PFK from *A. pernix*. The assay mixture contained 10 µg of enzyme, 50 mM sodium phosphate, pH 7, 2 mM ATP, 8 mM $MgCl_2$ and 2 mM fructose 6-phosphate

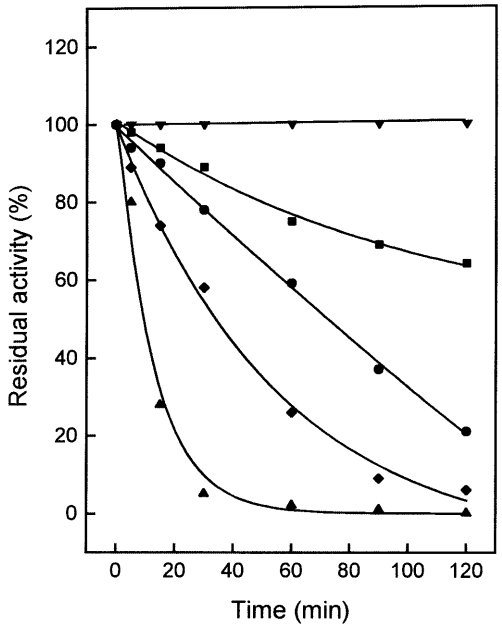

Fig. 3 Thermostability of ATP-dependent PFK from *A. pernix*. Ten µg of enzyme were incubated in 100 µl 50 mM potassium phosphate buffer, pH 7.0, between 80 °C and 100 °C: ▼ 80 °C, ■ 85 °C, ● 90 °C, ◆ 95 °C, ▲ 100 °C. At the times indicated, samples were cooled on ice for 10 min and assayed for residual activity at 85 °C; 100% activity corresponded to a specific activity of 5 U/mg

A

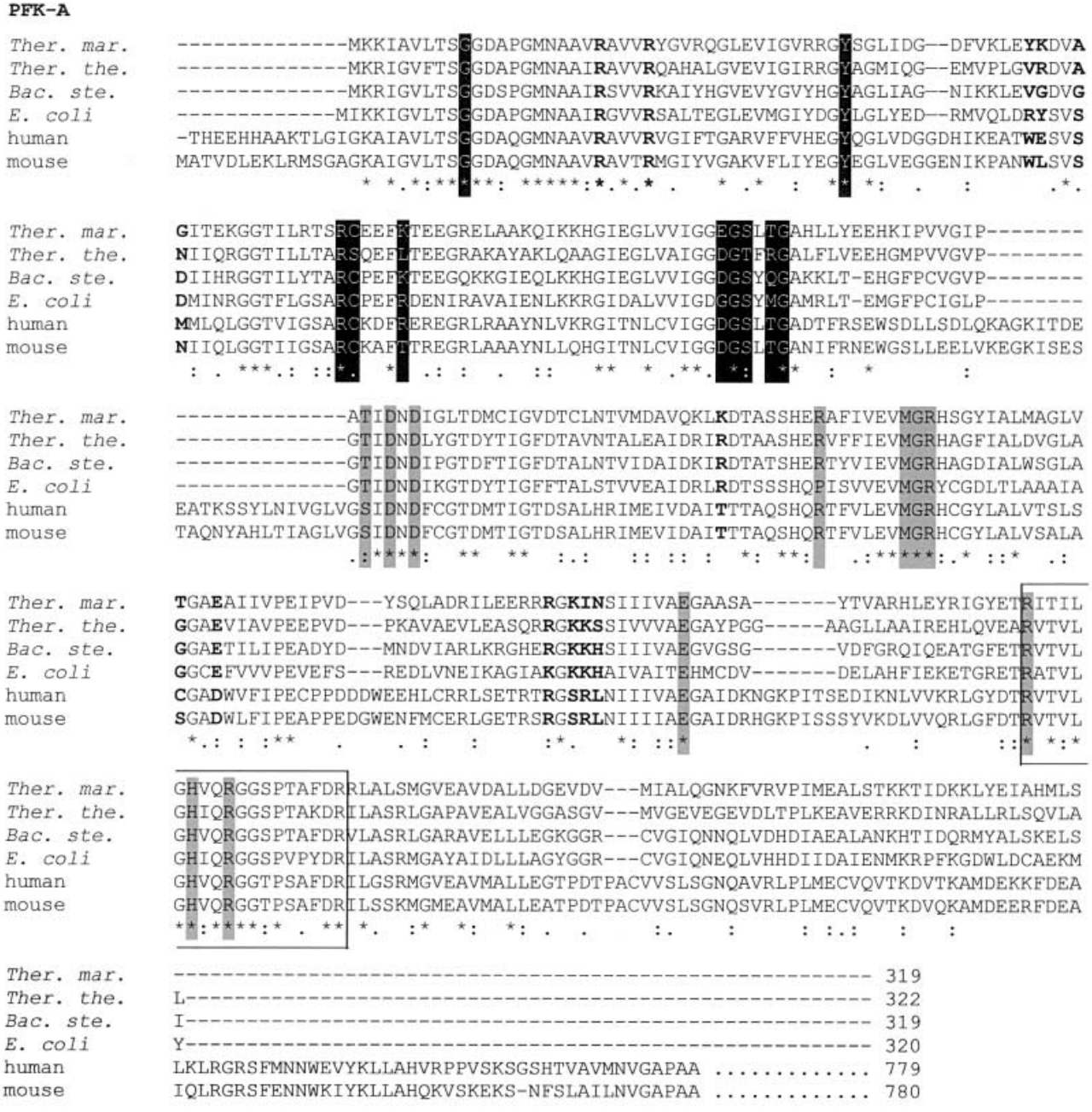

Fig. 4 A,B Multiple amino acid sequence alignments of selected PFKs of the PFK-A and PFK-B family. **A** ATP-dependent PFKs of the PFK-A family: *Ther mar*, *Thermotoga maritima* (Nelson et al. 1999; Hansen and Schönheit, submitted); *Ther the*, *Thermus thermophilus* (Xu et al. 1991); *Bac ste*, *Bacillus stearothermophilus* (French et al. 1987); *E. coli*, *Escherichia coli* (Hellinga and Evans 1985); human (Sharma et al. 1989); and mouse (Nakajima et al. 1994) were aligned. The sequences of the eukaryotic enzymes were truncated. Amino acids that form putative substrate-binding as well as effector-binding sites in accordance with the crystal structures of *B. stearothermophilus* and *E. coli* PFKs (Schirmer and Evans 1990) are shaded *black* (substrate binding for MgATP, site A), shaded *dark gray* (substrate binding for F-6-P, site B), and marked *bold* (effector binding site for ADP/PEP, site C). The consensus pattern [RK]-x(4)-G-H-x-Q-[QR]-G-G-x(5)-D-R is highlighted by a *box*. **B** Multiple sequence alignment of selected proteins of the PFK-B family of sugar kinases. Deduced amino acid sequences: ATP-PFK *Ape*, ATP-PFK *Aeropyrum pernix* (this work); ATP-PFK/SK *Mj*, ATP-PFK/sugar kinase *Methanococcus jannaschii* (Bult et al. 1996; Hansen and Schönheit unpublished); SCRK tomato, fructokinase tomato (Kanayama et al. 1997); RBSK, *E. coli*, ribokinase (Hope et al. 1986); KDGK *Ha ali*, 2-dehydro 3-deoxyglucokinase *Haloferax alicantei* (Holmes and Dyall-Smith 2000); adenosine kinase (ADK) human (Spychala et al. 1996); PFK-B *E. coli* (Daldal 1984). The two signature patterns [AG]-G-X(0,1)-[GAP]-X-N-X-[STA]-X(6)-[GS]-X(9)-G and [DNSK]-[PSTV]-X-[STAG](2)-[GD]-D-X(3)-[SAGV]-[AG]-[LIVMFY]-[LIVMSTAP] are highlighted by *boxes*. Deviations from the signature pattern are shaded *gray*. Conserved AGD motif, given in *bold letters*, is suggested to form an anion hole important for phosphoryl transfer (Schumacher et al. 2000)

B

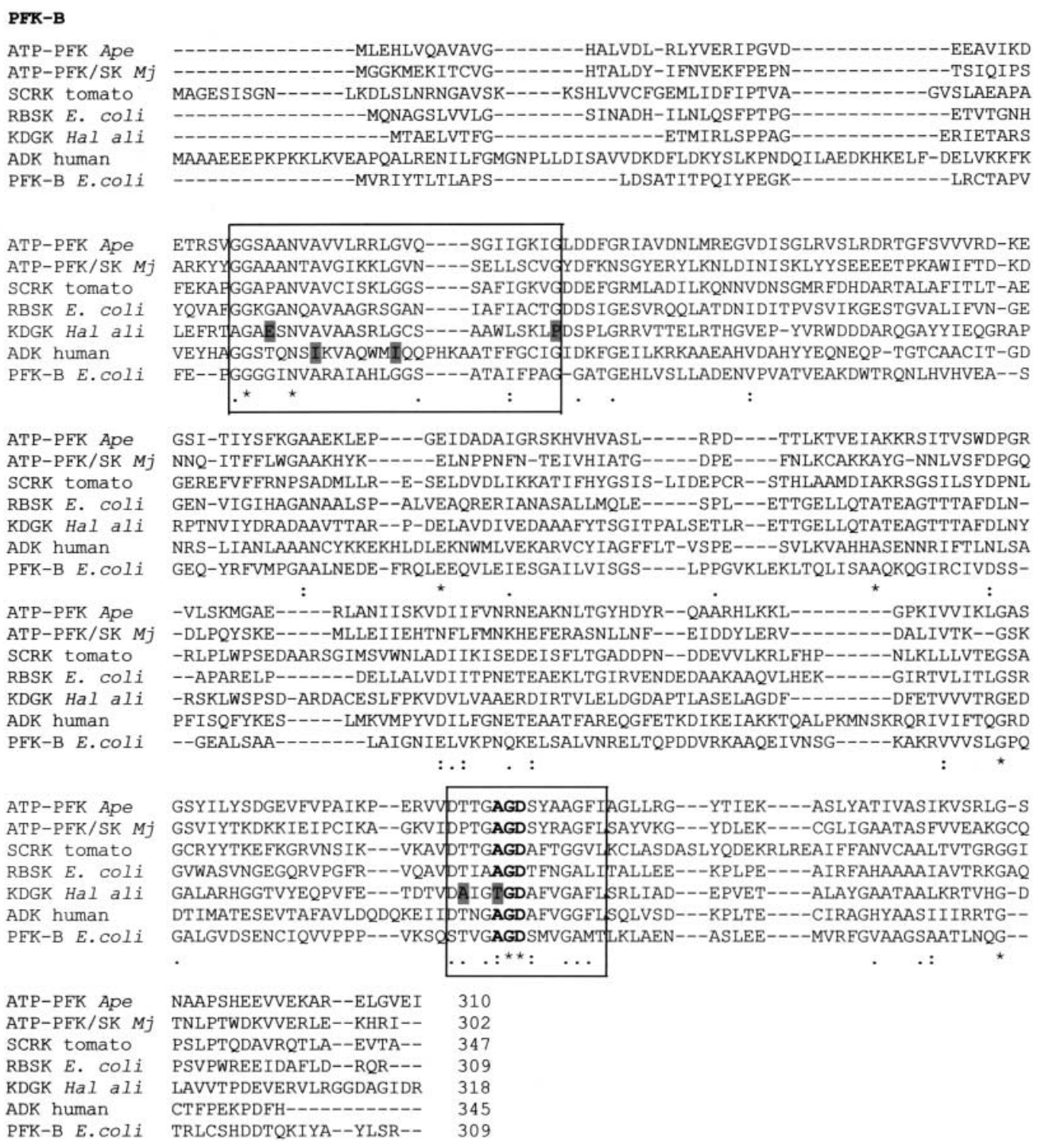

Fig. 4 B

(9%). The pH optimum of ATP-PFK was at pH 6.7; 60% of activity was found at pH 5.0 and at pH 8.5. ATP-PFK activity was sensitive to salts: activity was reduced by 50% (K_i values) in the presence of 50 mM ammonium sulfate, 100 mM KCl, and 1 M NaCl.

Effect of allosteric effectors on ATP-PFK activity

We tested the classical effectors of bacterial and eukaryal ATP-PFKs of the PFK-A family, such as ADP and phosphoenolpyruvate. Activity was assayed at 85 °C in the di-

rection of F-6-P phosphorylation at an F-6-P concentration near the apparent K_m and close to V_{max} conditions. Under these and all other conditions tested, no specific effect of any regulator could be detected. Furthermore, we could not detect an inhibitory effect of ATP at high concentrations (1–10 mM tested) as described for the *E. coli* PFK-B (Kotlarz and Buc 1981; Guixe and Babul 1985).

Temperature optimum and stability

The temperature dependence of ATP-PFK is shown in Fig. 2. At 40 °C the enzyme showed little activity; however, activity increased exponentially and showed an optimum at 90 °C. From the linear part of the Arrhenius plot, between 40 °C and 90 °C, an activation energy of 57 kJ/mol was calculated. The temperature stability of ATP-PFK was tested between 80 °C and 100 °C in 50 mM sodium phosphate, pH 7, by incubating the enzyme up to 120 min, followed by measuring residual activity at 50 °C (Fig. 3). After a 120-min incubation, the enzyme did not lose activity at 80 °C. At 95 and 100 °C, an almost complete loss of activity was observed after 120 min and 30 min, respectively. Addition of KCl and, to a lesser extent, NaCl (each 1 M) effectively stabilized ATP-PFK against heat inactivation at 100 °C; almost 90% residual activity was retained after 30 min in the presence of KCl and 55% in the presence of NaCl (not shown).

ATP-PFK activity in *A. pernix*

In cell extracts of *A. pernix* an ATP-PFK activity of about 2 mU/mg was measured at 80 °C. From gel filtration experiments with cell extracts an apparent molecular mass of about 115 kDa was calculated., which is identical to the value obtained for the purified recombinant ATP-PFK.

Discussion

In this communication we report the identification, expression and characterization of a gene coding for an archaeal ATP-PFK from the crenarchaeote *A. pernix*. The recombinant ATP-PFK had a native molecular mass of 115 kDa and was composed of a single 34 kDa subunit, indicating a homotetrameric structure. Both subunit size and oligomeric structure are typical properties of most ATP-PFKs from bacteria (PFK-A family) (Morgan and Ronimus 1998; Uyeda 1979), as well as of the first identified archaeal ATP-PFK from *Desulfurococcus amylolyticus* (Hansen and Schönheit 2000). *A. pernix* ATP-PFK showed a high temperature optimum and a pronounced thermostability up to 100 °C, which is in accordance with its function under the hyperthermophilic growth conditions of *A. pernix*. Like the ATP-PFK from the archaeon *D. amylolyticus* and the PFK-B from *E. coli* (Kotlarz and Buc 1981), the *A. pernix* ATP-PFK was not allosterically regulated by classical effectors of ATP-PFKs; e.g. enzyme activity was neither stimulated by ADP nor inhibited by phosphoenolpyruvate. In contrast, most bacterial and eukaryal ATP-PFKs (PFK-A family), including ATP-PFK from the hyperthermophilic bacterium *Thermotoga maritima* (Hansen and Schönheit, submitted), showed the classical allosteric response to these compounds. In accordance, sequences of the PFK-A family are conserved with respect to substrate binding and effector sites for ADP and phosphoenolpyruvate (Fig. 4A).

A. pernix ATP-PFK showed a broad substrate specificity for phosphoryl acceptors; besides fructose 6-phosphate, which is converted at the highest affinity, the enzyme also phosphorylates glucose 6-phosphate, adenosine, fructose, ribose 5-phosphate, and ribose. This property of the *A. pernix* ATP-PFK, together with its non-allosteric behaviour, indicates a similarity to members of the PFK-B sugar kinase family.

Indeed, BLASTP and TBLASTN searches of the nonredundant database with the sequence of the ATP-PFK from *A. pernix* revealed 266 hits (July 15, 2001); most of them were members of the PFK-B family including several putative archaeal sequences. The nine best scores were obtained with putative members of the PFK-B family from archaea (PAU82374, VNG1851, AF0356, PH1845, MTH1544, PAB0280, Pf 1740248, MJ0406, MTH1841), with PAU82374 from *Pyrobaculum aerophilum* giving the best match. We cloned and expressed the ORF MJ0406 from *Methanococcus jannaschii* in *E. coli* (Hansen and Schönheit, unpublished results). The recombinant *M. jannaschii* protein has an apparent molecular mass of 110 kDa as judged by gel filtration (subunit 33 kDa) and shows an ATP-dependent phosphorylation of F-6-P and ribose (substrate specificity and other substrate not yet tested) at 85 °C, defining the protein as an ATP-dependent PFK or sugar kinase. A multiple sequence alignment of the ATP-PFK from *A. pernix* and the ATP-PFK/sugar kinase from *M. jannaschii* with selected PFK-Bs from archaea, bacteria, and eukarya is given in Fig. 4B. Though all sequences annotated as PFK-B fall into a single family (Wu et al. 1991; Hofmann et al., 1999), the overall similarity of the selected sequences is rather low and only a few residues are conserved. Interestingly, the sequence similarity of the *A. pernix* ATP-PFK and the *E. coli* PFK-B is only 29% vs 26% between the *A. pernix* ATP-PFK and the PFK-A from *E. coli*. However, both the ATP-PFK from *A. pernix* and the ATP-PFK/sugar kinase from *M. jannaschii* contain the two signature patterns of the PFK-B family (Fig. 4B) (Hofmann et al. 1999). This includes the AGD motif (position 252–254 of the *A. pernix* sequence) which is conserved in most members of the PFK-B family and which has been suggested to form an anion hole important for phosphoryl transfer, as concluded from the crystal structure of *Toxoplasma gondii* adenosine kinase (Schumacher et al. 2000). The *A. pernix* ATP-PFK sequence contained neither the conserved substrate or effector binding sites nor the conserved consensus pattern of the PFK-A family (Fig. 4A), which is in accordance with the non-allosteric behaviour of the enzyme.

The data indicate that the described archaeal ATP-PFKs/sugar kinases belong to the PFK-B family. However, further evidence for evolutionary relationship of the archaeal ATP-PFKs/sugar kinases and PFK-B homologues requires the elucidation and comparison of crystal structures, as performed for the *E. coli* ribokinase and the human adenosine kinase (Mathews et al. 1998).

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