Cloning, expression and characterisation of a Family B ATP-dependent phosphofructokinase activity from the hyperthermophilic crenarachaeon Aeropyrum pernix

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Abstract

We have cloned a Family B sugar kinase gene from the aerobic hyperthermophilic crenarchaeon Aeropyrum pernix and have subsequently expressed the protein in Escherichia coli. The enzyme was purified with its associated histidine-tag by affinity chromatography with a nickel-nitrilotriacetic acid column followed by cation exchange chromatography and possesses a high degree of thermostable ATP-dependent phosphofructokinase activity. The enzyme has an estimated apparent $K_m$ for ATP and fructose-6-phosphate of 0.027 and 1.212 mM, respectively, that were determined in discontinuous assays at 95°C. The Family B ATP-dependent phosphofructokinase has a half-life of approximately 30 min at 95°C and is indicated to be monomeric. The implications of the presence of a Family B phosphofructokinase in the Crenarchaeae are discussed with reference to the origins of the Embden–Meyerhof pathway. ß 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Phosphofructokinase (PFK) catalyses the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate, a key enzymatic activity of the Embden–Meyerhof pathway. Due to its position within the metabolic pathway it is often highly regulated, especially amongst more evolved organisms [1]. Although PFKs have been extensively investigated over the years both within the bacterial and eukaryal domains, much less is known about the distribution and properties of PFKs within hyperthermophilic archaea. PFKs can now be assigned to three sequence families [1]. The well-known textbook Family A PFK contains both ATP- and pyrophosphate-dependent enzymes and is present throughout the domains Bacteria and Eukarya, much less is known about the distribution and properties of PFKs within hyperthermophilic archaea. PFKs can now be assigned to three sequence families [1]. The well-known textbook Family A PFK contains both ATP- and pyrophosphate-dependent enzymes and is present throughout the domains Bacteria and Eukarya, it is also present in the anaerobic crenarchaeon Thermoproteus tenax [1]. The best example of a biochemically characterised Family B ATP-dependent PFK (ATP-PFK) is that from Escherichia coli and has otherwise been indicated to be present by biochemical assay in only a few enterobacterial genera [2]. The Family C PFKs are ADP-dependent and are present only within some members of the Euryarchaeota, in the order Thermococcales, and probably also in Methanococcus jannaschii and Methanosarcina mazei [1,3,4].

Aeropyrum pernix is an aerobic, organotrophic, crenarchaeal hyperthermophile that grows optimally at 95°C and lies phylogenetically between the aerobic Sulfolobus shibatae and the anaerobes Pyrodictium occultum and Desulfovoccus amylolyticus based on sequence analysis of the small ribosomal subunit rDNA gene (16S) [5]. Examination of the annotated genome sequence of A. pernix strain K1 did not reveal any homologues of Family A or Family C PFKs. However, a Family B sugar kinase (gene APE0012) with probable phosphofructokinase activity was found [1,6]. In order to obtain a deeper understanding of the origins of the Embden–Meyerhof pathway it is important to determine the distribution, properties and phylogenetic origins of its constituent enzymatic activities. In this paper we describe the cloning, expression and characterisation of a Family B ATP-PFK of a known sequence from the archaea.
2. Materials and methods

2.1. Determination of ATP-PFK activity

The standard assay for determining activity of the *Aeropyrum* ATP-PFK was conducted using a modification of a discontinuous method, or stopped assay [7], with ATP as the phosphoryl donor and fructose-6-phosphate (F-6-P) as the other substrate. Reactions were incubated for 10 min (unless otherwise stated), at either 90°C or 95°C, and then stopped by immediate addition of EDTA to 50 mM and placement in an ice-water bath. The incubated reaction volume was always 100 μl and assays were performed in duplicate. The standard reaction contained 50 mM 3-[N-morpholino]propane sulfonic acid (MOPS), pH 6.5 (pH adjusted at both 90°C and 95°C using 0.025 mM KH2PO4, 0.025 mM Na2HPO4 as a reference buffer for pH standardisation), 5 mM ATP, 10 mM F-6-P, 100 mM KCl and 20 mM MgCl2. The amount of fructose-1,6-bisphosphate (F-1,6-P2) formed was measured by addition of NADH (Sigma; final concentration 0.2 mM), fructose-1,6-bisphosphate aldolase (Sigma; 0.07 units), triosephosphate isomerase (Sigma; 2.1 units) and α-glycerophosphate dehydrogenase (Sigma; 0.27 units). Complete oxidation of NADH at 37°C was followed at 340 nm (NADH extinction coefficient = 6.22 mM⁻¹ cm⁻¹), using an Ultraspec 3000 UV/Visible Spectrophotometer (Pharmacia Biotech), and the difference in absorbance between start of the reaction and the final absorbance (∆A340) was determined. To determine specific activities (units mg⁻¹ protein), the recorded ∆A340 values were corrected to account for the differences in volumes of the incubated reaction (100 μl) and the final assay volume.

2.2. Cloning, expression and enzyme purification

The gene APE0012 encoding the sugar kinase was amplified using a plasmid containing the full length open reading frame for the gene in an insert of 2499 bp designated pUC118 clone A2GR7315. Based on the annotated sequence of gene APE0012 two primers were designed. The two oligonucleotide primers were: 5’-GCATGATTCATGCTAGACATCTGGTGCAAG (forward primer with EcoRV restriction in bold and start codon underlined) and 5’-GGTACAGCTTCTAAATCTCCACCCC-GAGCTCT (reverse primer with HindIII recognition site in bold and stop codon underlined). The amplification reaction (35 cycles with an annealing temperature of 55°C) with Taq polymerase (Perkin Elmer) utilised 10 pg of plasmid from clone A2GR73125 and 0.2 μM each primer and a PCR product of approximately 930 bp was obtained (not shown). After purification of the PCR product according to standard methods [8] it was cut with EcoRV and HindIII, the digest products cleaned by phenol:chloroform extraction followed by ethanol precipitation and ligated into SfiI and HindIII-restricted pProEX HTb (Life Technologies). The resulting ligation mixture was used to electroporate *E. coli* strain DH 5α and plated on Luria–Bertani (LB) agar plates supplemented with ampicillin (100 μg ml⁻¹). Colonies were screened for the presence of inserts and those with inserts were analysed by restriction enzyme analysis using BamHI and SacI to confirm their identity.

Clones with inserts were examined for the ability to express ATP-PFK activity by growing the cultures in LB broth (10 ml) with 100 μg ampicillin ml⁻¹ to an OD600 of 0.3 followed with induction by adding isopropylthio-β-d-galactoside to 0.625 mM. Growth was allowed to proceed for 16 h at 30°C before harvesting the cells by centrifugation (4000×g for 10 min). The cells were resuspended in 50 mM MOPS, pH 7.0, 100 mM KCl, 7 mM β-mercaptoethanol, 1.0 mM MgCl2, sonicated, and cell debris removed by centrifugation (4000×g for 10 min). The cell-free supernatants were heat-treated for 40 min at 80°C and the denatured proteins removed by centrifugation at 8000×g for 15 min. Large-scale culturing was conducted essentially as described by Ronimus et al. (2001) except that the fermenter volume was 2.0 l and induction was allowed to continue for 16 h at 30°C [9].

Cells from the fermenter production run were centrifuged and resuspended in 50 mM Tris–HCl, pH 7.5, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated until near-complete lysis was achieved. Cell debris was removed by centrifugation (10 000×g for 40 min) and the supernatant heat-treated for 40 min at 80°C. Denatured protein was removed by centrifugation (10 000×g for 40 min) and the supernatant applied to a 25 ml nickel-nitrioltriacetic acid (Ni-NTA) column equilibrated in 20 mM Tris–HCl, 100 mM KCl, 10% glycerol (v/v) (Qiagen, Germany). The expressed protein was eluted with 100 mM imidazole buffer according to the manufacturer’s instructions. The imidazole was removed by dialysis and the sample re-applied to the Ni-NTA column, after regeneration performed according to the manufacturer’s instructions, and the protein eluted again with 100 mM imidazole buffer. The eluted protein was dialysed against HiLoad Q (Pharmacia Biotech) start buffer (25 mM MOPS, pH 7.0, 50 mM KCl, 10% glycerol (v/v), 0.2 mM MgCl2). The expressed protein was eluted in a 20 column volume linear salt gradient in the same buffer but with a final NaCl concentration of 1.0 M. The protein was ultrafiltered, primarily to remove the NaCl, and stored in 50% glycerol (v/v), 50 mM MOPS, pH 7.17 (at room temperature), 100 mM KCl, 0.1 mM MgCl2 and 7 mM β-mercaptoethanol.

2.3. Family B ATP-PFK characterisation

The enzyme preparations were checked for their relative purity by running aliquots on 8–25% polyacrylamide phast gels (Pharmacia Biotech) followed by silver staining conducted according to the manufacturer’s instructions. Pro-
tein concentrations were assayed according to the Coomassie blue dye-binding method with modifications using bovine serum albumin as standard [10]. The native structure of the expressed enzyme was assessed using both Superdex 200 and Superdex 75 (Pharmacia Biotech) in 50 mM MOPS, pH 7.0, 200 mM KCl at 1.0 ml min⁻¹. Blue dextran (molecular mass of 2000 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa) were used as standards. The half-life of the enzyme was assessed by incubating the enzyme at 95°C under a mineral oil overlay to prevent evaporation and aliquots were removed at 10, 20, 30, 40, 60, 90, 120 180 and 240 min. A sample (0 min) was taken for control purposes.

The determination of the optimum pH (50 mM MES for pH 5.5, 6.0 and 6.5 at 95°C and 50 mM MOPS for pH 6.5, 7.0 and 7.5 at 95°C), KCl concentration (50, 100, 200, 300, 400, 500 mM) and Mg²⁺ concentration (1.25, 2.5, 5, 10, 20, 30, 40 mM) utilised otherwise standard conditions while varying the concentration of the parameter being tested. For the determination of the forward reaction apparent $K_m$ values the ATP or the F-6-P concentrations were varied while the other substrates were held constant using standard assay conditions at 95°C. The ATP-PFK preparation used for determination of kinetic parameters was first concentrated approximately 5-fold (YM-10; Amicon) and a 2 min assay format used to obtain initial velocities. The ATP-PFK was added after a 20 s preincubation at 95°C and samples were processed in parallel. For examination of the effects of potential enzyme modulators the concentration of ATP (0.2 mM) and F-6-P (1.5 mM) were adjusted close to their apparent $K_m$ values to maximise the chance of observing an inhibitory or activation effect and were done at 90°C. For determining the ability of cations to replace MgCl₂, the concentrations of the ions were 10 mM in the assay (at 90°C) and were compared to a control with MgCl₂, also at 10 mM. The inhibitory effects of Cu²⁺ were examined using standard assay conditions at 90°C and the activity with alternate phosphoryl donors was assessed at 1 mM donor compared to a control with 1 mM ATP at 90°C. The stability of the reaction product F-1,6-P₂ (0.05 mM at start of incubation) was examined by incubating F-1,6-P₂ in standard assay buffer at 95°C under a mineral oil overlay, withdrawing aliquots and measuring the decrease in the $A_{340}$ obtained compared to that obtained with the unincubated sample.

3. Results

3.1. Cloning and expression

A total of 48 alkaline lysis plasmid DNA isolations were conducted, each derived from a single transformant, to check for the presence of inserts and of these seven had inserts of the appropriate size (approximately 930 bp).

These were subsequently checked by restriction enzyme analysis using BamHI–SacI and XhoI–SacI double digests and all produced identical digest profiles with fragments of the expected size (not shown). All of the isolates were then checked for expression levels in small-scale experiments and all but one produced a similar level of expression within a 2-fold range. The one displaying the highest level of expression was picked for larger-scale production. A total of 100 g of E. coli cells (wet weight) were recovered and processed as described above. Expression levels were indicated to be very low with an estimated 12.2 mg of the ATP-PFK being recovered in the 100 mM imidazole elution fraction after the first Ni-NTA purification step. The expression levels in E. coli were low possibly due to the fact that Aeropyrum and E. coli have large differences in their codon usages for arginine, which represents almost 11% of the amino acids in the APE0012-encoded Family B ATP-PFK [6].

3.2. Protein characterisation

The protein was purified after the three steps to near homogeneity as judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; not shown) and migrated with a molecular mass of 29 kDa with the histidine-tag present (representing an additional 2 kDa; see Table 1 for a summary of the properties of the A. pernix Family B ATP-PFK). Interestingly, gel filtration chromatography under non-denaturing conditions indicated that the native structure is likely to be monomeric, with similar estimated molecular masses being obtained whether Superdex 200 or Superdex 75 were used for the analysis.

The pH optimum for activity of the enzyme was analysed only between the pH values of 5.5 to 7.5 due to concerns about instability of the ATP at more acid pH values and was found to be maximal between 5.5 and 6.5, but possessed 69% of maximal activity at pH 7.5 (Table 1). The optimal MgCl₂ concentration for activity was found to be 20–30 mM and the enzyme possessed 6, 36 and 74% activity compared to the maximal activity when the MgCl₂ concentrations were 1.25, 2.5 and 5.0 mM, respectively. The enzyme was relatively unaffected by the KCl concentration with optimal activity between 50 and 100 mM, and possessed 85% of maximal activity when the KCl concentration was 500 mM. The product F-1,6-P₂ was relatively stable with 68% remaining after 120 min at 95°C and was estimated to have a half-life of approximately 150 min, and thus would not have constituted a significant factor over the 10 min assay period. The substrate ATP and product ADP reportedly have half-lives of 115 and 750 min at 90°C, respectively [11]. The enzyme itself was found to have a half-life of approximately 30 min at 95°C. Triton X-100 (0.1% final concentration), bovine serum albumin (1 mg ml⁻¹) and KCl (200 mM) did not stabilise the enzyme (not shown). The enzyme activity...
at varying temperatures was measured and was found to decrease approximately 2-fold every 10°C with change to lower temperatures (Table 1). Attempts were made to measure activity continuously at 55°C, the maximum temperature at which the mesophilic enzymes are useful, but were unsuccessful.

The ability of alternate phosphoryl donors to replace ATP was examined and several were found to partially support activity including GTP (84.1% of control activity with ATP), CTP (20.6%), UTP (9.5%), TTP (7.1%) and polyphosphate (5.1%). No detectable activity was found with either ADP or pyrophosphate thus defining the enzyme as an ATP-dependent PFK. A variety of cations could partially replace Mg^{2+} including Mn^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+} and Ca^{2+} (Table 1). The A. pernix ATP-PFK was not particularly inhibited by Cu^{2+} and retained 47.6% of control activity in the presence of 1.0 mM CuCl_{2} (84.6% in 0.1 mM CuCl_{2}).

The reaction kinetics at 95°C were hyperbolic for both cosubstrates and appeared to follow classic Michaelis-Menten behaviour (Fig. 1A and B). The apparent $K_m$ for ATP was found to be low, 0.027 mM, while the apparent $K_m$ for F-6-P was 1.212 mM. The specific activity was somewhat low compared to most other PFKs that have been characterised [1] and was 0.53 units mg$^{-1}$ protein. The following potential modulators of the Aeropyrum ATP-PFK were checked for their ability to produce either an activating or inhibitory effect (% control activity): cyclic AMP (108%), AMP (111.3%), UDP (111%), TDP (105%), acetyl-CoA (110%), glucose-6-phosphate (92%), iso-citrate (111%), co-enzyme A (111%), NH$_4$Cl (10 mM; 92%), inorganic phosphate (10 mM; 98%), CDP (104%), phosphoenolpyruvate (89%), citrate (89%), succinate (81%), GDP (54.2%), pyrophosphate (79%), polyphosphate (94%) and ADP (1.0 mM; 17.6%). Thus, the enzyme appeared to display product inhibition mediated by ADP, and secondarily by the similar purine-based GDP, but is otherwise nonallosteric. The inhibition by the reaction product, ADP, was examined at two other concentrations in the assay used for determining potential allosteric effects and the following results were found (% control activity with 1 mM ATP): with 0.5 mM ADP (32.2%) and with 0.1 mM (72.7%). Under standard control assay conditions with 5.0 mM ATP (25 times higher than that used for determining the effects of potential modulators) where a total $A_{340}$ of approximately 0.3 for a 10

![Graphical presentation of data used for determining the kinetic parameters of the A. pernix Family B ATP-PFK. A: Data for determining the apparent $K_m$ for ATP and respective $V_{max}$ with the ATP substrate concentration ([S]) plotted against the $A_{340}$ (2 min assay format). Inset shows corresponding Lineweaver-Burk plot with $r^2$ value calculated from linear regression analysis. B: Data for determining the apparent $K_m$ for F-6-P and corresponding $V_{max}$. Inset shows the double-reciprocal transformation of the data.](https://academic.oup.com/femsle/article-abstract/202/1/85/579459)
min assay was usually obtained (corresponding to a concentration of approximately 0.025 mM ADP), the inhibition would have been relatively minimal.

4. Discussion

We have shown here that the Family B sugar kinase gene APE0012 in *A. pernix* encodes for a thermostable enzyme with ATP-dependent PFK activity. This represents the first confirmed description of a Family B ATP-PFK outside the domain bacteria and the first confirmed example within a crenarchaeon. A gene, similar to APE0012 (random expectation value \( < 10^{-27} \)), has been found in *Halobacterium* strain NRC-1, raising the possibility that these aerobic archaea may also contain a Family B ATP-PFK activity [1,12]. In addition, the N-terminal amino acid sequence of the ATP-dependent PFK recently characterised from the anaerobic *Desulfurococcus amylolyticus* possesses a limited degree of homology suggesting that it may represent another Family B ATP-PFK [1,7]. Thus the Family B ATP-PFKs are likely to be more widely distributed than previously indicated. Family B ATP-PFKs, to our knowledge, have only been found in *E. coli* and in some related enterobacterial genera including *Shigella*, *Salmonella* and *Klebsiella* [1,2].

The *Aeropyrum* ATP-PFK is indicated to be nonallosteric with traditional effectors of regulated PFKs having no significant effect. However, it is likely to be autoregulated by its reaction product ADP and this may represent a simple control mechanism for controlling the glycolytic flux. Although the specific activity is low compared to the Family B ATP-PFK of *E. coli* (199) and most Family A and Family C PFKs [1], it is not low compared to the specific activities of other kinases with the Family B sequence signature. For example, the range of specific activities for pyridoxal kinases (EC 2.7.1.35) vary between 0.473 and 2.105, between 0.1 and 18 for adenosine kinases (EC 2.7.1.20) and between 2.7 and 11.2 for fructokinases (EC 2.7.1.4; http://www.brenda.uni-koeln.de). A relatively low specific activity for the ATP-PFK may not represent a significant problem for *A. pernix*, which is presumably able to obtain much higher ratios of ATP due to its aerobic respiration capabilities, combined with the organism’s 200 min doubling time [5]. The relative lack of inhibition by CTP is similar to that found for the ADP-PFK of *Thermococcus zilligii* and generally much less than that seen with Family A PFKs [13]. The enzyme has a very low apparent *Km* for its energy-driving cosubstrate ATP and thus the enzyme would likely be saturated under in vivo conditions. Perhaps of significance, the ATP-PFK is indicated by the gel filtration chromatography results to be monomeric. It is hard to draw any firm conclusion about these latter data though, as only a single Family B ATP-PFK has been characterised from *E. coli* and the enzyme is homodimeric (it forms homotetramers in the presence of high levels of ATP) [14].

The discovery of an ATP-PFK activity in *A. pernix* is significant in that it allows for a key gap in the otherwise nearly-complete Embden–Meyerhof pathway of this organism to be filled. Other enzymes of the pathway are indicated to be potentially present including a fructose-1,6-bisphosphate aldolase (of the DhnA family; APE0011; [15]), triosephosphate isomerase (APE1538), glyceraldehyde-3-phosphate dehydrogenase (phosphorylating; APE0171), phosphoglycerate kinase (APE0173), phosphoglycerate mutase (2,3-bisphosphate-independent form; APE1616 [16]), enolase (APE2458) and pyruvate kinase (APE0489) (http://pedant.gsf.de/) [12,15,16].

The most significant implications from the work presented here is that associated with the distribution of Family A, B and C PFKs within the universal tree of life based on small ribosomal subunit rDNA gene sequence analysis and its implications for the origins of the Embden–Meyerhof pathway. Family A PFKs, both ATP- and pyrophosphate-dependent are distributed almost entirely within the bacterial and eukaryal domains, and the only confirmed exception being the pyrophosphate-dependent PFK in the anaerobic crenarchaeon *T. tenax* [1]. On the other hand, Family C ADP-PFKs have been found only in a limited number of Euryarchaea [1,3,4]. Family B ATP-PFKs have previously only been identified in a few enterobacteria but are now indicated to be scattered within the kingdom Crenarchaeota [2]. Taken together, the multiplicity of gene sequence families encoding for PFKs at the top of the Embden–Meyerhof pathway combined with the fact that enzymes of the lower trunk pathway (enzymes involved in conversion of glyceraldehyde-3-phosphate to pyruvate) are more conserved can be taken as support for an autotrophic origin for the pathway [16,17].

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References


