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MINI-REVIEW

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The biochemical properties and phylogenies of phosphofructokinases from extremophiles

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Abstract The enzyme phosphofructokinase (PFK) is a defining activity of the highly conserved glycolytic pathway, and is present in the domains Bacteria, Eukarya, and Archaea. PFK subtypes are now known that utilize either ATP, ADP, or pyrophosphate as the primary phosphoryl donor and share the ability to catalyze the transfer of phosphate to the 1-position of fructose-6-phosphate. Because of the crucial position in the glycolytic pathway of PFKs, their biochemical characteristics and phylogenies may play a significant role in elucidating the origins of glycolysis and, indeed, of metabolism itself. Despite the shared ability to phosphorylate fructose-6-phosphate, PFKs that have been characterized to date now fall into three sequence families: the PFKA family, consisting of the well-known higher eukaryotic ATP-dependent PFKs together with their ATP- and pyrophosphate-dependent bacterial cousins (including the crenarchaeal pyrophosphate-dependent PFK of *Thermoproteus tenax*) and plant pyrophosphate-dependent phosphofructokinases; the PFKB family, exemplified by the minor ATP-dependent PFK activity of *Escherichia coli* (PFK 2), but which also includes at least one crenarchaeal enzyme in *Aeropyrum pernix*; and the tentatively named PFKC family, which contains the unique ADP-dependent PFKs from the euryarchaeal genera of *Pyrococcus* and *Thermococcus,* which are indicated by sequence analysis to be present also in the methanogenic species *Methanococcus jannaschii* and *Methanosarcina mazei*.

Key words Phosphofructokinase · Glycolysis · Thermophile · Pyrophosphate · *Aeropyrum* · *Thermococcus* · Evolution

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Introduction

Glycolysis is a highly conserved catabolic pathway catalyzing the degradation of glucose to pyruvate in a sequence usually involving ten enzymatic reactions, and is present within the domains Archaea, Bacteria, and Eukarya (Fothergill-Gilmore and Michels 1993; Woese et al. 1990; Selig et al. 1997; Schäfer and Schönheit 1993; Schönheit and Schäfer 1995). The enzyme phosphofructokinase (PFK) catalyzes a critical priming reaction in the glycolytic pathway, the phosphoryl transfer to the 1-position of the substrate fructose-6-phosphate. Due to its strategic position at the top of the pathway and the ATP-dependence of the reaction, it is in a prime position to control the flux through the overall enzymatic sequence. The allosterically controlled PFKs of higher eukaryotes are a standard biochemical textbook topic because they represent one of the most highly regulated enzymes known, with at least 23 effectors (reviewed by Bloxham and Lardy 1973; Uyeda 1979; Sols 1981; Tejwani 1978; Goldhammer and Paradies 1979). A complete account of the allosteric regulation observed among this large collection of characterized enzymes is beyond the scope of this review. Instead, the primary focus is on the impact that newly identified and characterized PFKs from extremophiles have on scenarios concerning the early evolution of metabolism and the Embden-Meyerhof pathway.

The Family A ATP-PFKs (EC 2.7.1.11) of higher eukaryotes are related by sequence to the Family A PFKs of bacteria that are either ATP- or pyrophosphate-dependent (PP_i-PFKs; EC 2.7.1.90), and to the Family A PP_i-PFKs of plants (Mertens et al. 1998; Michels et al. 1997). They are also related to a single sequence from the crenarchaeal kingdom of the domain Archaea, that of *Thermoproteus tenax* PP_i-PFK (Siebers et al. 1998). Together these proteins constitute what has been termed the PFK A family (Wu et al. 1991), which has been shown to be a monophyletic group by various authors (Michels et al. 1997; Mertens et al. 1998; Alves et al. 1996; Siebers et al. 1998; Wu et al. 1991; Ding et al. 2000). The higher eukaryotic ATP-PFKs are generally homotetrameric enzymes that are usually highly regulated by a number of effectors, including citrate, fructose-2,6-

bisphosphate, fructose-1,6-bisphosphate, AMP, ADP, cyclic-AMP, P_i, phosphoenolpyruvate, and 3-phosphoglycerate (Kemp and Foe 1983; Uyeda 1979). These enzymes are approximately twice as large as the bacterial ATP-dependent Family A PFKs, probably the result of a gene duplication event, with the N-terminal half retaining the highest degree of homology with their bacterial relations (Poorman et al. 1984). The C-terminal portion, in contrast, is relatively more diverged and has evolved additional regulatory roles (Wu et al. 1991). The yeast ATP-PFK is somewhat unusual in that it is octomeric, containing two different subunits ($\alpha_4\beta_4$), a situation likely to have arisen by gene duplication (Heinisch et al. 1989). The bacterial Family A ATP-PFKs are also tetrameric, with monomer sizes of 33–38 kDa, and they tend to be regulated, but generally by fewer effectors than the higher eukaryotic ATP-PFKs (Ronimus et al. 1999a). The PP_i-PFK enzymes, which are also members of the Family A PFKs, have been broadly classified by Mertens et al. (1998) into two groups based on their sensitivities to the common higher eukaryotic PFK regulator fructose-2,6-bisphosphate and the size of their subunits. Under this classification scheme, Type I PP_i-PFKs include those enzymes from the bacteria, the crenarchaeon *Thermoproteus tenax*, the extremely thermophilic *Dictyoglomus thermophilum*, and some protists (Siebers et al. 1998; Ding et al. 1999, 2000). These PP_i-PFKs contain only a single type of subunit (homomeric) and are not activated by the effector fructose-2,6-bisphosphate, and the monomer sizes vary from the smallest of 36 kDa to the largest of 64–67 kDa. Type II PP_i-PFKs are activated by fructose-2,6-bisphosphate, contain two different subunits in a tetrameric native structure ($\alpha_2\beta_2$), and have a subunit size of 55–68 kDa (Mertens et al. 1998). These enzymes are found in plants and in the photosynthetic protist *Euglena gracilis* (Enomoto et al. 1988; Mertens et al. 1998). PP_i-PFKs catalyze a reaction that is nearly reversible in vivo, and the enzyme is able to operate bifunctionally, i.e., it is able to replace the gluconeogenic enzyme fructose-1,6-bisphosphatase (Reeves et al. 1974). Crystal structures are now available for both the *Bacillus stearothermophilus* and *E. coli* Family A ATP-PFKs (Shirakihara and Evans 1988; Rypniewski and Evans 1989; Evans and Hudson 1979; Shirmer and Evans 1990).

For some time, *Escherichia coli* has been known to possess two PFKs (both classified as EC 2.7.1.11), one of the Family A and a second of the Family B type (Kotlarz and Buc 1981; Fraenkel et al. 1973; Daldal 1984). These two enzyme families were originally thought not to share significant homology, leading the author to suggest that they were phylogenetically unrelated (Daldal 1984). However, others have suggested that Family A and Family B PFKs may potentially be related, on the basis of their predicted secondary structures, subunit sizes, similar functions, a limited degree of homology found between some members from the two families, and the fact that the genes encoding the two *E. coli* PFKs are on opposite sides of the chromosome (Wu et al. 1991). The PFKB enzyme family is interesting in that the tertiary structure appears to have enabled the enzyme to adapt easily to a variety of sugar-based substrates (Schumacher et al. 2000). Other substrates that have been shown to be phosphorylated by enzymes of the PFKB family include tagatose-6-phosphate (EC 2.7.1.144), fructose (producing fructose-1-phosphate; EC 2.7.1.4), fructose-1-phosphate (producing fructose-1,6-bisphosphate; EC 2.7.1.56), adenosine (EC 2.7.1.20), ribose (EC 2.7.1.15), pyridoxine (EC 2.7.1.35), myo-inositol, phosphomethyl pyrimidine (EC 2.7.1.7), 2-dehydro-3-deoxygluconate (EC 2.7.1.45), inosine-guanosine (EC 2.7.1.173), and ADP-heptose (Hoffman et al. 1999; Sigrell et al. 1998). Crystal structures are available for three PFKB family kinases, the human and *Toxoplasma gondii* adenosine kinases and the *E. coli* ribokinase (Mathews et al. 1998; Schumacher et al. 2000; Sigrell et al. 1998; Hoffman et al. 1999).

In 1994, Kengen et al. discovered unique ADP-dependent kinase activities in the hyperthermophilic euryarchaeal *Pyrococcus furiosus,* including an ADP-glucokinase, which phosphorylates glucose to glucose-6-phosphate, and an ADP-PFK. A related ADP-PFK was subsequently purified from the New Zealand freshwater hyperthermophilic species *Thermococcus zilligii* (Ronimus et al. 1997) and characterized biochemically, and the enzyme was found to be nonallosteric (Ronimus et al. 1999b). The gene encoding the *T. zilligii* enzyme has been cloned and ultimately expressed in *E. coli* and also characterized (Ronimus et al. 2001a). The *P. furiosus* ADP-PFK has been cloned, expressed, and characterized, and was also found to be nonallosteric (Tuininga et al. 1999).

As can be seen from the above synopsis of PFKs, in the last few years some interesting variants of PFK have been identified, especially among hyperthermophiles, organisms that are considered to represent the closest modern-day relatives of the last universal common ancestor, generally considered to have given rise to all life (Woese et al. 1990). These additional and relatively unique enzymes have the potential to alter our view of the way evolution of the Embden-Meyerhof pathway proceeded and has prompted us to review the biochemical features of PFKs in general and their phylogenies. The recent findings are summarized and their impact on the interpretation of events leading to the early evolution of metabolism is discussed.

Materials and methods

Representative amino acid sequences from the PFK families were used in BLASTP, Gapped-BLAST, PSI-BLAST, and TBLASTN searches of the nonredundant database (Altschul et al. 1990, 1997) and retrieved for analysis. Sequence alignments and phylogenetic trees were produced using the neighbor-joining method of Clustal W, version 1.6 (Thompson et al. 1994). For the construction of the Family A PFK phylogenetic tree, only approximately 220 of the most highly conserved residues, predominantly in the N-terminal portions of the proteins, were used (and only the respective residues in the N-terminal halves of the higher eukaryal sequences). For the PFKB and ADP-dependent phylogenetic constructions, the entire full-length sequences were used because these families do not possess the variation in length that is present within the Family A PFKs.

Statistical validation of the tree branch nodes and lengths for all phylogenies were assessed using 100 bootstraps. Comparison of the crystal structures was performed using the VAST algorithm (Madej et al. 1995; Gibrat et al. 1996). Determination of the total number of PFKB and Family A sequences available in the database was conducted using the BLASTP (nonredundant database with daily updates) and the Pfam database (Sonnhammer et al. 1997).

Results

Biochemical characteristics of Family A PFKs

The biochemical properties of all bacterial and protist Family A PP_i-PFKs that have been characterized to date, and of representative eukaryal Family A ATP-PFKs, bacterial ATP-PFKs, and plant PP_i-PFKs, are summarized in Table 1. Although Family A ATP-PFKs are usually thought of as being highly regulated, there are several exceptions where nonallosteric enzymes have been described, for example, in *Lactobacillus delbrueckii* (*bulgaricus*), *Lactobacillus plantarum*, *Trypanosoma brucei*, *Dictyostelium discoideum*, and *Arthrobacter crystallopoietes*, and, indeed, even the highly regulated muscle ATP-PFK is nonallosteric at pH 8.0 (Le Bras et al. 1991; Branny et al. 1993; Simon and Hofer 1978; Cronin and Tipton 1985; Martínez-Costa et al. 1994; Ferdinandus and Clark 1969; Kemp and Foe 1983). In one study, the allosteric yeast ATP-PFK was replaced by the nonallosteric *Dictyostelium discoideum* ATP-PFK (by expression of the nonallosteric enzyme in an ATP-PFK-deficient yeast strain), and the resulting mutant strain grew with a similar generation time on glucose-containing medium (Estévez et al. 1995). Thus, although the general trend is to regulate PFK at the top of glycolysis, it is by no means an absolute requirement for cells to possess this control mechanism.

Some trends in the biochemical properties of Family A ATP-PFKs can be discerned from Table 1, although it should be mentioned that that data set is not totally inclusive (i.e., some eukaryotic tissue ATP-PFK subtypes are not included). For example, the pH optima of the ATP-PFKs for the forward reaction tend to be slightly alkaline, whereas for the PP_i-PFKs, it is acidic (see also Bloxham and Hardy 1973). In addition, the apparent K_m ($S_{0.5}$) values for fructose-6-phosphate are higher than those for ATP in most cases, except for the *Bacillus stearothermophilus*, *E. coli,* and *Lactobacillus plantarum* enzymes (Byrnes et al. 1994; Blangy et al. 1968; Simon and Hofer 1978). The reversibility of the ATP-driven reaction has been examined only in a few cases. The apparent K_m value for the *E. coli* enzyme for fructose-1,6-diphosphate is 3.5 mM, which is much higher than those, in general, reported for PP_i-PFKs. For the *Saccharomyces cerevisiae* enzyme, the rate of the reverse reaction was approximately 10% that of the forward reaction (Sols and Salas 1966), and the K_{cat} for the *B. stearothermophilus* PFK was found to be 35-fold lower (Byrnes et al. 1994). The bacterial Family A ATP-PFKs have a fairly narrow size range, between 33.5 and 38 kDa. Eukaryotic Family A ATP-PFK enzymes possess a much greater subunit size range, the smallest being that of the *Trypanosoma brucei* ATP-PFK (53 kDa) and the largest being that of the *Saccharomyces cerevisiae* ATP-PFK (108 kDa) (Michels et al. 1997; Heinisch et al. 1989).

Some general trends can also be gleaned from the data presented in Table 1 for PP_i-PFKs. For example, where the specific activities of the reverse reaction have been examined (12 reverse-reaction data points), the values are only marginally higher than those of the forward reaction in 2 cases (*Rhodospirillum rubrum* and *Methylomonas methanica*); in 1 case, they are equal (*Entamoeba histolytica*), and in the other 9 cases, significantly, they are often two-threefold lower. In the majority of cases, then, the forward glycolytic reaction is favored. In partial support of the gluconeogenic direction being favored in some cases, however, data from studies of PP_i-PFKs using crude cell-free extracts from *Clostridium innocuum*, *Streptococcus pleomorphus,* and *Erysipelothrix rhusiopathiae* suggest that in these organisms there may be a slight preference for the gluconeogenic direction, although the individual enzymes were not purified (Petzel et al. 1989). The absolute values of the forward reaction specific activities range from a high of 840 U mg^{-1} protein for *Methylomonas methanica* to a low of 2.8 for the *T. tenax* enzyme, but this latter value partly reflects the suboptimal temperature used for the assay of this enzyme (at least 30°C below the optimal temperature for growth; Siebers et al. 1998).

With regard to pH optima for the two reaction directions, there is a tendency for the PP_i-PFKs to have a slightly to moderately acidic optimum (especially for the protist PP_i-PFKs) for the forward reaction and a slightly alkaline optimum for the reverse reaction. For example, in the 19 cases where the forward direction optima have been determined, the number having acidic optima was 12. In contrast, of the 14 cases where data on reverse direction optima are available, only 2 had acidic optima, and in 3 cases the values were equal with no apparent preference for either direction. In only 2 cases were the pH optima for the reverse reaction less than that for the forward reaction, and then only slightly so (*Propionibacterium freudenreichii* and *Giardia lamblia*). The average overall difference is approximately 0.8 pH units more acidic for the forward reaction based on the 14 cases where both values were reported.

With regard to the apparent K_m values for substrates, the K_m values for fructose-6-phosphate are higher than those for the phosphoryl donor in 19 of the 20 cases where data is available, the exception being the PP_i-PFK from *Naegleria fowleri*, an enzyme activated by AMP (Mertens et al. 1989). The ratio of apparent K_m for fructose-6-phosphate to that for PP_i ranges from about 14.6:1 in the case of the *Thermotoga maritima* PP_i-PFK to 2.3:1 in the case of *Thermoproteus tenax*. In all cases, the apparent K_m for fructose-6-phosphate is submillimolar and those for PP_i are equal to or below 0.2 mM. The apparent K_m values for fructose-1,6-bisphosphate are all submillimolar except for that from *Dictyoglomus thermophilum,* and in 13 out of 14 cases, it is lower than that reported for fructose-6-phosphate. The apparent K_m values for inorganic phosphate

Table 1. Summary of the properties for biochemically characterized purified and partially purified phosphofructokinases

PFK	Subunit size (kDa)[a]	Structure	Forward reaction			Reverse reaction			SA (U mg^{-1}) (For/Rev)[c]	Reference(s)
			K_m (mM) F-6-P[b]	K_m (mM) P donor[b]	pH opt	K_m (mM) F-1,6-P_2[b]	K_m P_i[b]	pH opt		
Prokaryotic PP_i-PFKs										
Spirochaeta thermophila	61	dimer	0.24	0.11	5–6.4	0.038	0.4	7–7.5	446/239	Ronimus et al. 1999a, 2001b
Dictyoglomus thermophilum	37.4	dimer	0.228	0.022	5.7–6.3	2.9	4.3	7–7.5	6.2/0.6	Ding et al. 1999, 2000; Ding 2000
Thermotoga maritima	46.5	dimer	0.98	0.067	5.6–5.8	–	–	–	203	Ding et al. 2001
Propionibacterium freudenreichii (formerly *P. shermanii*)	43 (48)	dimer	0.100	0.069	7.5	0.051	0.6	7.0–7.4	258/232	O'Brien et al. 1975; Ladror et al. 1991; Mertens et al. 1989
Amycolatopsis methanolica	36.2	tetramer	0.4	0.200	7.5	0.025	0.84	7.5	107	Alves et al. 1996
Borrelia burgdorferi	62	dimer	0.109	0.015	6.4–7.2	–	–	–	82.9	Deng et al. 1999
Treponema pallidum	62.4	dimer	0.529	0.042	7.5–8.5	0.267	1.39	7.25–7.75	125/42.4	Roberson et al. 2001
Acholeplasma laidlawii	37	dimer	0.65	0.110	7.4	–	–	–	12	Pollack and Williams 1986; DeSantis et al. 1989
Rhodospirillum rubrum	40	dimer	0.38	0.025	7.2	0.02	0.82	8.6	20/24.2	Pfleiderer and Klemme 1980
Thermoproteus tenax	37	dimer(?)	0.053	0.023	–	0.033	1.43	–	2.8/2.1	Siebers and Hensel 1993; Siebers et al. 1998
Methylomonas methanica	45	dimer	0.38	0.051	8.0	0.1	1.7	8.0	840/870	Beschastnyi et al. 1992
Ancient Eukaryal PP_i-PFKs										
Toxoplasma gondii	45	dimer	0.27	0.033	6–7.0	–	–	7–8.0	48.3/22	Peng and Mansour 1992
Giardia lamblia	64	monomer	0.25	0.039	7.2	0.072	1.09	7.0	146/98	Li and Phillips 1995; Phillips and Li 1995; Mertens 1990
Trichomonas vaginalis	48	dimer	0.08	0.012	acidic	0.01	1.25	–	–	Mertens et al. 1989
Trichomonas foetus	–	–	0.12	0.014	acidic	0.03	0.8	–	–	Mertens et al. 1989
Isotricha prostoma	48	dimer	0.08	0.012	5	0.01	1.25	7.0	59	Mertens et al. 1989
Entamoeba histolytica	42 (48)	dimer	0.038 (0.04)	0.014	5	0.018	0.8	7.0	46 (35)/46	Reeves et al. 1974; Huang et al. 1995; Bruchhaus et. al. 1996; Wood et al. 1977
Entamoeba histolytica	60.5	dimer	0.085	0.04	6.4–7.2	–	–	–	316	Deng et al. 1998
Eimeria tenella	–	–	0.077	–	6.8	–	–	7.5	–	Denton et al. 1994
Euglena gracilis[d]	110	monomer	0.300 (2.5)	0.100	6.5–6.7	0.017	1.2	6.5–6.7	18.5/12	Enomoto et al. 1988; Miyatake et al. 1984
Naegleria fowleri[e]	48 (51)	tetramer	0.010	0.015	6.0	0.035	0.59	7.5	65/32	Wessberg et al. 1995; Mertens et al. 1993
Cryptosporidium parvum	–	–	0.320	–	–	–	–	–	–	Denton et al. 1996
Hexamita inflata	67–70	monomer	0.800	0.110	–	0.02	1.14	–	300	Phillips et al. 1997
Representative Family A Plant PP-PFKs										
Solanum tuberosum[f]	67/57	$\alpha_2\beta_2$	0.32 (0.3)	0.007	7.3	0.005	0.2	–	6.4 (41)/5.4	Agosti et al. 1992; Yuan et al. 1988; Stitt 1989
mung bean[g]	–	–	0.1	0.1	7.8	–	–	–	24.2	Anderson and Sabularse 1982
Ananas comosusi[h]	61.5	dimer	0.89	0.011	7.7–7.9	0.094	0.149	6.7–8.0	43.8/8.9	Trípodi and Podestá 1997
Family C ADP-PFKs (euryarchaeal)										
Pyrococcus furiosus	52	tetramer	2.3	0.11	6.5	–	–	–	88	Tuininga et al. 1999
Thermococcus celer	–	–	2.5	0.200	–	–	–	–	–	Selig et al. 1997
Thermococcus litoralis	–	–	4.0	0.400	–	–	–	–	–	Selig et al. 1997
Thermococcus zilligii	52.7	tetramer	1.47 (3.77)	0.60 (0.11)	6.4	0.56	12.5	7.5–8.0	197/2.9	Ronimus et al. 1999b, 2001a

Representative Family A ATP-PFKs										
Thermus thermophilus[i]	36.5	tetramer	0.060–0.1	0.02–0.03	8.1	–	–	–	155	Xu et al. 1990; Yoshida et al. 1971; Cass and Stellwagen 1975
Bacillus stearothermophilus	33.9	tetramer	0.30	0.100	8.2	–	–	–	–	Byrnes et al. 1994
Lactobacillus bulgaricus	34	tetramer	0.300	0.200	6–6.5	–	–	–	–	Le Bras et al. 1991; Branny et al. 1993
Trypanosoma brucei[j]	53 (49)	tetramer	0.830	0.026	–	–	–	–	164	Cronin and Tipton 1985, 1987; Misset et al. 1986; Michels et al. 1997
Streptomyces coelicolor A3(2)[k]	36.7	dimer(?)	1.0	0.4	7.5–8.0	–	–	–	137	Alves et al. 1997
Escherichia coli[l]	34.7	tetramer	0.013	0.060	8.2	3.5	–	–	–	Kotlarz and Buc 1982; Blangy et al. 1968; Kemerer et al. 1982; Babul 1978
Thermotoga maritima[m]	34.5	tetramer	0.437	0.009	7.2–8.0	–	–	–	432	Ding et al. 2001
Schistosoma mansoni[n]	86	–	3.5	0.1–0.25	7.5–9.0	–	–	–	18	Su et al. 1996
Dictyostelium discoideum	95	tetramer	0.022	0.016	7.6	–	–	–	83.5	Martínez-Costa et al. 1994
Lactobacillus plantarum	38	tetramer	0.376	0.708	7–7.5	–	–	–	92.2	Simon and Hofer 1977, 1978
Streptococcus lactis	33.5	tetramer	0.25	0.18	7.2–7.8	–	–	–	161	Fordyce et al. 1982
Family B Prokaryotic ATP-PFKs										
Desulfurococcus amylolyticus	33 (37)	tetramer	1.17 (0.37)	0.28 (0.036)	5–5.5 (7.6–8.5)	–	16.7	0.5	47 (27)/4.5	Hansen and Schönheit 2000; Ding 2000
Escherichia coli	32.4	tetramer	0.011	0.05	–	–	0.14	–	–	Kotlarz and Buc 1982; Daldal 1984
Aeropyrum pernix	33.3	–	–	–	–	–	–	–	–	Ronimus and Morgan, unpublished data

PP_i-PFKs have also been identified in the following species: *Amycolatopsis orientalis*, *A. mediterranei*, *A. azurea,* and *A. saturnea* (Alves et al. 1994); *Streptococcus pleomorphus* (Petzel et al. 1989); *Acholeplasma axanthum, Acholeplasma equifetale* (DeSantis et al. 1989) and *Acholeplasma florum* (Pollack and Williams 1986; DeSantis et al. 1989); *Anaeroplasma intermedium* (Petzel et al. 1989); *Rhodopseudomonas gelatinosa* (Pfleiderer and Klemme 1980); *Alteromonas communis* (Sawyer et al. 1977); *Pseudomonas marina* (Sawyer et al. 1977); *Alcaligenes pacificus*, *A. cupidus, A. venustus,* and *A. aestus* (Sawyer et al. 1977); *Naegleria gruberi* (Mertens et al. 1993)*; Streptococcus pleomorphus* (Petzel et al. 1989); *Erysipelothrix rhusiopathiae and Anaeroplasma bactoclasticum* (Petzel et al. 1989); *Clostridium innocuum* (Petzel et al. 1989); *Bacteroides fragilis* (Macy et al. 1978; Roberton and Glucina 1982); *Bacteroides ruminicola*, *B. melanogenicus* and *B. succinogenes* (Roberton and Glucina 1982); *Actinoplanes* spp.(Seiler et al. 1996); *Actinomyces naeslundii* (Takahashi et al. 1995); *Propionibacterium arabinosum* (Wood and Goss 1985) and *Spirochaeta* species, including *S. alkalica*, *S. asiatica*, *S. isovalerica*, *S. litoralis*, *S. zeulzerae*, *S. halophila*, *S. alkalphila* (Ronimus et al. 1999a) and *S. stenostrepta* (Morgan and Ronimus 1998); *Methylomonas methanica*, *Methylosinus trichosporium,* and *Methylococcus capsulatus* (Trotsenko and Shishkina 1990); *Clostridium* sp. (Cruden et al. 1983); *Trimyena compressum* (Mertens et al. 1993); *Prevotella intermedia* and *P. nigrescens* (Takahashi and Yamada 2000)

PFK, phosphofructokinase; PP_i-PFK, pyrophosphate-dependent PFK; F-6-P, fructose-6-phosphate; F-1,6-P_2, fructose-1,6-diphosphate; ND, not determined

[a] Molecular masses are derived from gene sequence data where possible (those in parentheses are alternate values reported)

[b] apparent forward recation (For) K_m values for nonallosteric enzymes and $S_{0.5}$ for allosteric enzymes; values for ADP for reverse reaction (Rev) by ATP-PFKs or AMP by ADP-PFKs

[c] specific activities are those reported in the literature or extrapolated from relevant data

[d] *Euglena gracilis* F-6-P and P_i in the presence of F-2,6-diphosphate

[e] *N. fowleri* values determined in the absence of AMP

[f] *Solanum tuberosum* forward kinetic values determined in the presence of 10 µM fructose-2,6-diphosphate and reverse with no added NaCl

[g] in the presence of 17 µM fructose-2,6-diphosphate

[h] in the presence of 2 µM fructose-2,6-diphosphate at pH 7.7

[i] pH optimum is that for strain X-1 and reported kinetic parameters were determined with no phosphoenolpyruvate present

[j] *T. brucei* F-6-P $S_{0.5}$ in the presence of 1 mM ATP and that for ATP with 2.0 mM F-6-P

[k] $S_{0.5}$ for *S. coelicolor* with no phosphoenolpyruvate and $S_{0.5}$ for ATP with 5 mM F-6-P

[l] *E. coli* K(R) values

[m] *T. maritima* values with no PP_i or polyphosphate present

[n] apparent K_m for F-6-P with no ATP and $S_{0.5}$ for ATP with 1 mM AMP at pH 8.2

range from 0.4 mM to 4.3 mM. In the 10 out of 11 cases of bacterial PP_i-PFKs where the subunit and native structures have been determined, the enzymes are indicated to be dimeric except that for *Amycolaptosis methanolica* (Alves et al. 1996). For the protist PP_i-PFKs, examples of monomers have been reported in *Hexamita inflata* and *Giardia lamblia*, while the remainder are either dimeric (on average) or tetrameric in the case of *Naegleria fowleri*. The individual sizes for the bacterial subunits vary from 37 to as high as 62.4 kDa, while those reported for the protists range between 42 and 110 kDa. Plant PP_i-PFKs possess a narrower range of sizes, varying between 56 and 68 kDa with the smaller β subunit possessing catalytic properties and the α subunit having regulatory properties (Mertens et al. 1998; Blakeley et al. 1991). The PP_i-PFKs of bacteria and protists are generally nonallosteric with the exception of the *E. gracilis* and *N. fowleri* enzymes, which are activated by fructose-2,6-bisphosphate and AMP, respectively (Enomoto et al. 1988; Mertens et al. 1993). The plant PP_i-PFKs are universally activated by fructose-2,6-bisphosphate (Mertens et al. 1998).

Phylogeny of Family A PFKs

A BLASTP search of the nonredundant database and the Pfam database revealed the presence of 69 and 71 Family A PFK sequences, respectively (data not shown; Altschul et al. 1990; Sonnhammer et al. 1997). Initially, a total of 69 sequences were used to construct a near totally inclusive phylogenetic tree for Family A PFK sequences, but the topologies and the bootstrap values for key branchings of the tree were not strongly supported (not shown). To produce a tree with stronger bootstrap support for the major lineages, a number of sequences were excluded from the analysis and the lengths of the sequences themselves were reduced to approximately 220 residues, with the last residue being a strictly conserved glutamic acid, a strategy employed by other authors (Siebers et al. 1998; Mertens et al. 1998). An amino acid sequence alignment showing the regions of sequences that were used in the analysis is shown in Fig. 1. Residues shown to be involved in catalysis and secondary structure elements are indicated on the basis of analysis of the crystal structures (Shirakihara and Evans 1988; Rypniewski and Evans 1989; Evans and Hudson 1979; Shirmer and Evans 1990). Two of the most highly conserved motifs are a fructose-6-phosphate binding site P(K/G)TID(N/A/G)D at position 124–130 of the *E. coli* sequence and the amino acid motif MGR at position 170–172. For an analysis of the alteration of kinetic effects associated with the in vitro mutagenesis of key residues, see Deng et al. (1999), Chi and Kemp (2000), Mertens et al. (1998), Siebers et al. (1998), Ding et al. (2000) and Michels et al. (1997). Several sequences were excluded from the analysis, including that of the highly divergent *Propionibacterium freudenreichii* (Mertens et al. 1998) in addition to those of *Haemonchus contortus*, *Mastigamoeba balamuthi*, *Candida albicans, Spiroplasma*, *Mycoplasma*, *Mycobacterium*, multiple *Chlamydia* (3 sequences), *Aspergillus*, *Oryctolagus*, *Mus musculus*, *Clostridium,* and *Paenibacillus*. A phylogenetic tree produced using the neighbor-joining method and including the selected 55 Family A sequences is shown in Fig. 2. There are three large clusters of PFKs within the Family A sequences, similar to clusters found by several other authors, which have been defined as Groups I, II, and III by Siebers et al. (1998). This designation will be used here as well (see Fig. 2; Mertens et al. 1998; Alves et al. 1997; Ding et al. 2000). The Group I division contains two large subgroups, each with only either bacterial or eukaryal ATP-PFKs, and this separation between the bacterial and eukaryal ATP-PFKs has high bootstrap support (92%). The Group II enzyme division contains all of the protist PP_i-PFKs that have been characterized to date (Table 1) in addition to the plant PP_i-PFKs. There is a deep division in the group with the *Entamoeba*, *Trypanosoma*, *Oriza,* and α subunits of the *Treponema* and *Borrelia* clustering together, and this separation has strong support (97%). Group III enzymes are divided into two subclusters with the *Dictyoglomus* and *Thermoproteus* enzymes on one arm and the remainder, including the characterized *Amycolaptosis* and *Streptomyces* on the other, although this division is not well supported by the bootstrap value (43%). The *T. tenax* enzyme is the only archaeal (crenarchaeal) sequence within the Family A PFKs. The Group III enzymes of the thermophiles *T. tenax* and *Dictyoglomus* have short lineages, a result supported by the work of Mertens et al. (1998), Siebers et al. (1998), and Ding et al. (2000). Interestingly, the shortest branch lengths, although only marginally so, are those for the ATP-PFKs of the thermophiles *Thermotoga maritima*, *Thermus aquaticus,* and *Aquifex aeolicus*, in addition to those of the bacilli. Finally, two authors have previously noted the inclusion of ATP-dependent enzymes in *Trypanosoma brucei* and *Streptomyces coelicolor* clustering with PP_i-dependent enzymes, leading the authors to suggest that these ATP-PFKs must have evolved from a PP_i-PFK ancestral phenotype (Alves et al. 1997; Michels et al. 1997).

Biochemical characteristics of Family B PFKs

The best-characterized example by far of a Family B PFK is the minor PFK of *Escherichia coli*, termed PFK 2, which accounts for 5%–10% of the total enzyme activity and is encoded for by the *pfkB* gene (see Table 1; Fraenkel et al. 1973; Kotlarz and Buc 1982; Daldal 1984). Although PFK 2 is generally considered to be a minor PFK, mutant strains of *E. coli* lacking the main PFK 1 enzyme grow as fast as the wild-type strain with both PFKs present when grown on either glycolytic or gluconeogenic carbon sources (Robinson and Fraenkel 1978; Kotlarz and Buc 1981; Torres et al. 1997). This enzyme was originally reported to be nonallosteric but has been subsequently found to be inhibited by ATP when fructose-6-phosphate concentrations are low (Guixé and Babul 1985, 1998). In addition, ATP affects the oligomeric structure of the enzyme, inducing the formation of inactive tetramers in the presence of high concentrations of ATP, a process that is reversed by the presence of fructose-6-phosphate (Guixé and Babul 1988). Another feature that separates PFK 2 from Family A PFKs is that it is not activated by potassium or ammonium ions, and, at

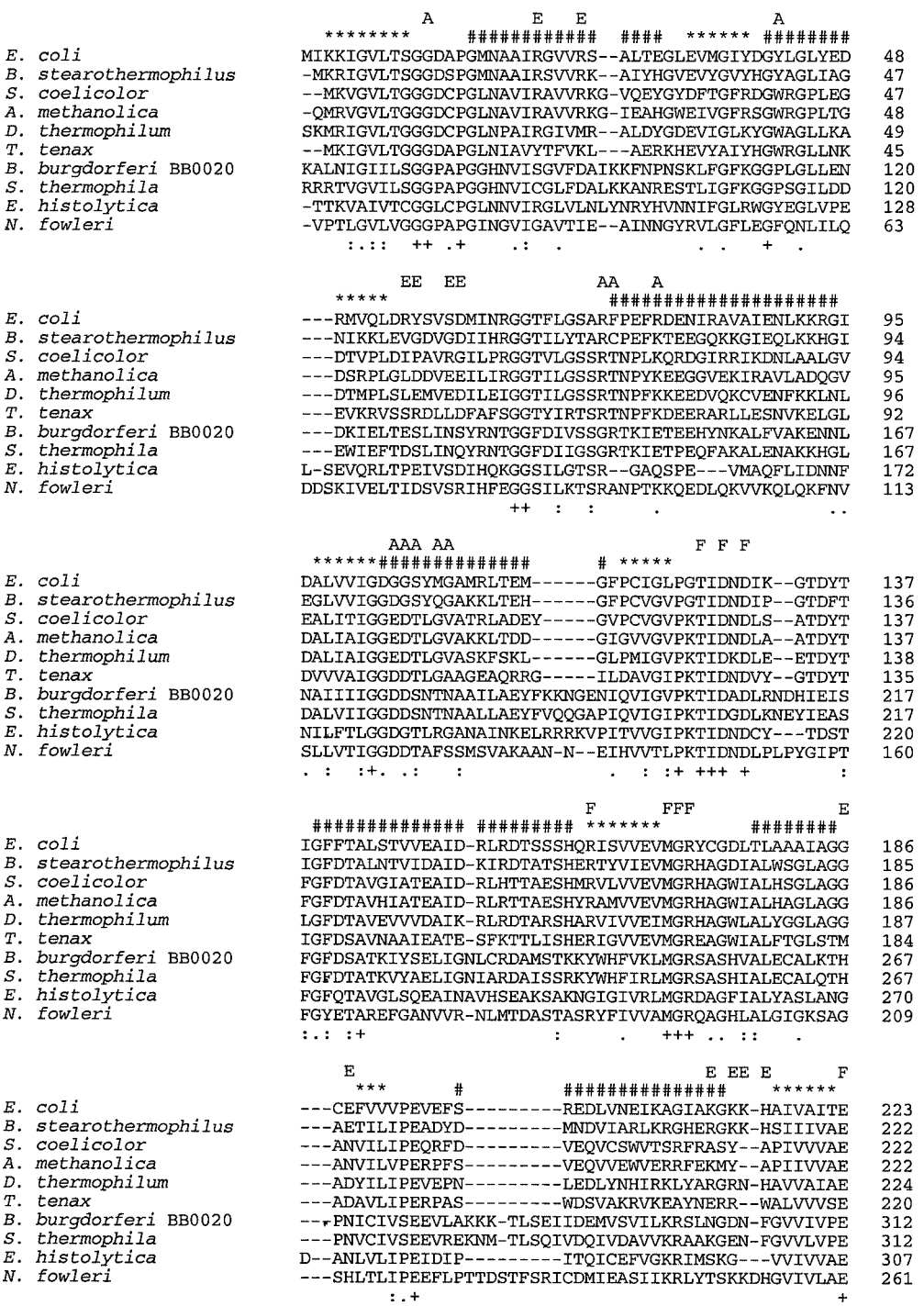

Fig. 1. Alignment of representative Family A phosphofructokinases (PFKs). The letters *A*, *F*, and *E* above the sequence blocks denote ATP, fructose-6-phosphate, and effector binding site residues, respectively. *Plus signs* below the sequences indicate strictly conserved residues, a *single dot* and *double dots* represent strong and weak conserved residues, respectively, according to Thompson et al. (1994). *Asterisks* above the sequences denote β sheet structures and *hash marks* α helical structures

least in *E. coli*, it is expressed constitutively (Kotlarz and Buc 1982; Uyeda 1979). Antibodies have been raised independently to both the major *E. coli* Family A and Family B PFK enzymes. The antibodies do not share any cross-reactivity (i.e., antibodies made to PFK 2 do not detect the major ATP-PFK and vice versa), and they have been used to screen various species of bacteria. Proteins reacting with the PFK 2 antibodies were detected in *Shigella boydii*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Klebsiella oxytocans* (Kotlarz and Buc 1982). A phosphoenolpyruvate-insensitive ATP-PFK activity was also detected in these species, strongly suggesting that a Family B PFK was also present, akin to the *E. coli* scenario described above (Kotlarz and Buc 1982).

Interestingly, a nonallosteric ATP-PFK displaying Michaelis-Menten kinetics has been purified and characterized from the hyperthermophilic crenarchaeal *Desulfurococcus amylolyticus* (Hansen and Schönheit 2000; Ding 2000). An N-terminal sequence was obtained from the protein, but no significant homology to other PFKs

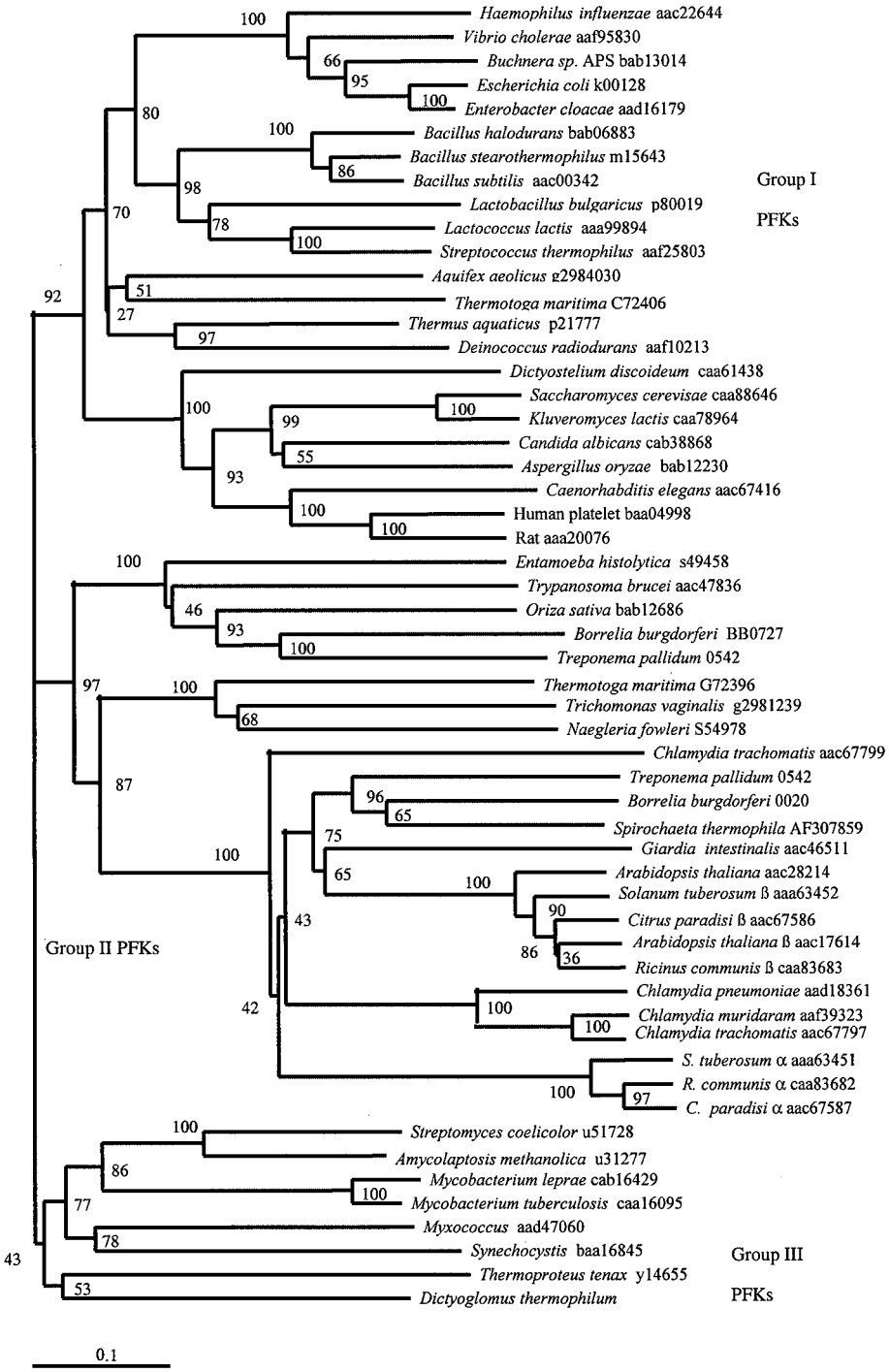

Fig. 2. Phylogenetic tree of Family A PFKs

was reported after searching the database (Hansen and Schönheit 2000). The release of the *Aeropyrum pernix* genome sequence, however, prompted us to use this N-terminal data to search the genome of this organism for homologues (Kawarabayasi et al. 1999). This crenarchaeal isolate is an aerobic, heterotrophic marine hyperthermophile able to grow at temperatures up to 100°C that is placed phylogenetically on a branch between the aerobic *Sulfolobus shibatae* and the anaerobes *Desulfurococcus amylolyticus* and *Pyrodictium occultum* (Sako et al. 1996). The best match obtained using an Expect value of 1,000 in a BLASTP search of the *Aeropyrum* database with the *Desulfurococcus* ATP-PFK N-terminus was with a putative sugar kinase with a PFKB family sequence signature (Altschul et al. 1990; Hoffman et al. 1999; Kawarabayasi et al. 1999). We have subsequently cloned and expressed this gene (APE 0012) in *E. coli* and have shown the protein to have an ATP and fructose-6-phosphate-dependent enzymatic activity that is active at 80°–95°C with a half-life of approximately 30 min at 95°C (unpublished data), thus confirming the presence of Family B ATP-PFKs within the crenarchaeal kingdom of the domain Archaea. Whether

the *Desulfurococcus* enzyme also falls into the PFKB family of enzymes is unknown at present, but the cloning of this enzyme is underway (Hansen and Schönheit 2000). If it does, then the current view that the Family B members of PFKs are only minor PFKs, both from a biochemical and phylogenetic perspective, should be reevaluated.

Phylogeny of Family B PFKs

When the *Aeropyrum pernix* gene APE 0012 was used to search the nonredundant database for homologues with TBLASTN and BLASTP search algorithms, 105 and 100 hits were obtained, respectively (results not shown; Altschul et al. 1990). A total of 119 sequences were retrieved using the Pfam database (Sonnhammer et al. 1997). A number of PFKB homologues were identified in archaea and hyperthermophilic bacteria, including *Pyrobaculum aerophilum* (1), *Pyrococcus horikoshii* (4), *Pyrococcus abyssi* (4), *A. pernix* (2), *Methanococcus jannaschii* (2), *Methanobacterium thermoautotrophicum* (3), *Archaeoglobus fulgidus* (2), *Sulfolobus solfataricus* (1), *Haloferax* sp. (1), *Halobacterium* strain NRC-1 (2), *Thermotoga maritima* (4), *Aquifex aeolicus* (1), and one in *Thermoplasma acidophilum* (unpublished results; Sonnhammer et al. 1997; Ruepp et al. 2000). By far the best match (random expectation value $>10^{30}$) was with a gene in *Pyrobaculum aerophilum*, suggesting that this organism is also likely to contain a Family B ATP-PFK. Another high-scoring homologue was found in *Halobacterium* strain NRC-1 (random expectation value $>10^{27}$). An amino acid sequence alignment with representative sequences from the multisubstrate PFKB enzyme family is shown in Fig. 3. The strictly conserved AGD at position 250–252 within the motif DTXGAGD (248–252) of the *Aeropyrum* sequence has been shown to form an anion hole within the *Toxoplasma gondii* adenosine kinase (Schumacher et al. 2000). Analysis of the three-dimensional structure of the human adenosine kinase suggests that the aspartic acid residue (D) of the motif plays a role in the deprotonation of the 5′-hydroxyl group during phosphotransfer (Schumacher et al. 2000; Sigrell et al. 1998). In addition, the strictly conserved glycine–glycine dipeptide (position 42–43 of the *A. pernix* gene 0012 gene product sequence) has been shown to help form the base of a pocket for binding the adenosine in the *T. gondii* adenosine kinase (Schumacher et al. 2000). Although all the PFKB signature sequences are clustered into a single family (Hoffman et al. 1999), the proteins themselves, as mentioned earlier, can catalyze a variety of reactions within the cell. Unfortunately, only two known Family B ATP-PFKs have been demonstrated to date (*E. coli* and *A. pernix*), and thus it would be speculative to produce a phylogenetic comparison of the homologues identified above among archaea and hyperthermophilic bacteria. In addition, there are several reasons, including that one could easily expect these enzymes to be under different evolutionary pressures, dependent on the individual organisms, their metabolic requirements, and their physiologies, which would result in differential rates of evolution (see also Wu et al. 1991). The organisms themselves are also likely to reflect different rates of evolution, particularly the aerobic hyperthermophiles shown, as these species of archaea appeared relatively late and evolved more rapidly than organisms such as *P. furiosus*, as indicated by the small ribosomal subunit branch lengths within the universal tree of life (Woese et al. 1990). A comparison of the *E. coli* ribokinase and *Bacillus stearothermophilus* crystal structures using the VAST algorithm combined with a direct comparison of known secondary structures (not shown) strongly suggests that the Family A and B PFKs are likely to have evolved independently (Shirakihara and Evans 1988; Sigrell et al. 1998; Madej et al. 1995; Gibrat et al. 1996; Schumacher et al. 2000).

Biochemical characteristics of the ADP-PFKs (Family C PFKs)

The biochemical characteristics of ADP-dependent PFKs (AMP-forming) that have been studied to date are shown in Table 1. The enzymes are specific for ADP as the phosphoryl donor, possessing little, if any, activity with either ATP (<10% and 6% control activity with ADP for the *P. furiosus* and *T. zilligii* enzymes, respectively) or PP_i (or polyphosphate) and are nonallosteric (Tuininga et al. 1999; Ronimus et al. 1999b, 2001a). Other nucleotide diphosphates, particularly guanosine diphosphate (GDP), are able to support significant levels of activity varying between 5% and 28% control activity (Tuininga et al. 1999; Ronimus et al. 2001a). The *T. zilligii* enzyme is, interestingly, far less sensitive to Cu^{2+} than most Family A PFKs (Gebhard et al. 2001). Polyphosphate and tripolyphosphate cannot support activity for either the *Thermococcus* ADP-PFK, the *P. furiosus* ADP-PFK, or the *P. furiosus* ADP-glucokinase (Ronimus et al. 1999b; Tuininga et al. 1999; Kengen et al. 1995). Several potential benefits have been proposed to accrue from using ADP instead of ATP. These include that ADP is slightly more thermostable than ATP, that ADP is produced during anabolic reactions involving kinases (with the ADP becoming available for the ADP-dependent kinases), and that an advantage would be gained during low energy charge conditions for the activation of sugars and their subsequent degradation in glycolysis (Kengen et al. 1994, 1995). The amounts of energy gained through the hydrolysis of ATP and ADP are equal and thus there is no direct energetic advantage achievable (Lehninger 1982; Kengen et al. 1994; Ronimus et al. 1999b). The apparent K_m values for fructose-6-phosphate range between 1.47 and 4.0 mM, values that are considerably higher on average than those for the Family A PFKs. The apparent K_m value for glucose is relatively high, 0.73 mM, for the ADP-glucokinase from *P. furiosus* (Kengen et al. 1995) and 0.033 mM for ADP. The optimum pH for the ADP-PFK reaction is slightly acidic, in contrast to the slightly alkaline optima for the Family A ATP-PFKs.

Phylogeny of ADP-PFKs (Family C PFKs)

An amino acid sequence alignment is shown in Fig. 4, and the respective relationships of Family C ADP-PFKs

Fig. 3. Amino acid sequence alignment for representative PFKB homologues. *Hash marks* above the sequence blocks denote α helices; *asterisks* above denote β sheets. *Plus* symbols below identify strictly conserved amino acid residues; *two dots* mark strong group conservation; *one dot* denotes weak group conservation (Thompson et al. 1994). Secondary structure is based on the structure of the *E. coli* ribokinase (Sigrell et al. 1998). *R. capsulatus, Rhodobacter capsulatus*; *X. campestris, Xanthomonas campestris*; *S. citri, Spiroplasma citri*; *P. aerophilum, Pyrobaculum aerophilum*; *A. pernix, Aeropyrum pernix*; *S. solfataricus, Sulfolobus solfataricus*; *P. horikoshii, Pyrococcus horikoshii*

homologues and related ADP-glucokinases that are available in the database are shown in the phylogram presented in Fig. 5 (Ronimus et al. 2001a). These enzymes possess a limited phylogenetic distribution, and thus far ADP-PFKs and their glucokinase counterparts have been found only within the kingdom Euryarchaea in both mesophilic and thermophilic members of the Methanococcales, and also in the orders Thermococcales and Methanosarcinales (Ronimus et al. 2001a; Verhees et al. 2000). As seen in the alignment, there are some strictly conserved residues that are likely to play key roles in the catalysis of this unique set of enzymes. There is only a very limited hint of homology with PFKs of the PFKB family, in the last C-terminal conserved **TVG**(I/L)**GD**TIS motif (corresponding identical residues are in bold), which shares some homology with the conserved D**TXGAGD** motif, also at the C-termini of the PFKB sequences (see Fig. 3; Schumacher et al. 2000; Henikoff and Henikoff 1994; Ronimus et al. 2001a). An additional strictly conserved glycine–glycine dipeptide at position 101–102 of the *T. zilligii* ADP-PFK sequence) may represent a counterpart to the *A. pernix* Family B ATP-PFK sequence and could be significant. A crystal structure will be required to demonstrate any connected evolutionary past between Family B ATP-PFKs and Family C ADP-PFKs. It is fairly obvious from the tree that a gene duplication event has led to the ADP-glucokinases and the ADP-PFKs (Fig. 5; Ronimus et al. 2001a).

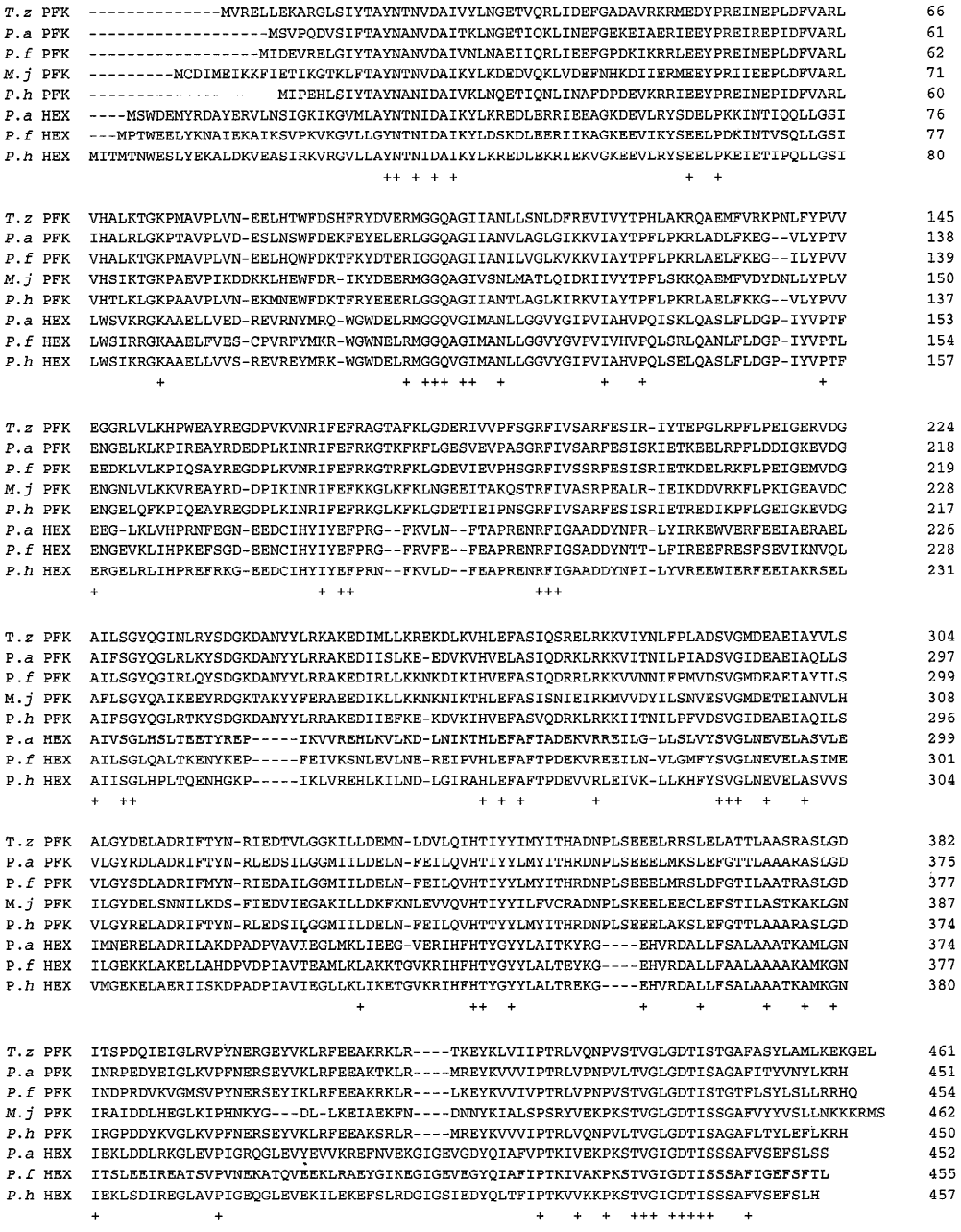

Fig. 4. Amino acid sequence alignment of ADP-PFKs and ADP-glucokinases (reproduced from Ronimus et al. 2001a). *Plus signs* identify residues that are strictly conserved. *T. z, Thermococcus zilligii*; *P. a, Pyrococcus abyssi*; *P. f, P. furiosus*; *P. h, P. horikoshii*; *M. j, Methanococcus jannaschii*; *HEX,* glucokinase

Discussion

We have summarized here the biochemical properties and phylogenetic relations of Family A (ATP- and PP_i-dependent), B (ATP-dependent), and C (ADP-dependent) PFKs. The phylogenetic trees presented, and their respective amino acid sequence alignments, highlight the presence of three sequence families for PFK activity. This has significant ramifications concerning the evolution of the glycolytic pathway. Three recent genome comparison studies (Dandekar et al. 1999; Galperin et al. 1998; Cordwell 1999), and phylogenetic/biochemical analyses of enzymes of the gluconeogenic and glycolytic pathways (Fothergill-Gilmore and Michels 1993; Brown and Doolittle 1997; Galperin et al. 1998; Schönheit and Schäfer 1995; Ding et al. 2000; Ronimus et al. 2001a) lend fairly strong support to the notion that the Embden-Meyerhof pathway most likely originated in a gluconeogenic direction, as suggested by Wächterhäuser (1988). Overall, the lower portion of the glycolytic pathway, or trunk pathway as it is also termed, involved with triose-phosphate transformations is more conserved (Galperin et al. 1998, 2000; Dandekar et al. 1999; Ronimus et al. 2001a). This is certainly supported by the multiplicity of PFK sequence types and ADP-glucokinases presented here (Ronimus et al. 2001a). Examination of the global distribution of the various PFK families within the small ribosomal subunit tree of life is instructive (Woese et al. 1990). As shown in the phylogenetic tree, the PFK Family A (Fig. 2) is distributed almost in its entirety between the

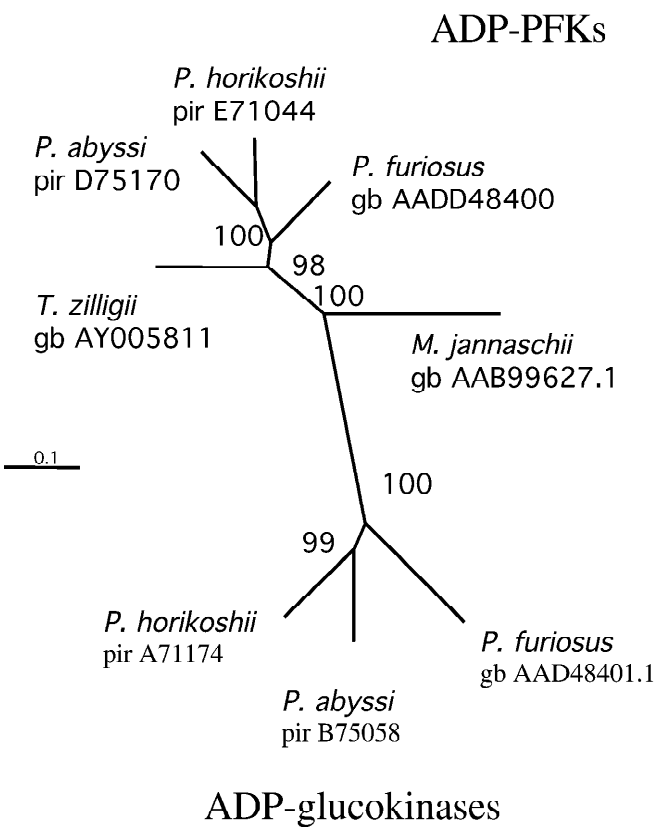

Fig. 5. Phylogenetic tree showing the relationship between the ADP-PFKs and the ADP-dependent glucokinases (reproduced from Ronimus et al. 2001a)

domains Bacteria and Eukarya (Woese et al. 1990). Only a single Family A sequence has so far been identified in the archaea, in the crenarchaeal *Thermoproteus tenax* (Siebers et al. 1998). The origin of the *T. tenax* enzyme is of interest because the enzyme may have resulted from a lateral gene transfer event, although the distribution of PFK subtypes in the Archaea remains relatively underinvestigated. Interestingly, an ATP-dependent PFK activity has been identified in the mesophilic *Methanococcus maripaludis* and partially characterized (Yu et al. 1994). The enzyme has a slightly unusual pH optimum of 6.0 and is apparently allosterically controlled because it is activated by inorganic phosphate, AMP and ADP, although it was not activated by the common eukaryal activator fructose-2,6-diphosphate. In the presence of inorganic phosphate, the enzyme was also activated by GDP and citrate. The phylogenetic origin of this enzyme is unknown, but given its allosteric nature, it resembles a Family A ATP-PFK. However, several other members of the orders Methanococcales, Thermococcales, and Methanosarcinales possess a Family C PFK, so the presence of a Family A PFK in *M. maripaludis* might also reflect a lateral gene transfer event. The presence of Family B ATP-PFKs in the crenarchaeal kingdom, exemplified now by the *Aeropyrum pernix* ATP-PFK (unpublished data), and probably also by *Desulfurococcus amylolyticus* and *Pyrobaculum aerophilum*, suggests that the PFKB ATP-PFK family is distributed more widely than recognized previously. The potential for a Family B ATP-PFK to be present in some halophilic archaea is suggested by the high-scoring homologue found in *Halobacterium* strain NRC-1. However, a recent study of glycolytic pathways of *Halococcus saccharolyticus* utilizing ^{13}C-nuclear magnetic resonance combined with screening for enzymatic activities has demonstrated a modified Embden-Meyerhof pathway involving the utilization of fructose via ketohexokinase and fructose-1-phosphate kinase, both traditional enzymes belonging to the PFKB family of sequences (Johnsen et al. 2001). In addition, in *Haloarcula vallismortis*, a PFK is not apparently present, and the organism instead uses a fructose-1-phosphate kinase with a molecular mass of approximately 60 kDa under denaturing conditions (approximately twice the molecular mass of PFKB enzymes), but whether this enzymes possesses a PFKB family sequence signature is unknown (Altekar and Rangaswamy 1990). Whether the PFKB homologues that have been identified through database searches in other archaeal species are genuine ATP-PFKs remains to be determined, but research in this area could prove fruitful. The determination of the crystal structure for the ADP-PFK would also be useful to remove all doubt of a phylogenetic linkage to other PFKs. In contrast to the wider distributions of the Family A and B PFKs within the domains, the Family C ADP-dependent PFKs are apparently limited thus far only to species of three orders of the kingdom Euryarchaea (Kengen et al. 1995; Ronimus et al. 1999b; Tuininga et al. 1999; Verhees et al. 2000; Ronimus et al. 2001a).

From energetic and kinetic perspectives, the PP_i-PFK is the only enzyme that would appear to have been capable of operating in both gluconeogenesis and glycolysis, i.e., it would have been able to serve nearly equally as well as a fructose-1,6-bisphosphatase. In the few cases where the reverse reaction catalyzed by either an ATP-PFK (both Family A and B) or an ADP-PFK has been examined, the specific activities of the reaction are on the order of 10- to 100-fold less than the corresponding forward direction suggesting in metabolic terms that the only reaction of relevance is in the glycolytic direction (Table 1). This is not unexpected given the large energy drop of 7.3 kcal mol^{-1} (30.5 kJ mol^{-1}; Lehninger 1982). As such, the PP_i-PFK, on these theoretical grounds and in line with the presumed gluconeogenic origin for the Embden-Meyerhof pathway (Wächterhäuser 1988), is the only PFK subtype able to have played a role in the development of gluconeogenesis without a fructose-1,6-bisphosphatase being present initially. Fructose-1,6-bisphosphatase (FBPase) is commonly present in both the bacterial and eukaryal domains, except in some cases where only a PP_i-PFK is present, for example in the *Thermotoga maritima*, *Borrelia burgdorferi*, and *Treponema pallidum* genomes (Nelson et al. 1999; Fraser et al. 1997, 1998). Recent BLASTP and PSI-BLAST sequence database searches revealed the presence of 100 FBPases, with only one being identified amongst the archaea, in the *Halobacterium* sp. NRC-1 (unpublished results; Altschul et al. 1990, 1997). Although a FBPase activity has been detected in the hyperthermophilic euryarchaeal *P. furiosus* (Schönheit and Schäfer 1995), and in *M. thermoautotrophicum* (Fuchs et al. 1983), *Methanococcus maripaludis* (Yu et

al. 1994), and *Methanothrix* strain FE (Pellerin et al. 1987), a classic FBPase sequence could not be detected in any of the other eight completely sequenced archaeal genomes, thus raising the possibility that another gene encoding for an enzyme with FBPase activity is present in these archaea. Recently, however, a gene product of *Methanococcus jannaschii* (MJ0109) was found to possess both a fructose-1,6-bisphosphatase and an inositol monophosphatase activity. A homologous gene for MJ0109 is found in each of the nine sequenced archaeal genomes (unpublished results; Altschul et al. 1997). If the presence of an alternate gene for FBPase is substantiated in archaea, then this would add further support, in addition to that resulting from the multiple sequence types within the Family C sugar kinases, to the notion that the Embden-Meyerhof pathway evolved from the bottom up.

Much discussion concerning whether PP_i, polyphosphate or ATP could have served as the original phosphoryl donor, especially for Family A PFKs has been published (Baltscheffsky 1996; Siebers et al. 1998; Alves et al. 1994, 1996; Michels et al. 1997; Morgan and Ronimus 1998; Mertens 1991; Ding et al. 2000, 2001). In light of the results presented here, equal consideration must now be given to ADP as a phosphoryl donor. The discussion relevant to the original energy source and phosphoryl donor has been recently been reinvigorated by an analysis of kinetic and in vitro mutagenesis data reported using the *E. coli* Family A ATP-PFK and the *Entamoeba histolytica* PP_i-PFK (Chi and Kemp 2000). In this study, a mutant *E. histolytica* PP_i-PFK was generated that differed from the wild-type enzyme by a single residue, which then altered its kinetic preference for PP_i to ATP. This was used to suggest that a latent nucleotide binding site was present and that the nucleotide-dependent enzyme type is the more primitive (Chi and Kemp 2000). In addition, in at least two cases, those of *Trypanosoma brucei* and *S. coelicolor*, only small changes were apparently required to convert a presumed PP_i-PFK into an ATP-PFK (Michels et al. 1997; Alves et al. 1997). Several pieces of data would be helpful in clarifying the apparent enigmatic evolution of Family A PFKs. Despite the knowledge that PP_i can serve as a phosphoryl donor within the PFKA family, no crystal structure is yet available for a PP_i-PFK, although diffractable crystals have been recently reported for the BB 0020 gene product encoding a *Borrelia burgdorferi* PP_i-PFK (Deng et al. 1999). Although this data would certainly be of major assistance, it would also be helpful to obtain a crystal structure from the minimally sized hyperthermophilic Family A PP_i-PFKs, for example, from either *Thermoproteus tenax* or *Dictyoglomus thermophilum*. As mentioned earlier, these two thermophile-derived enzymes have very short branches within the Family A PFK tree (Fig. 2). Also in support of the *T. tenax* and *D. thermophilum* PP_i-PFKs being ancient is the fact that three other phylogenetic analyses have indicated the early divergence of the Group III subgroup (Siebers et al. 1998; Mertens et al. 1998; Ding et al. 2000). The last universal common ancestor (LUCA) is generally considered to have arisen in a thermophilic, nearly anaerobic (microaerophilic) environment in which a PP_i-PFK could have represented a selective advantage. The advantage, however, might have equally related to the requirement of a PFK activity functioning in the gluconeogenic direction than for a system efficiently utilizing energy under anaerobic conditions. It is conceivable that, since the large *E. histolytica* enzyme is relatively more diverged (see Fig. 2), the original Family A PFK ancestor could have been PP_i-dependent. This ancestral enzyme could later have evolved into an ATP-dependent enzyme (as seen with the thermophilic bacterial and bacilli ATP-PFKs) but then later developed into a PP_i-PFK again (and in some cases, i.e., the mutated *E. histolytica* enzyme and the *Trypanosoma brucei* and *S. coelicolor* enzymes, back to an ATP-dependent form). Somewhat in opposition to this scenario, however, the ATP-PFKs of thermophiles and those of the bacilli also have short branch lengths (Fig. 2).

Unfortunately, there has been no systematic comparison of the ability of polyphosphate to support activity amongst PP_i-PFKs (Kemp and Tripathi 1993) or, to our knowledge, among Family B PFKs. The *Spirochaeta thermophila*, *Dictyoglomus thermophilum*, *Thermotoga maritima*, and *Toxoplasma gondii* PP_i-PFKs are able to utilize polyphosphate (Ding et al. 2000, 2001; Ronimus et al. 1999a, 2001b; Peng and Mansour 1992). During the characterization of the *Spirochaeta thermophila* PP_i-PFK, no activity was observed with polyphosphate at 1 mM unless additional magnesium was added to the reaction to overcome the extensive chelation (one magnesium bound per two phosphates) by the polyphosphate (Ronimus et al. 1999a). In contrast, polyphosphate was not able to replace PP_i with the *Propionibacterium*, *Entamoeba*, *Methylomonas,* and *Amycolatopsis* enzymes (Wood and Goss 1985; Beschastnyi et al. 1992; Alves et al. 1996). In addition, tripolyphosphate could not replace PP_i in the case of the *Isotricha prostoma* enzyme (Mertens et al. 1989). The potential regulation by polyphosphate (1.0 mM) was examined with the *Toxoplasma gondii* enzyme (Peng and Mansour 1992). It was reported to inhibit activity by 69% when the PP_i concentration was 50 μM (only 2% inhibition was observed when the PP_i concentration was increased to 1 mM), but no additional magnesium was added for these assays. The ATP-PFK of *Desulfurococcus* does not utilize PP_i or polyphosphate (Hansen and Schönheit 2000; Ding 2000). The Family C ADP-dependent PFKs of the euryarchaea (and the ADP-glucokinase of *P. furiosus*) do not seem to be able to use either PP_i or polyphosphate (Tuininga et al. 1999; Ronimus et al. 1999b; Kengen et al. 1995), even if additional magnesium is added to overcome the chelation effects of polyphosphates, for example, in the case of the *Thermococcus zilligii* ADP-PFK (Ronimus et al. 1999b), and the *P. furiosus* and the *T. zilligii* ADP-PFKs cannot use tripolyphosphate.

Recently, the two PFKs, one ATP- and the other PP_i-dependent (both activities are present in cells simultaneously), from the hyperthermophilic bacterium *Thermotoga maritima* were characterized (Ding et al. 2001). Remarkably, the data suggest that in *T. maritima* the ATP-PFK is not allosterically controlled as much by the usual bacterial ATP-PFK effectors such as citrate, GDP, or phosphoenolpyruvate, as it is by PP_i and polyphosphate. In addition, the nonallosteric PP_i-PFK has a lower apparent K_m (18-fold lower) and a higher V_{max} (1.5-fold higher) for poly-

phosphate compared with PP_i (Ding et al. 2001). The apparent K_m for tripolyphosphate is nearly 7-fold lower than that for PP_i. This raises the possibility that the enzyme may function in vivo as a polyphosphate-dependent PFK akin to the polyphosphate glucokinases characterized from *Mycobacterium tuberculosis* and *Propionibacterium* (Phillips et al. 1993; Hsieh et al. 1993; Wood and Goss 1985). Together, the data strongly suggest that PP_i and polyphosphate have an important regulatory role in this hyperthermophile. Whether this represents the vestige of a primordial metabolism is, however, somewhat in doubt because the PP_i-PFK seems to be more related to Group II Family A PFKs (see Fig. 2) and is thus possibly the result of a lateral gene transfer event.

Mertens (1991) proposed that the presence of PP_i-PFKs was a general reflection of the metabolic consequences of anaerobiosis (Mertens 1991), primarily owing to the gains in energy achieved by recycling PP_i instead of wasting the residual energy of hydrolysis by pyrophosphatase. Examination of Table 1 shows that there is, indeed, a good correlation between an anaerobic way of life and the presence of PP_i-PFKs. Examples, however, in which this correlation is not supported include *T. maritima*, with both an ATP- and a PP_i-PFK, the aerobic methanol-utilizing *Amycolaptosis methanolica*, and the aerobic methane oxidizer *Methylomonas methanica*, with PP_i-PFKs, and the ATP-PFK indicated to be present in the anaerobic hyperthermophilic *Aquifex aeolicus*. In addition, although bacilli and *E. coli* are generally known to be aerobes, these species also grow very well anaerobically with ATP-PFKs. Finally, *Spirochaeta halophila* is a facultative anaerobe, and it possesses a primarily PP_i-PFK-based activity (Ronimus et al. 1999a).

What has become clear from the summary presented here is that investigations concerning the origins of phosphofructokinase activity should now be cast more widely and take into account the multiple evolutionary origins of catalysis of fructose-6-phosphate by PFKs to their product fructose-1,6-bisphosphate. From the data presented, the Family A PFKs are seen to be distributed almost solely amongst the domains Bacteria and Eukarya as ATP- and PP_i-PFKs (Woese et al. 1990). The Family B ATP-PFKs possess a more limited distribution, being operative as PFKs in some enterobacterial genera and, at least, in the crenarchaeal genus *Aeropyrum* (probably also in *Desulfurococcus* and *Pyrobaculum*), but perhaps also in some euryarchaea. The ADP-PFKs are, to date, limited to the kingdom Euryarchaea. Overall, the data suggest that perhaps biochemical (and microbiology) textbooks should now reserve a few lines of text for these alternate PFKs, many of which are nonallosteric and thus counter to the norm, when they summarize the enzymes involved in glycolysis.

Note added in proof

While this paper was in the publication process, Ito et al. (2001) reported the crystal structure for a monomeric ADP-dependent glucokinase from *Thermococcus litoralis*. The structure, unexpectedly, shows similarities to both the human and the *Toxoplasma gondii* adenosine kinase, members of the ribokinase family to which PFKB enzymes are related (Ito et al. 2001). Very low levels of sequence identity were evident in a structural-based alignment between the *T. litoralis* glucokinase and members of the ribokinase family, and yet a common ancestral origin for the ADP- and the Family B ATP-dependent kinases was suggested. In addition, a Family B ATP-PFK from *Aeropyrum pernix* has been cloned and expressed in *E. coli*, and was found to possess ATP-specific PFK activity, confirming the presence of Family B enzymes within the crenarchaea (Ronimus et al. 2001c).

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