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Purification, microsequencing and cloning of spinach ATP-dependent phosphofructokinase link sequence and function for the plant enzyme

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Database
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Despite its importance in plant metabolism, no sequences of higher plant ATP-dependent phosphofructokinase (EC 2.7.1.11) are annotated in the databases. We have purified the enzyme from spinach leaves 309-fold to electrophoretic homogeneity. The purified enzyme was a homotetramer of ~52 kDa subunits with a specific activity of 600 $mU \cdot mg^{-1}$ and a K_m value for ATP of 81 μM. The purified enzyme was not activated by phosphate, but slightly inhibited instead, suggesting that it was the chloroplast isoform. The inclusion of adenosine 5′-(β,γ-imido)triphosphate was conducive to enzyme activitiy during the purification protocol. The sequences of eight tryptic peptides from the final protein preparation, which did not utilize pyrophosphate as a phosphoryl donor, were determined and an exactly corresponding cDNA was cloned. The sequence of enzymatically active spinach ATP-dependent phosphofructokinase suggests that a large family of genomics-derived higher plant sequences currently annotated in the databases as putative pyrophosphate-dependent phosphofructokinases according to sequence similarity is misannotated with respect to the cosubstrate.

Phosphofructokinase (PFK) catalyzes the phosphorylation of D-fructose 6-phosphate to D-fructose 1,6-bisphosphate. The enzyme has been extensively studied in a wide spectrum of prokaryotes and eukaryotes [1–7]. At least three forms of PFK are known that differ with respect to the phosphoryl donor. The classical PFK of mammals, yeast and eubacteria, a key enzyme of glycolysis, is ATP-dependent and subject to extensive allosteric regulation by various metabolites [8,9]. In plants, various protists, and some prokaryotes, pyrophosphate (PP_i)-dependent forms of PFK are known (EC 2.7.1.90) [10–13]. These enzymes share sequence similarity with ATP-dependent PFK (ATP-PFK) and are designated either as PP_i-PFK or as pyrophosphate:fructose-6-phosphate 1-phosphotransferase. They differ markedly with respect to their regulatory properties across species. Plant PP_i-PFK is subject to extensive allosteric regulation, in particular by fructose 2,6-bisphosphate [14], whereas the enzyme from various anaerobic protists is not [10,15]. Notably, ATP-dependent and PP_i-dependent PFKs interleave in molecular phylogenies, indicating that several independent changes of cosubstrate specificity have occurred during PFK evolution among eubacteria [16–18], among archaebacteria [2], and among eukaryotes [4]. A third form of PFK has been reported only from archaebacteria. It is ADP-dependent (ADP-PFK), belongs to the the ribokinase superfamily, typically occurs among archaebacteria that lack an Embden–Meyerhof pathway [19,20], and can accept acetyl phosphate as the phosphoryl donor [21].

Abbreviations
ATP-PFK, ATP-dependent phosphofructokinase; PFK, phosphofructokinase; PP_i, pyrophosphate; *So*PFK, *Spinacia oleracea* phosphofructokinase.

Higher plant ATP-PFK remains more elusive than its counterparts from other sources. In 1975, Latzko & Kelly [22] reported the existence of chloroplast- and cytosol-specific isoenzymes in spinach. Since then, the isoforms of ATP-PFK from various plant sources have been studied [23–34]. Spinach cytosolic ATP-PFK is activated by 25 mM phosphate, whereas the chloroplast enzyme is slightly inhibited [27,28,30–32]. Various effectors, including ADP, phosphoenolpyruvate, 3-phosphoglycerate, and phosphoglycolate, have been reported [27–29], and both the chloroplast and the cytosolic enzymes can accept ribonucleoside triphosphates other than ATP as the phosphoryl donor [35,36]. Chloroplast and cytosolic ATP-PFKs have also been partially purified and characterized from various green algae [29,37,38].

Higher plants also possess PP_i-PFK [23,24], which occurs only in the cytosol [33,34]. The subunit structure and sequence of higher plant $\alpha_2\beta_2$ heterotetrameric PP_i-PFK are known [12,39], but corresponding information about plant ATP-PFK is not available. ATP-PFK from potato tubers was purified to apparent homogeneity; the final prepartion was reported to consist of four different subunits (PFK_{a-d}) with molecular masses of 46 300, 49 500, 50 000 and 53 000 kDa, respectively [33]. More recently, two isoforms of ATP-PFK from banana fruit with native molecular masses of 210 and 160 kDa, respectively, but of unknown subunit composition, were partially purified [34]. However, plant ATP-PFK activity has never been experimentally linked to any specific protein sequence, because no purified ATP-PFK from any plant source has been sequenced to date, and nor has ATP-PFK activity been demonstrated for any putative plant ATP-PFK gene product by recombinant expression in heterologous systems. Although sequence comparisons have suggested that some database entries currently annotated as putative PP_i-PFK might in fact correspond to ATP-dependent enzymes [40], experimental evidence to support this suggestion is lacking. Here we report the purification of ATP-PFK from spinach leaves to electrophoretic homogeneity, its sequence, subunit composition, and putative chloroplast localization, and comparison with PFK sequences from other sources.

Results and Discussion

Purification and microsequencing of spinach PFK

The present purification protocol combined elements from previously published PFK purification protocols [6,33–35]. Anion exchange chromatography of crude extract from whole cells on DEAE Fractogel yielded only one peak of enzyme activity. This activity was further purified by gel filtration, sucrose gradient centrifugation, reactive dye affinity chromatography, and a MonoQ column (Table 1). A final step of hydroxylapatite chromatography was necessary to completely remove ribulose-1,6-bisphosphate carboxylase/oxidase from the sample, the large subunit of which comigrated with ATP-PFK in SDS/PAGE (Fig. 1). This step yielded 309-fold purified ATP-PFK, but was accompanied by the loss of 93% of total activity (Table 1).

Table 1. Purification of spinach ATP-PFK.

Purification step	Total activity (mU)	Total protein (mg)	Specific activity (mU·mg^{-1})	Purification (fold)
Crude extract	60 000	30 000	2	–
DEAE-Sepharose	520	200	2.6	1.3
Sephacryl S-400	420	100	4.2	2.1
Sucrose gradient	214	22.8	9.4	4.7
Reactive red	214	4.64	46	23
Mono Q	166	0.63	264	132
Hydroxylapatite	13	0.021	619	309

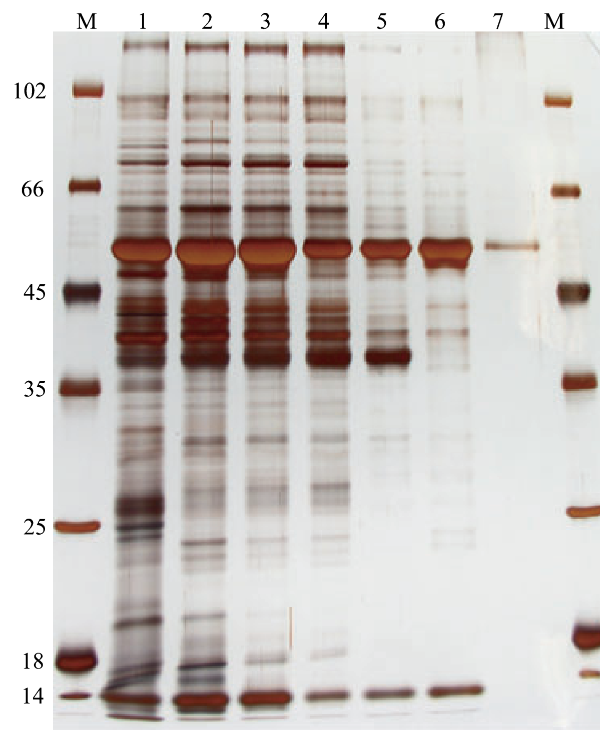


Fig. 1. Silver-stained 12% SDS/PAGE of spinach PFK from different purification steps. Lane 1: crude extract. Lane 2: DEAE fractogel eluate. Lane 3: Sephacryl S-400 HR eluate. Lane 4: concentrated protein after sucrose gradient centrifugation. Lane 5: reactive red eluate. Lane 6: Mono Q eluate. Lane 7: hydroxylapatite eluate. M: molecular mass standard (size indicated).

The final preparation contained a single, electrophoretically homogeneous, protein of 52 kDa (Fig. 1).

The 52 kDa protein was excised from a Coomassie-stained gel and digested with trypsin, and the resulting peptides were analyzed by ESI-Q-TOF MS/MS, yielding the sequences of eight different internal fragments (Fig. 2). The sequence of fragment NLEGGSLLGTSR is incomplete at the N-terminus, due to low resolution of the spectrum (data not shown). Database searches with the peptide sequences confirmed the purified protein as a member of the PFK family. Sequencing revealed no peptides from ribulose-1,6-bisphosphate carboxylase/oxidase, or peptides from any other protein, indicating that the 52 kDa band harbored ATP-PFK as the single major constituent.

PCR with degenerate oligonucleotides based on the sequences of peptides TIDNDILLMDK and YIDPTY (Fig. 2) yielded a 500 bp amplification product that was used as a hybridization probe for screening a *Spinacia oleracea* cDNA library [41]. One positive clone, p*So*PFK2, was detected, sequenced, and found to be N-terminally truncated, so its sequence was completed by 5′-RACE PCR. The conceptionally translated sequence encoded by p*So*PFK2 contained only one of the eight peptide sequences determined from the purified protein (Fig. 2). New degenerate primers were designed against the peptides LSGNAVLGDIGVHFK and EIYFEPTK, and produced a PCR fragment of 750 bp that was cloned and sequenced. The coding sequence was completed by 5′- and 3′-RACE, yielding *So*PFK1, which contained all eight peptides determined from the purified protein (Fig. 2). An *in silico*-generated mass spectrum of tryptic peptides of *So*PFK1 predicted fragments with masses corresponding to all eight sequenced peptides, confirming that the purified ATP-PFK was identical with *So*PFK1. Database searching with *So*PFK1 as a query revealed strongest similarity to sequences annotated as putative PP_i-dependent PFKs from higher plants.

Properties of spinach ATP-PFK

The sequence of p*So*PFK1 was 1829 bp long with an ORF of 1509 nucleotides, encoding a predicted protein

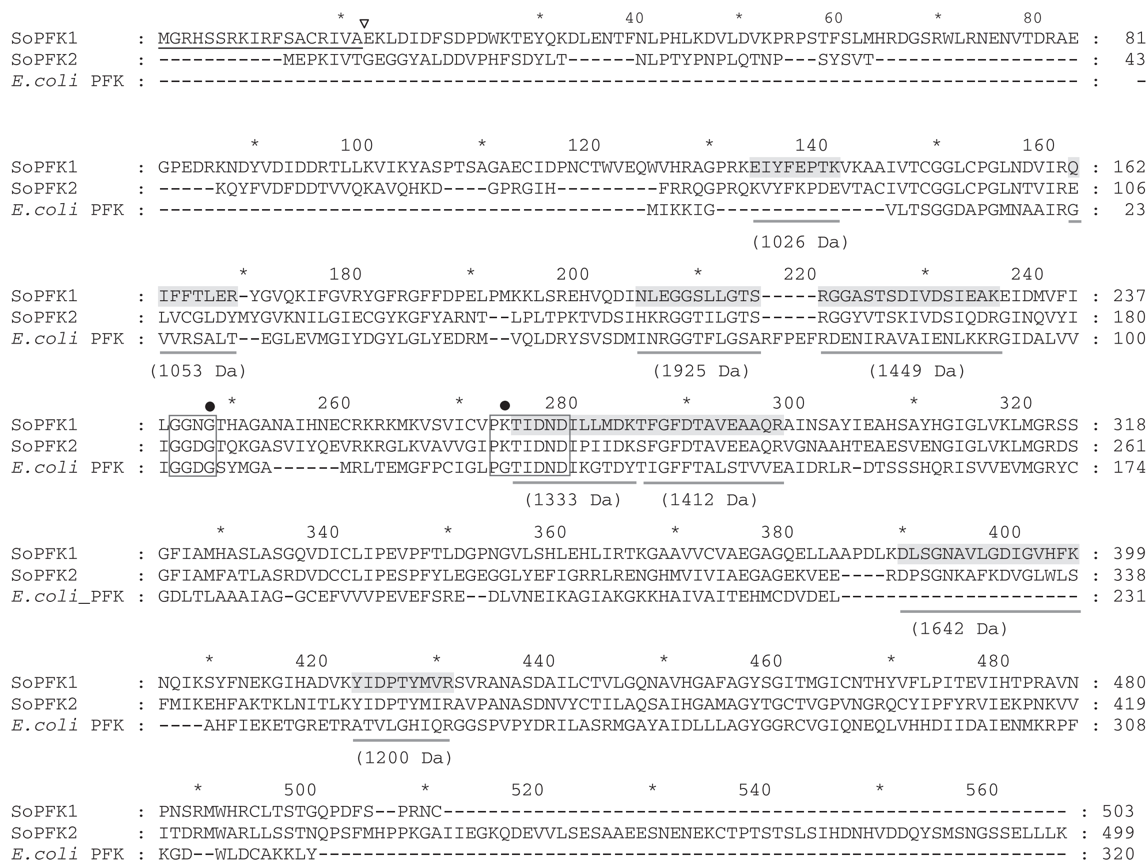


Fig. 2. Deduced protein sequencees of PFK from *Spi. oleracea* cDNA clones. Peptide sequences directly determined by MS are highlighted. The potential transit peptide is underlined, and the potential cleavage site is indicated by an arrow. Conserved regions for domains of atypical ATP-PFK are borderd and supported by the *Esc. coli* PFK sequence [39].

of 503 residues (Fig. 2). The predicted molecular mass of *So*PFK1 was 55.5 kDa, which was slightly larger than the 52 kDa determined for the purified protein. This difference could be due to a cleaved transit peptide for chloroplast targeting. *So*PFK1 has an N-terminal extension compared to *So*PFK2 and *Escherichia coli* PFK. The transit peptide-prediction programs CHLOROP [43] and IPSORT [42] did not recognize this extension as a chloroplast targeting sequence, but SIGNALP [42] identified a potential peptidase cleavage site (Fig. 2). Cleavage of the protein at this site would yield a mature protein of 53 kDa, which would be in better agreement with the size of the purified protein. The protein encoded by p*So*PFK2 had a calculated molecular mass of 55.3 kDa. *So*PFK2 and *So*PFK1 shared 45% amino acid identity, predominantly due to a conserved core region, but *So*PFK1 was about 50 amino acids longer at the N-terminus and 57 amino acids shorter at the C-terminus than *So*PFK2 (Fig. 2). Gel filtration of partially purified spinach PFK yielded a molecular mass ~200 kDa for the native enzyme (Fig. 3), consistent with the native mass of 210 kDa reported for banana PFKI [34].

Purified spinach PFK had a specific enzyme activity of 600 $mU \cdot mg^{-1}$ with ATP as phosphoryl donor. The enzyme did not utilize PP_i as a cosubstrate (Fig. 4). The K_m was 1.7 mM for D-fructose 6-phosphate and 81 µM for ATP (supplementary Fig. S1), which was significantly higher than the K_m of 30 µM reported for the cytosolic isoenzyme [28]. PFK activity was inhibited by the addition of 25 mM phosphate (Fig. 4), a typical feature of chloroplast ATP-PFK [27,28,32], and in contrast to the stimulation of the cytosolic isoenzyme observed in spinach and other plants [27]. These data suggest that the purified protein was the chloroplast isoenzyme, although the level of inhibition was only 17%, and thus considerably lower than the 50% reported previously [27,28]. This discrepancy could be due to the fact that the protein was eluted with phosphate from the final hydroxylapatite column. Instability of the protein prevented removal of the phosphate from the PFK preparation by dialysis prior to activity measurements and led to complete loss of activity, so this question could not be answered.

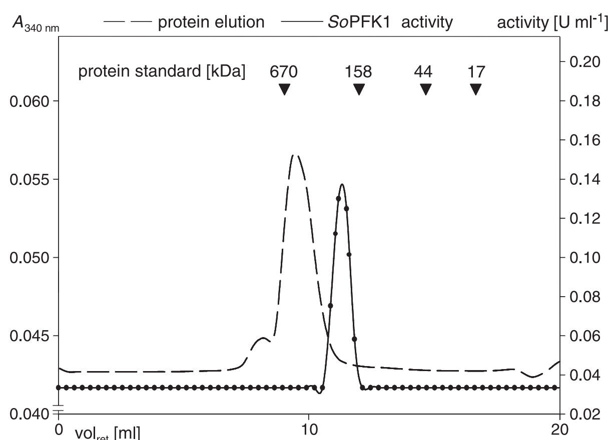


Fig. 3. Elution profile for *So*PFK1 after gel filtration chromatography on a Superdex 200 HR 10/30 column. Marker protein sizes are indicated by arrows.

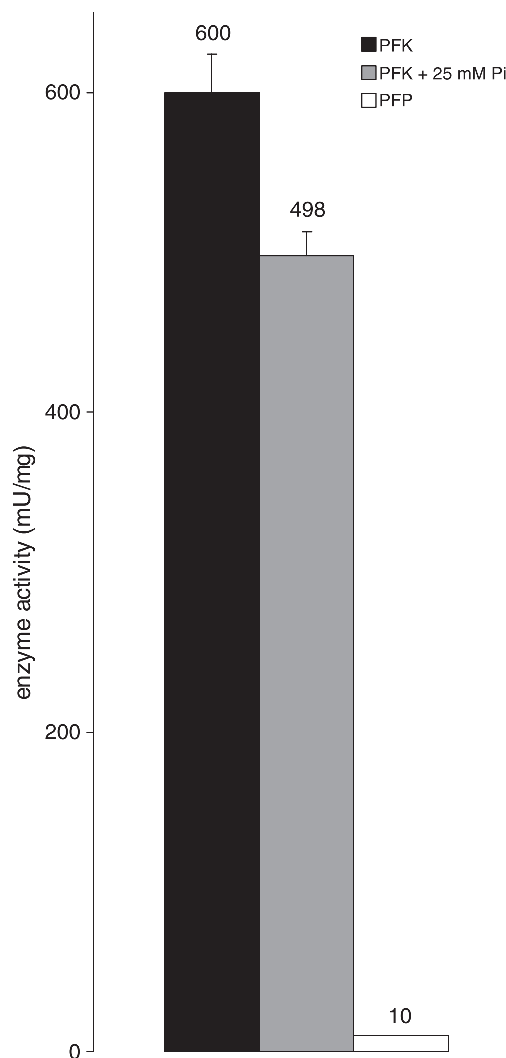


Fig. 4. Spinach PFK activity assayed in the absence (black bar) or presence (gray bar) of 25 mM phosphate. The white bar shows pyrophosphate:fructose-6-phosphate 1-phosphofructokinase activity.

Subunit composition

Previous purifications of plant ATP-PFK suggested that the enzyme consists of two [33] or four [32,34]

subunits with molecular masses between 50 and 70 kDa. Our final active preparation revealed a single 52 kDa subunit (Fig. 1). Eight peptides determined from that protein all mapped to the cDNA sequence of *So*PFK1, and importantly, we obtained no sequences of tryptic fragments that did not map to *So*PFK1. Together with the finding that gel filtration yielded a molecular mass for the active enzyme of 200 kDa, this indicates that the purified spinach enzyme is a homotetramer of ~ 52 kDa subunits, similar to the putative chloroplast enzyme from banana fruit [34]. However, the specific activity of the electrophoretically homogeneous spinach enzyme is 10-fold lower than that of banana, and 300-fold lower than that of the PFKs from potato tuber, which consisted of four different subunits [33]. It would seem likely that the elution by phosphate, an inhibitor of the chloroplast enzyme, contributes to the lower activity in the final purification step [27,28,30]. Hence, we cannot exclude the possibility that additional subunits, as observed in potato, interact in the spinach tetramer *in vivo* in such a way as to increase the specific activity, and that these were removed during purification. *So*PFK2 could be such an additional subunit, but the homogeneity of our final active preparation does not suggest a heteromeric composition of the 200 kDa enzyme purified here.

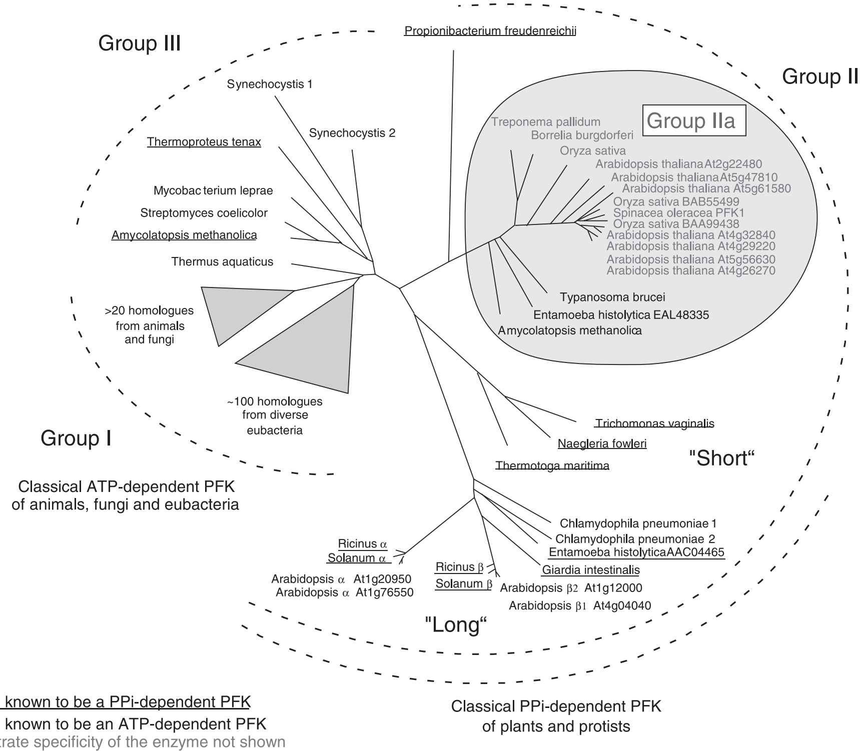
Our attempts to express the *So*PFK1 subunit in active form in the PFK-deficient *PFK2/PFK1* double mutant yeast strain HD114-8D [5] under the control of the Gal promoter in the plasmid pYES2/CT failed to generate strains possessing detectable ATP-PFK activity (data not shown), although we obtained immunologically detectable C-terminally His-tagged *So*PFK1 in the soluble fraction of transformants. The heat activation treatment that was successfully used to restore highly specific ATP-dependent activity of the *Entamoeba* enzyme [6] also failed for the heterologously expressed spinach protein. We are aware of no reports in which plant ATP-PFK activity has been obtained via heterologous expression in any system; the reasons for this remain obscure. It is conceivable that the potential N-terminal targeting peptide interfered with correct folding of the protein or subunit interaction in the heterologous system.

Higher plant ATP-PFK sequence comparisons

Spinach ATP-PFK clustered within the class of PFK sequences that have been previously designated as group II [1,2] in the larger scheme of PFK sequence diversity, as sketched in Fig. 5A, where it is evident that some organisms, such as the actinomycete *Amycolatopsis methanolica*, possess two very distinct PFK types [7]. Within group II, *So*PFK1 clustered with *Oryza sativa* and *Arabidopsis thaliana* PFK homologs, as does *So*PFK2 (Fig. 5B). In sequence alignments, many of the group II plant enzymes show a long N-terminal extension (labeled 'Nex' in Fig. 5B), which in some cases are predicted as a chloroplast import signal (labeled 'pCp') by CHLOROP 1.1 and IPSORT [42,43], but these sequences interleave with other homologs that lack N-terminal extensions. *So*PFK1 has an N-terminal extension relative to prokaryotic homologs that is reminiscent of a chloroplast transit peptide, but the protein was not predicted to be chloroplast targeted by CHLOROP, although the N-terminal extension present in some rice and *Arabidopsis* homologs did predict chloroplast targeting (Fig. 5B). Nevertheless, SIGNALP [44] predicted a potential cleavage site between residues 18 and 19 (Fig. 2). The distribution of the presence of N-terminal extensions and predicted chloroplast transit peptides for plant PFK homologs did not correspond with sequence similarity (Fig. 5B).

Among the published sequences that fall within the cluster of sequence similarity designated here as group IIa, there are few with demonstrated function, and only ATP-dependent activity has been shown for members of this group (Fig. 5B). PFK enzymes can change their cosubstrate specificity for PP_i or ATP through mutations at a very few specific residues [1–4,39], and within group II, both PP_i- and ATP-dependent enzymes are known (Fig. 5A,B), as are the residues that confer PP_i or ATP dependence by virtue of cosubstrate interactions at the active site [3,4]. The crucial residues, Gly105 and Lys124, in the *Esc. coli* enzyme [45] are conserved in the atypical and previously uncharacterized ATP-dependent PFK sequences [3,4,6,39], and they are also conserved in *So*PFK1 and *So*PFK2 (Fig. 2). From these observations, we conclude that annotation of PFK sequences with respect to their phosphoryl donor specificity cannot be done on the basis of general sequence similarity but has to be based on the amino acid constellation at positions corresponding to *Esc. coli* 105 and 124 and biochemical data.

With the notable exception of the *Thermoproteus tenax* PP_i-PFK [2], archaebacteria usually possess an ATP- or ADP-dependent PFK that is highly distinct from the eubacterial and eukaryotic enzyme, but instead is related to ribokinases [19,20]. Perhaps the most striking aspect of PFK gene diversity is the general absence of the eukaryotic- and eubacterial-type PFK among archaebacteria, and vice versa. This supports earlier conclusions [46], despite reports to the contrary [47] that eukaryotes generally possess a eubacterial type of glycolytic pathway [48].


A
Group III
Propionibacterium freudenreichii
Group II
Synechocystis 1
Synechocystis 2
Thermoproteus tenax
Mycobacterium leprae
Streptomyces coelicolor
Amycolatopsis methanolica
Thermus aquaticus
>20 homologues from animals and fungi
~100 homologues from diverse eubacteria
Group I
Classical ATP-dependent PFK of animals, fungi and eubacteria
Group IIa
Treponema pallidum
Borrelia burgdorferi
Oryza sativa
Arabidopsis thaliana At2g22480
Arabidopsis thaliana At5g47810
Arabidopsis thaliana At5g61580
Oryza sativa BAB55499
Spinacea oleracea PFK1
Oryza sativa BAA99438
Arabidopsis thaliana At4g32840
Arabidopsis thaliana At4g29220
Arabidopsis thaliana At5g56630
Arabidopsis thaliana At4g26270
Typanosoma brucei
Entamoeba histolytica EAL48335
Amycolatopsis methanolica
Trichomonas vaginalis
Naegleria fowleri
Thermotoga maritima
"Short"
Chlamydophila pneumoniae 1
Chlamydophila pneumoniae 2
Entamoeba histolytica AAC04465
Giardia intestinalis
Ricinus α
Solanum α
Arabidopsis α At1g20950
Arabidopsis α At1g76550
Ricinus β
Solanum β
Arabidopsis β2 At1g12000
Arabidopsis β1 At4g04040
"Long"
Enzyme known to be a PPi-dependent PFK
Enzyme known to be an ATP-dependent PFK
Cosubstrate specificity of the enzyme not shown
Classical PPi-dependent PFK of plants and protists

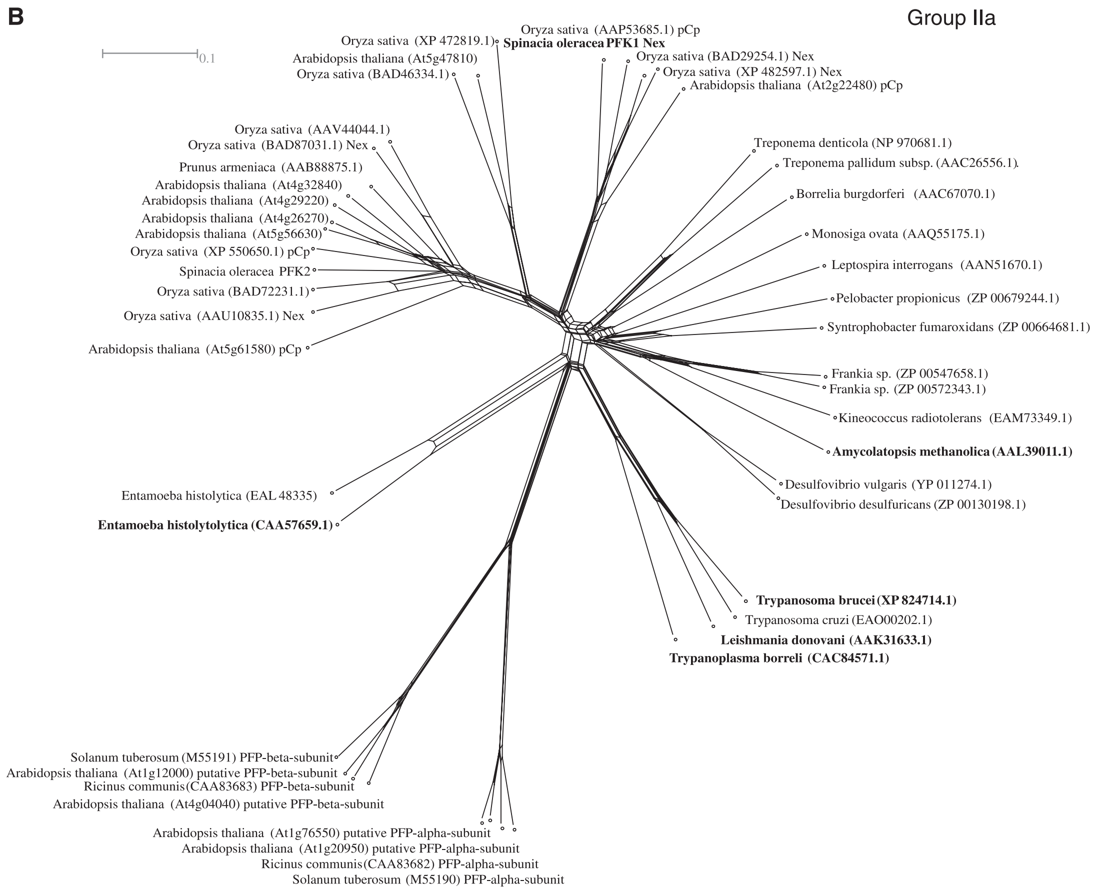

B
Group IIa
0.1
Oryza sativa (AAP53685.1) pCp
Oryza sativa (XP 472819.1)
Spinacia oleracea PFK1 Nex
Arabidopsis thaliana (At5g47810)
Oryza sativa (BAD46334.1)
Oryza sativa (BAD29254.1) Nex
Oryza sativa (XP 482597.1) Nex
Arabidopsis thaliana (At2g22480) pCp
Oryza sativa (AAV44044.1)
Oryza sativa (BAD87031.1) Nex
Prunus armeniaca (AAB88875.1)
Arabidopsis thaliana (At4g32840)
Arabidopsis thaliana (At4g29220)
Arabidopsis thaliana (At4g26270)
Arabidopsis thaliana (At5g56630)
Oryza sativa (XP 550650.1) pCp
Spinacia oleracea PFK2
Oryza sativa (BAD72231.1)
Oryza sativa (AAU10835.1) Nex
Arabidopsis thaliana (At5g61580) pCp
Treponema denticola (NP 970681.1)
Treponema pallidum subsp. (AAC26556.1)
Borrelia burgdorferi (AAC67070.1)
Monosiga ovata (AAQ55175.1)
Leptospira interrogans (AAN51670.1)
Pelobacter propionicus (ZP 00679244.1)
Syntrophobacter fumaroxidans (ZP 00664681.1)
Frankia sp. (ZP 00547658.1)
Frankia sp. (ZP 00572343.1)
Kineococcus radiotolerans (EAM73349.1)
Amycolatopsis methanolica (AAL39011.1)
Desulfovibrio vulgaris (YP 011274.1)
Desulfovibrio desulfuricans (ZP 00130198.1)
Entamoeba histolytica (EAL 48335)
Entamoeba histolytolytica (CAA57659.1)
Trypanosoma brucei (XP 824714.1)
Trypanosoma cruzi (EAO00202.1)
Leishmania donovani (AAK31633.1)
Trypanoplasma borreli (CAC84571.1)
Solanum tuberosum (M55191) PFP-beta-subunit
Arabidopsis thaliana (At1g12000) putative PFP-beta-subunit
Ricinus communis(CAA83683) PFP-beta-subunit
Arabidopsis thaliana (At4g04040) putative PFP-beta-subunit
Arabidopsis thaliana (At1g76550) putative PFP-alpha-subunit
Arabidopsis thaliana (At1g20950) putative PFP-alpha-subunit
Ricinus communis(CAA83682) PFP-alpha-subunit
Solanum tuberosum (M55190) PFP-alpha-subunit

Fig. 5. Sequence similarity among PFK homologs. Sequences that have been shown to specify PP_i-dependent PFK activity are indicated by black underlined text, sequences that specify ATP-PFK activity are in black, and sequences without biochemical characterization are in gray. (A) Schematic representation of sequence similarities among the larger family of PFK enzymes following the group I, II and III nomenclature of Siebers *et al.* [2] and Müller *et al.* [1], including the 'long' and 'short' families [1]. The scheme in (A) is not intended to represent evolutionary relationships, but is instead intended to show where the previously uncharacterized plant sequences within group IIa fit into the overall diversity of biochemically characterized PFK sequences. (B) NEIGHBORNET planar graph of sequence similarities among representatives from the fuller spectrum of currently available database sequences that fall within group IIa. The scale bar indicates substitutions per site.

Materials and methods

Strains and media

Escherichia coli strain XL1-Blue MRF′ (Stratagene, Heidelberg, Germany) was used for plasmid handling. *Saccharomyces cerevisiae* strain AST9-1B (*Mata pfk::LEU2 PFK1::TRP1 ura3-52 his3-11, 15 leu2-3, 112 trp1::loxP Mal2-8c SUC 2 GAL*), kindly provided by J J Heinisch (University of Osnabrück, Germany), was used as a recipient strain for heterologous expression of spinach PFK enzymes.

Enzyme assay

PFK activity was determined spectrophotometricaly at 30 °C in 50 mM Hepes/NaOH (pH 7.8), 0.5 mM $MgCl_2$, 0.15 mM NADH, 0.6 mM ATP, 2 mM dithiothreitol, 10 U of triose-phosphate isomerase, 1 U of glycerol-3-phosphate dehydrogenase and 1 U of aldolase [49,50] in a final assay volume of 200 μL (GENios Microplate Reader; Tecan Instruments, Crailsheim, Germany). The reaction was initiated by addition of 0.4 mM D-fructose 6-phosphate, and activity was determined by decrease in absorbance at 340 nm. For discrimination between the chloroplast and cytosolic isoforms, 25 mM NaH_2PO_4 was added to the assay [31,32]. For measurement of PP_i utilization, ATP was substituted with 0.6 mM PP_i, and 10 μM fructose 2,6-bisphosphate was added, because PP_i-PFK is stimulated by fructose 2,6-bisphosphate [51]. The K_m for ATP was measured at a D-fructose 6-phosphate concentration of 0.4 mM in the presence of 3 U of creatine kinase and 1 mM creatine phosphate for continuous ATP regeneration. K_m values were determined with Hanes–Woolf plots.

Purification of spinach ATP-PFK

All procedures were carried out at 4 °C unless indicated otherwise. Raw extract was prepared from 1.3 kg of 5–8-week-old spinach leaves (*Polka*) by homogenization in a Waring Blender (Torrington, CT, USA) using 250 mL of 50 mM Tris/HCl (pH 7.8), 2 mM dithiothreitol, 5 mM MgOAc, 1 mM ATP, 0.1% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 1 mM iodoacetate, 10 g of Polyclar AT and 2.5 mL of 100 × Protease Inhibitor Mix (Sigma, Taufkirchen, Germany). The homogenate was filtered through two layers of cheesecloth, and the filtrate was centrifuged twice at 31 000 ***g*** for 30 min (RC5B plus with SLA 1500 rotor; Sorvall, Hanau, Germany). The supernatant was applied to a 3 × 16 cm column of DEAE Fractogel 650 S (Merck, Darmstadt, Germany) previously equilibrated with buffer A [50 mM Tris/HCl, pH 7.8, 2 mM dithiothreitol, 5 mM MgOAc, 1 mM ATP, 1 mM iodoacetate, 10% (v/v) glycerol]. After washing of the column with 86 mL of buffer A, proteins were eluted in a 100 mL gradient of 0–900 mM KCl in buffer A and collected in 2.5 mL fractions.

Fractions with ATP activity were pooled, and the volume was reduced to 10 mL by ultrafiltration on Amicon Ultra filter devices (Millipore, Eschborn, Germany). The concentrated protein was applied to a 3 × 60 cm Sephacryl S-400 HR column (Pharmacia, Freiburg, Germany) equilibrated with buffer C [50 mM Tris/HCl, pH 7.8, 2 mM dithiothreitol, 5 mM MgOAc, 1 mM iodoacetate, 150 mM NaCl, 10% glycerol, 1 mM adenosine 5′-(β,γ-imido)triphosphate tetralithium salt hydrate]. Proteins were eluted with 2 L of buffer C in fractions of 2% of the column bed volume. Fractions containing ATP-PFK activity were pooled, concentrated as described above, and desalted into buffer D [20 mM Tris, pH 7.8, 2 mM dithiothreitol, 5 mM MgAc, 10% glycerol, 1 mM adenosine 5′-(β,γ-imido)triphosphate tetralithium salt hydrate, 5% (w/v) sucrose] on two PD-10 columns (Amersham Biosciences, Freiburg, Germany). Protein samples were layered on 10 mL 5–20% sucrose gradients in 20 mM Tris (pH 7.8), 2 mM dithiothreitol, 5 mM MgAc, 1 mM adenosine 5′-(β,γ-imido)triphosphate tetralithium salt hydrate and 10% glycerol.

After centrifugation for 19.5 h in an SW 40 Ti (Beckman, Munich, Germany) rotor at 100 000 ***g***, 500 μL fractions were collected from the gradient. Fractions with ATP-PFK activity were pooled, concentrated to a volume of 2.5 mL on Amicon Ultra Centrifugal filter units (Amicon, Witten, Germany), and desalted into buffer A on PD-10 columns. The protein was applied to a 0.7 × 9 cm Reactive Red120 column (Sigma) equilibrated in buffer A, and eluted with a 30 mL gradient of 0–1 M KCl in buffer B. Fractions with ATP-PFK activity were collected, concentrated, and dialyzed against buffer E (20 mM Tris, pH 7.8, 2 mM dithiothreitol, 5 mM MgAc, 10% glycerol) as above. The concentrated protein was loaded onto a 1 mL MonoQ HR 5/5 anion exchange column (Amersham Biosciences) equilibrated in buffer E and eluted into 0.3 mL fractions with a 15 mL 0–400 mM KCl gradient in buffer E. Samples with

ATP-PFK activity were pooled and dialyzed against buffer F (20 mM Tris, pH 7.2, 2 mM dithiothreitol, 5 mM MgAc, 10 mM KH_2PO_4). In a final chromatographic purification step, protein was loaded on a Bio-Gel HT hydroxylapatite column (Bio-Rad, Munich, Germany) equilibrated in buffer F. The eluate of a 12 mL gradient of 10–500 mM KPO_4 in buffer F was collected in 0.3 mL fractions and assayed for PFK activity. Active fractions were pooled, and purity of the preparation was determined by SDS/PAGE.

Molecular mass determination

ATP-dependent PFK was partially purified by DEAE Fractogel 650 S, Sephacryl S-400 HR and Reactive Red120 chromatography as described above. Fractions with ATP activity were pooled and dialyzed against buffer G (150 mM NaCl, 20 mM Tris, pH 7.8, 1 mM ATP, 2 mM dithiothreitol, 5 mM MgAc, 5% glycerol). The sample was applied to a Superdex 200 HR 10/30 column (Amersham Biosciences) equilibrated with buffer G. Proteins were eluted with 40 mL of buffer G. Fractions of 0.5 mL were collected and assayed for ATP-PFK activity. The gel filtration mass standard (Bio-Rad) was eluted under the same conditions.

In-gel tryptic digestion, peptide sequencing and protein identification

Purified ATP-PFK was cut out of a SDS/PAGE gel and digested with trypsin [52]. Peptides were sequenced by nanoelectrospray tandem MS on a QSTAR XL mass spectrometer (Applied Biosystems, Darmstadt, Germany) as previously described [53].

Cloning of PFK genes

Degenerate primers were designed on the basis of peptide sequences determined from the purified protein. For *So*PFK1, the peptide fragments EIYFEP and GNAVLG were selected. For *So*PFK2, TIDNDI and YIDPTY were used for primer generation. Degenerate oligonucleotide pairs (5′-GARATYTAYTTYGARCCT-3′/5′-WCCVARAACAGCRTTTCC-3′, *So*PFK1; and 5′-ACHATYGAYAAYGATATT-3′/5′-RTABGTDGGTRCTATGTA-3′, *So*PFK2) were incubated with 10 ng of cDNA substrate for 10 min at 98 °C, and this was followed by 30 cycles of 1 min at 55 °C (*So*PFK1) or 50 °C (*So*PFK2), 1 min at 94 °C, and 1 min at 72 °C, with a final step of 10 min at 72 °C in the presence of 2 mM MgOAc, 0.25 mM dNTP, and 2.5 U of Triple-Master polymerase (Eppendorf, Hamburg, Germany) in the supplier's buffer. The PCR fragment was cloned into pBluescript SK+ (Stratagene), sequenced, and used as a hybridization probe to screen recombinant clones of an *Spi. oleracea* cDNA library according to the manufacturer's instructions.

Phylogenetic analysis

Sequences of 49 represenative PFK homologs were retrieved from GenBank (http://www.ncbi.nlm.nih.gov) and aligned with CLUSTALW [54]. Protein LogDet distances were calculated with the program LDDIST [55]. NEIGHBORNET planar graphs of LogDet distances were constructed with NEIGHBOR-NET55 and visualized with SPLITSTREE [56].

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Supplementary material

The following supplementary material is available online:

Fig. S1. K_m values for ATP and D-fructose 6-phosphate of *Spinacea oleracea* PFK1 calculated by Hanes–Woolf plots.

Fig. S2. Hydroxylapatite FPLC elution profile of spinach phosphofructokinase.

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