Phosphofructokinase (PFK) is one of the key enzymes in glycolysis. The textbook version of ATP-dependent PFK (ATP-PFK) is present throughout the domains of Eucarya, Bacteria, and Archaea, although it is not yet known if the single ancestral ATP-PFK recently described is related by sequence (6, 11, 15). Since the first pyrophosphate-dependent PFK (PPi-PFK) was reported by Reeves et al., who identified it in Entamoeba histolytica (21), a large number of PPi-PFKs have been found in higher plants, primitive eukaryotes, and bacteria, and a single example has been found in archaea (14, 17, 22, 25, 28). It has been suggested previously that PPi-PFKs may represent a single example has been found in archaea (14, 17, 22, 25, 28). It has been suggested previously that PPi-PFKs may represent a single example has been found in archaea (14, 17, 22, 25, 28). It has been suggested previously that PPi-PFKs may represent a single example has been found in archaea (14, 17, 22, 25, 28). It has been suggested previously that PPi-PFKs may represent a single example has been found in archaea (14, 17, 22, 25, 28).
FIG. 1. Multiple alignment of amino acid sequences of PFKs from D. thermophilum and nine other species, carried out by using Clustal W (version 1.6).

D. thermophilum (pf1) LEMVIDIIEIGTLGSSRTNFPPKEEIVQKVENFKKNLNLIDIALIGGDEGGLYHVKFS
A. methanolica (pf1) LDVVIDIIIRGTLGSSRTNPPKEEGGVEKRIVALDQGVDAIAIGGDEGGLYHVKFS
T. tenax (pf1) SRDLLEFSGGYITIRSRNTNFPPKEEERLESKVLGLDQGVDAIAIGGDEGGLYHVKFS
T. maritima (pf1) IDEVRHIKLFSSGSKLRSTNFPPKEEERLESKVLGLDQGVDAIAIGGDEGGLYHVKFS
E. histolytica (pf1) PEITSIDNQKGSILGSRQGQ---PEVAQFLIDNNFLLTGDQGDDRGANAN
S. coelicolor (pfk) IPAVGILPFRTQTVLGERTSNPLQFQDRGIRKINDLALAEGYTEITIGDQGDRTLRA
T. brucei (pf1) RGVRTNHYGTNLGSSRTGQGD---PEEVDLTIRLDGGLNTGQDGDLGVVIS
T. pallidum (pf1) PEVIDNCNKHGTQLGSLRQGNNR---VVDIDQYERINLHILFIIGDSGSQGKAE
T. maritima (pf1) KYQAVGTEKHGTLKSRCECEFTEEBRGELAQKKHIIEGGLVIGGISQATAGLHY
E. coli (pfk) RYSVDINRGN6TFLSGARCPFREDNIRAVENLKKIGDNLVDGISGYMGMAKLT
B. stearothermophilus (pf1) VGDVQILHGRYTLACTCPEFKETFGEQKIGEQLWKHEGGLVIGGISQATAGLHY

D. thermophilum (pf1) KL-G----LWIVGVPKTIDKDE---ETTDYTGDFGTAVEVVAQIKRLDDTATISR-VI
A. methanolica (pf1) DD-G----IGVGVPKTIDKDE---ATDYTGDFGTAVEVVAQIKRLDDTATISR-VI
T. tenax (pf1) RRGI---LDGVIPKTDIDVY---GTDTYIFGSADNIAITESPTKTLISER-IG
T. maritima (pf1) ERSKQ---ELKHHPKTDIDMPLPENMTGFETARVHTELVNLQDSKTRNWY
E. histolytica (pf1) KELRKKVTVPGIPKTDINDC---YTDTSFGFQTVAGLSQAINBKHSAKSNQUNIG
S. coelicolor (pfk) DEYG---VPCGVIPKTDINDC---ATDYTFGDFDVAIGAEIDRLHDTAESHR-IVL
T. brucei (pf1) QAEREGRVDISVGVDPKTDINDC---FCHRRGFQTVAEQKQIAAAYEAASANYVG
T. pallidum (pf1) DEIKHRNLISIIGIPKTDINDC---FVQKSFQTVAEQKQIAAAYEAASANYVG
T. maritima (pf1) EEHK---IPVYPAIRIDIND----LTMCIGVDCLNTMDAVQKLDATISHER-AP
E. coli (pfk) EMG---FPICGLQPDIDK---GTDYTIGFDALTSTVAA1DRLDSSSHQPP-IS
B. stearothermophilus (pf1) EHG---FPICGLQPDIDP---GTDYTIGFDALTSTVAA1DRLDSSSHQPP-IS

D. thermophilum (pf1) VVEIMGRHAGWLALYGLGLAGD-ADYILIPFV---EPNLED---LYNHIBKLYARGHNDVAL
A. methanolica (pf1) VVEVSMRHAGWLALYGLGLAGD-ANVIILPVE---PV5VEQ---VVEVAVFRRFEMAYP
T. tenax (pf1) VVEVEMRHALYHYLGTSM---ADAVLPER---PSWDS---VAKRKEAYNE-RRW
T. maritima (pf1) FVAMRGREAGHIALYGLGKAS---ATI1IPFPEEKGTVLLECVDVLDAIKLKLGMRRDG
E. histolytica (pf1) IYRLMRDFAAFYISLASHLNDALVPI---DIPITQ---ICEFVQKRMISGKV
S. coelicolor (pfk) VVEMGRHAGWLALYGLGLAGD-ANVIILPQ---RFVQDQ---VCWMTSRSRFSRASYP
T. brucei (pf1) VKLMGMRHSFSFQMTAIVASAQAVASICLVPI---PISQ---VMILLERCFSRSSC
T. pallidum (pf1) LVKRMRGSGFQMTAIVASAQAVASICLPQ---PISQ---VMILLERCFSRSSC
T. maritima (pf1) IYVEVEMRHALYHYLGTSM---ADAVLPER---PSWDS---VAKRKEAYNE-RRW
E. coli (pfk) VVEVSMRHYCDDLIALAAAGC-CEPFFVEPFP---FSPRED---LVNEKAGIAGKKGKNS
B. stearothermophilus (pf1) VVEMGRHAGWLALYGLGLAGD-ANVIILPQ---RFVQDQ---VCWMTSRSRFSRASYP

FIG. 1. Multiple alignment of amino acid sequences of PFKs from D. thermophilum and nine other species, carried out by using Clustal W (version 1.6). * B. stearothermophilus, pfp and pfk, genes encoding PP-PFK and ATP-PFK, respectively; A, F, and E, residues involved in binding ATP, F-6-P, and phosphoenolpyruvate (PEP), respectively, for the E. coli ATP-PFK. Asterisks, identical residues; dashes, gaps; dots, highly conserved residues.

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D. thermophilum (pfk)  
A. methanolica (pfk)  
T. tenax (pfk)  
T. maritima (pfk)  
E. histolytica (pfk)  
S. coelicolor (pfk)  
T. brucei (pfk)  
T. pallidum (pfk)  
T. maritima (pfk)  
E. coli (pfk)  
B. stearothermophilum (pfk)  

FIG. 1—Continued.
retrieved from sequence databases and aligned (1) with the PP$_i$-PFK sequence from D. thermophilum (Fig. 1). The F-6-P binding sites were highly conserved in all PFKs examined. Dictyoglomus PFK has almost the same amino acid residues for F-6-P binding as those from Amycolatopsis methanolica, T. tenax, Mycobacterium tuberculosis, Streptomyces coelicolor, Propionibacterium freudenreichii, and E. coli (9, 23). The PFK from Dictyoglomus also possesses amino acid residues in the phosphoryl binding site identical to those for A. methanolica, T. tenax (PP$_i$-PFK), and S. coelicolor (ATP-PFK). A phylogenetic tree was generated from 22 representative amino acid sequences (Fig. 2). The D. thermophilum sequence is most homologous to the group III PFKs, as defined by Siebers et al. (25), including the PP$_i$-PFK from T. tenax and A. methanolica, the putative PP$_i$-PFK from Mycobacterium tuberculosis, and the ATP-PFK from S. coelicolor. The full sequence of the Dictyoglomus pfp gene agreed completely with the N-terminal sequence obtained from the purified native protein, except for the lack of the first methionine residue in the native enzyme, which supports our previous suggestion, based on the sequence, that the enzyme has homology with the T. tenax enzyme (8). The data from the alignment of 22 sequences and the phylogenetic tree strongly support the contention that the central carbohydrate metabolism of glycolysis was established before the segregation of the three domains of life. The fact that all of the sequences presented in the phylogenetic tree can be aligned, despite their varying phosphoryl

![Phylogenetic Tree](image-url)
Glu by Asp or Leu, the allosteric transition is abolished. The purified sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) from (Table 1). A single band was obtained by sodium dodecyl of cell pellet), indicating high-level expression of the enzyme from 800 ml of induced culture (approximately 5 g [wet weight] yield of the purified recombinant enzyme was 53 mg of protein prescribed for the purification of the native enzyme (8). The final recombinant enzyme were essentially the same as those de- zyme activity was checked hourly after induction for up to 5 h. The conditions for purification and characterization of the recombinant enzyme were essentially the same as those described for the purification of the native enzyme (8). The final yield of the purified recombinant enzyme was 53 mg of protein from 800 ml of induced culture (approximately 5 g [wet weight] of cell pellet), indicating high-level expression of the enzyme (Table 1). A single band was obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) from the purified Dicytogenous recombinant enzyme (Table 1; Fig. 3). The recombinant and native enzymes had the same estimated molecular weights (approximately 37,000) when both were run on the same SDS-PAGE gel (data not shown). In addition, a comparison of the native and recombinant Dicytogenous PPi-PFKs enzymes demonstrates that they possess a high degree of similarity (Table 2). Most of the biochemical and the kinetic properties of the recombinant enzyme were very similar to those of the native enzyme (8), including, for example, thermostability and the extreme sensitivity to Cu2+.

TABLE 1. Purification of the recombinant PPi-PFK from D. thermophilum Rt146 B.1

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Sp act (U/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heated supernatant</td>
<td>757</td>
<td>2,300</td>
<td>0.33</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>516</td>
<td>329</td>
<td>1.6</td>
<td>4.8</td>
<td>68</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>363</td>
<td>79</td>
<td>4.6</td>
<td>14</td>
<td>48</td>
</tr>
<tr>
<td>Red dye 120</td>
<td>328</td>
<td>53</td>
<td>6.2</td>
<td>19</td>
<td>43</td>
</tr>
</tbody>
</table>

TABLE 2. Comparison of some properties of native and recombinant D. thermophilum PPi-PFK

<table>
<thead>
<tr>
<th></th>
<th>Mg2+ (mM)</th>
<th>pl</th>
<th>Mol wt</th>
<th>Forward pH optimum</th>
<th>Km (mM)</th>
<th>Half-life (min) at 80°C</th>
<th>% Activity in response to 1 μM Cu2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant</td>
<td>1–3</td>
<td>4.3</td>
<td>37,000</td>
<td>5.8</td>
<td>0.136</td>
<td>165</td>
<td>40</td>
</tr>
<tr>
<td>Native</td>
<td>1–3</td>
<td>4.4</td>
<td>37,000</td>
<td>5.9</td>
<td>0.127</td>
<td>170</td>
<td>38</td>
</tr>
</tbody>
</table>

a Determined by SDS-PAGE.
b Native and recombinant enzymes (150 μg ml−1) were incubated at 80°C in buffer containing 50 mM MOPS, 2 mM MgCl2, and 0.02% Triton X-100, pH 6.4, under a mineral oil overlay for different lengths of time. Residual activity at 50°C was then assayed and compared to that of the unheated control.
context, Mertens (13) has suggested that the role of PP_i-PFK is that of a glycolytic enzyme adapted to anaerobiosis. However, other investigators have pointed out that PP_i-PFKs (especially those in Archaea and extremely thermophilic bacteria) are likely to be more ancient than ATP-PFKs (2, 16). The results from the Dicytoglomus PP_i-PFK strongly support the latter hypothesis. As seen with the enzyme from *T. tenax*, the Dicytoglo- mum enzyme is the first biochemically characterized extremely thermophilic bacterial PP_i-dependent enzyme and offers another opportunity to gain insight into the differentiation of PFK substrate specificities and the characteristics of the phenotype of the original ancestral PFK precursor. The group III enzymes are suggested to be of a more ancient origin than group II enzymes, so it follows that the PP_i-PFKs from *T. tenax* and extremely thermophilic bacteria may represent more ancient enzymes than the enzymes in mesophilic bacteria, primitive eukaryotes, and higher plants. In support of this latter contention, in two cases, that of *S. coelicolor* and *Tryp- anosoma brucei*, there is strong sequence evidence for the evolution of ATP-PFKs from PP_i-PFKs (2, 15). Finally, it is hoped that the X-ray crystallographic determination of the structure of PP_i-PFK from *D. thermophilum* Rt46 B.1, which is in progress, will help clarify the phylogenetic origins of PFKs.

**Nucleotide sequence accession number.** The *Dicytoglo- mum pfp* gene sequence data has been submitted to GenBank under accession number AF268276.

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**REFERENCES**


