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Purification and properties of the pyrophosphate-dependent phosphofructokinase from *Dictyoglomus thermophilum* Rt46 B.1

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Abstract The distribution of phosphofructokinase phosphoryl donor subtypes (ATP-, ADP-, and pyrophosphate) in the deeply rooted phylogenetic lineages of thermophiles is of interest with regard to the evolution of phosphofructokinase activity and of the Embden–Meyerhof pathway. In this article we present the first biochemical description of a thermostable pyrophosphate-dependent phosphofructokinase from the hyperthermophilic bacterium *Dictyoglomus thermophilum*. The enzyme was not allosterically controlled by traditional modulators of phosphofructokinases and has significant activity with tripolyphosphate and polyphosphate. Kinetic parameters of the enzyme suggest it plays primarily a glycolytic role. The enzyme required Mg^{2+} for optimal activity, was partially activated by some monovalent and divalent cations, and was strongly inhibited by Cu^{2+}. The sequence of the 21 N-terminal residues suggests that the enzyme is most similar to the pyrophosphate-dependent phosphofructokinases from *Amycolatopsis methanolica* and the hyperthermophilic crenarchaeon *Thermoproteus tenax*, enzymes which have been suggested to represent an ancient lineage of phosphofructokinases (Siebers et al. 1998). The unexpected finding of a pyrophosphate-dependent phosphofructokinase in *Dictyoglomus thermophilum*, which is phylogenetically related to *Thermotoga maritima*, previously shown to possess an ATP-dependent phosphofructokinase activity, is discussed.

Key words Phosphofructokinase · Pyrophosphate · *Dictyoglomus thermophilum* · Polyphosphate · Tripolyphosphate

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Introduction

Phosphofructokinase (PFK) is a definitive enzyme of the Embden–Meyerhof pathway and is present within the three domains Bacteria, Eukarya, and Archaea. Three subtypes of PFK, with respect to their phosphoryl donor, have been described: an ATP-, an ADP-, and a pyrophosphate-dependent form. The ATP-PFK is found in most highly evolved organisms (Mertens 1991), in many bacteria (Byrnes et al. 1994), and in the crenarchaeon *Desulfurococcus amylolyticus* (Selig et al. 1997). A unique ADP-PFK has been identified in the genera *Pyrococcus* (*P. furiosus*) and *Thermococcus* (*T. celer*, *T. litoralis*, and *T. zilligii*) of the archaeal kingdom Euryarchaeota (Kengen et al. 1994; Selig et al. 1997; Ronimus et al., in manuscript). This enzyme is likely to represent a new family of PFK sequences as no homologous open reading frame has been identified within the *P. horikoshii* genome sequence (Kawarabayasi et al. 1998) or is identifiable within the *P. furiosus* genome when searched with either ATP- or representative PP_i-PFK encoding sequences (unpublished data; Altschul et al. 1990). In 1974, Reeves et al. reported that there was a PP_i-PFK activity in the amoebic parasite *Entamoeba histolytica*, and subsequently PP_i-PFKs have been shown to be present in many anaerobic bacteria, primitive eukaryotes, and in some plants (Mertens et al. 1993; O'Brien et al. 1975; Yan and Tao 1984). In addition, Morgan and Ronimus (1998) have found that all the thermophilic and nonthermophilic spirochetes possess a PP_i-PFK activity. The only PP_i-PFK from an archaeal species, the crenarchaeon *Thermoproteus tenax*, has been described by Siebers et al. (1998), who also recently reported the sequence of the PP_i-PFK-encoding gene.

Siebers et al. (1998) and Morgan and Ronimus (1998) have suggested that a PP_i-PFK that utilizes pyrophosphate as the phosphoryl donor for PFK activity might represent the ancestral form of the enzyme, although others (e.g., Mertens 1991) have proposed that it represents only an adaptation to anaerobic metabolism and thus would have presumably arisen on multiple occasions from ATP-PFKs.

In support of PP_i possibly representing the original phosphoryl donor for PFK activity, there are at least two examples in which sequence comparisons of ATP-PFKs have strongly suggested that ATP-PFKs are derived from PP_i-PFK ancestors; the ATP-PFK of *Streptomyces coelicolor* and the glycosomal ATP-PFK of *Trypanosoma brucei* (Alves et al. 1997). The number of sequence changes required to convert a PP_i-PFK to an ATP-PFK are not known with certainty, partially because of the lack of a crystal structure for a PP_i-PFK, but may only require subtle changes. However, the ramifications of these changes are significant because the PP_i-PFK-mediated reaction is reversible whereas the ATP-driven reaction is irreversible under physiological conditions. In this context, the biochemical characteristics of the PFKs from Bacteria and Archaea from deeply rooted phylogenetic lineages are of particular interest. The genus *Dictyoglomus* represents a deeply rooted bacterial hyperthermophilic lineage, and this article describes the purification of a PP_i-PFK from *Dictyoglomus thermophilum* Rt46 B.1, an isolate from a New Zealand hot spring (Love et al. 1993; Patel et al. 1987).

Materials and methods

Bacteria and culture conditions

Dictyoglomus thermophilum Rt46 B.1 was obtained from the Thermophile Research Unit Culture Collection and was cultured anaerobically in the medium described by Patel et al. (1987). Large-scale cultures (80l) for protein purification were carried out in 20-l polypropylene containers. The medium was filter sterilized (0.2 μm) and gassed with 80% N_2 + 20% CO_2 for 60 min before inoculation with 0.5 l of log-phase culture per 20 l fresh medium at 73°C. Cells were harvested by diafiltration with a 0.1-μm hollow fiber system (Amicon, Beverly, MA, USA), recovered by centrifugation, and the resulting cell pellet then stored at −70°C before initiating the purification.

Chemicals and reagents

The following enzymes and reagents were purchased from Sigma (St. Louis, MO, USA): aldolase, α-glycerophosphate dehydrogenase, triosephosphate isomerase, phosphoglucoisomerase, glucose-6-phosphate dehydrogenase, fructose-1,6-diphosphate, fructose-2,6-diphosphate, pyrophosphate, tripolyphosphate, polyphosphate, potential activators and inhibitors, $NADP^+$, NADH, HEPES, and bis-Tris. All other buffers and chemical reagents used were of analytical grade quality.

Determination of forward and reverse PFK reaction rates and protein concentrations

The activity of the *Dictyoglomus* PFK was assayed spectrophotometrically using an Ultrospect 3000, UV/Visible Spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) by a variation of the method of Janssen and Morgan (1992). The final concentration of the reaction mixture in a total volume of 100 μl was 5 mM fructose-6-phosphate (F-6-P); 1.0 mM phosphoryl donor (PP_i, ATP, or ADP); 3.5 mM $MgCl_2$; 0.2 mM NADH; 0.04 U aldolase; 0.5 U α-glycerophosphate dehydrogenase, and 5.0 U triosephosphate isomerase. Coupling enzymes were desalted before use with either Amicon Centricon-3s (α-glycerophosphate dehydrogenase, triosephosphate isomerase) or Centricon-10s (aldolase). Assays were conducted by monitoring the decrease of absorbance at 340 nm at 50°C, the maximum temperature at which the mesophilic coupling enzyme assay can be utilized. The following enzymes were used as positive controls during the investigation: the ATP-dependent PFK-positive control was fructose-6-phosphate kinase (EC. 2.7.1.11) type III from rabbit muscle, and the PP_i-dependent PFK positive control was PP_i-PFK (EC 2.7.1.90) derived from *Propionibacterium freundenreichii*. The ADP-PFK-positive control consisted of a semipurified PFK from *Thermococcus zilligii* (shown to be ADP dependent; Ronimus et al., in manuscript). Other control reactions were performed using the following conditions: no F-6-P, no coupling enzymes, or no phosphoryl donors in the reaction mixture. All assays were conducted in duplicate except those for determining kinetic parameters, which were performed in triplicate and used optimal conditions for at least 2 min. Specific activities are expressed in units mg^{-1} of protein. One unit (U) is defined as that amount of enzyme required to convert 1 μmole of F-6-P into fructose-1,6-diphosphate (F-1,6-P_2) per minute, and for the reverse reaction, the conversion of F-1,6-P_2 into F-6-P per minute. The reverse reaction was assayed at 50°C in a total volume of 100 μl using phosphoglucoisomerase (0.4 U) and glucose-6-phosphate dehydrogenase (0.1 U) as described by Mertens et al. (l993), except that the phosphoglucoisomerase concentration was doubled. The concentrations of other components were 3.5 mM $MgCl_2$, 0.2 mM $NADP^+$, and 50 mM MOPS (pH 7.3 at 50°C). Control experiments were conducted for both forward (1.5 mM F-1,6-P_2) and reverse (1.5 mM F-6-P) reactions to ensure that the coupling enzymes were not rate limiting. Protein was determined by a modification of the method of Bradford using bovine serum albumin as the standard (Bollag and Edelstein 1993).

Purification of the *Dictyoglomus* PP_i-PFK

Step 1. Preparation of cell-free extract

Dictyoglomus cells (120 g wet weight) were suspended in 1.2 l of 0.1 M $MgCl_2$, 0.1 M KCl, 7 mM β-mercaptoethanol, 10% glycerol, 50 mM imidazole buffer, pH 7.5, and lysed by sonication in six 200-ml aliquots for three periods of 2 min each at full power (Heat Systems-Ultrasonics, Farmingdale, NY, USA). Cell debris was removed by centrifugation at 4°C at 15 000 × *g* for 40 min. Ammonium sulfate was added to the supernatant to a final concentration of 1.0 M.

Step 2. Phenyl-Sepharose hydrophobic interaction chromatography
The cell-free extract was applied to a 700-ml phenyl-Sepharose column (Pharmacia Biotech) preequilibrated with starting buffer A [50 mM Tris, 1 mM $MgCl_2$, 1.0 M $(NH_4)_2SO_4$, pH 7.5]. The fractions were eluted with a linear gradient (4.0 l) with buffer B (50 mM Tris, 1 mM $MgCl_2$, pH 7.5) and further eluted with a linear gradient (2.0 l) of buffer C (50 mM Tris, 1 mM $MgCl_2$, 50% ethanediol, pH 7.5). Active fractions were pooled and concentrated with a YM 30 ultrafiltration membrane (Amicon) and dialyzed overnight against buffer B.

Step 3. Q-Sepharose anion exchange chromatography
The dialyzed enzyme solution was applied to a 55-ml Q-Sepharose column 26/10 (Pharmacia Biotech) that was equilibrated with buffer B. The activity was eluted with a gradient (400 ml) of 50 mM Tris, 1 mM $MgCl_2$, 1 M NaCl, pH 7.5, at a flow rate of 4 ml min^{-1}. Active fractions were pooled, concentrated by ultrafiltration with a YM30 membrane (Amicon), and dialyzed overnight against buffer B.

Step 4. Chromatofocusing
The recovered fractions from the Q-Sepharose step were loaded onto a Mono P HR 5/20 column (Pharmacia Biotech). Proteins were eluted with a 40-ml pH gradient (6.5–5.5) in buffer D and E with a flow rate of 1.0 ml min^{-1}. Buffer D contained 0.025 M bis-Tris, 1 mM $MgCl_2$, at pH 6.7, and buffer E contained 25 mM bis-Tris, 1 mM $MgCl_2$, 4.0 ml polybuffer 96 (Pharmacia), and 6.0 ml polybuffer 74 in a total volume of 100 ml (Pharmacia Biotech), pH 5.5. Active fractions were pooled and concentrated by ultrafiltration and dialyzed overnight against buffer F (25 mM HEPES, 50 mM NaCl, 1 mM $MgCl_2$, pH 7.8).

Step 5. Gel filtration chromatography
The concentrated and dialyzed sample was applied to a BIOSEP-SEC3000 column (60 × 0.78 cm; Phenomenex, Torrance, CA, USA), which was equilibrated with buffer F as the mobile phase. Active fractions were pooled, concentrated, and dialyzed against buffer G containing 25 mM bis-Tris and 5 mM $MgCl_2$, pH 6.0.

Step 6. Red dye-120 dye-ligand chromatography
Dialyzed active fractions following gel filtration were loaded onto a red dye-120 column that was equilibrated with buffer G. The enzyme was eluted by three linear gradients of $MgCl_2$, pH, and PP_i utilizing buffers G and H. Buffer H contained 25 mM Tris, 1 mM $MgCl_2$, and 10 mM PP_i, pH 7.5. The purified PFK was concentrated and extensively dialyzed against 20 mM bis-Tris and 1 mM $MgCl_2$, pH 6.8, to remove PP_i from the final purification step. Aliquots of the purified enzyme were then stored in 50% glycerol, 7 mM β-mercaptoethanol, 0.1 mM EDTA, and 1 mM $MgCl_2$ at −70°C.

SDS-PAGE gel electrophoresis

Determination of the subunit molecular weight was carried out using 10%–15% SDS Phast Gels (Pharmacia Biotech). The isoelectric point was derived from comparison of the purified enzyme on pH 3–9 isoelectric focusing (IEF) gels (Pharmacia Biotech) with a pH 3.0–10.0 isoelectric focusing standard (Pharmacia Biotech). The SDS-PAGE and IEF separations and subsequent silver stainings were performed according to the manufacturer's instructions.

Determination of the native molecular weight and thermostability

The molecular mass of the purified PP_i-PFK was determined by gel filtration using a BIOSEP-SEC3000 column (Phenomenex) equilibrated with 25 mM bis-Tris, 200 mM NaCl, and 1 mM $MgCl_2$, pH 6.7. The standard proteins used for the calibration of the column were thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), lactate dehydrogenase (140 kDa), carbonic anhydrase (29 kDa), and α-lactate albumin (14 kDa), which were compared to the elution volume of the purified PFK. All injections were of constant size (25 µl), and the proteins were eluted at a flow rate of 1.0 ml min^{-1} in the buffer used for equilibration. Blue-dextran 2000 (2000 kDa) was used to determine the column void volume. The thermostability of the purified *Dictyoglomus* PFK was investigated by incubating the enzyme (150 µg ml^{-1}) at 80°C or 90°C in buffer containing 50 mM MOPS, 2 mM $MgCl_2$, and 0.02% Triton X-100, pH 6.4, under a mineral oil overlay for different incubation times. Residual activity was then assayed at 50°C and compared to the unheated control.

N-Terminal sequencing

Sequencing was carried out using Edman degradation chemistry on an Applied Biosystem Procise 492 protein sequencer. The first 21 residues were used for homology searching and incorporated the BLASTP search at the National Centre for Biotechnology Information, National Library of Medicine, National Institute of Health, Bethesda, MD, USA (Altschul et al. 1990).

Determination of kinetic parameters

Forward reaction kinetic parameters were determined at 50°C and pH 6.0 by varying the concentration of either F-6-P or PP_i (or other phosphoryl donors) in the presence of saturating quantities of PP_i (1 mM) or F-6-P (5 mM), respectively. Reverse reaction parameters were determined at 50°C and pH 7.2 by varying the concentration of F-1, 6-P_2 or phosphate (P_i) in the presence of saturating quantities of P_i (10 mM) or F-1,6-P_2 (10 mM), respectively. Possible activators or inhibitors of PP_i-PFK were added

separately (each adjusted to pH 6.0) to the assay mixture with the purified enzyme, using near-apparent K_m concentrations of the substrates F-6-P (0.5 mM) and PP_i (0.3 mM).

Results

Purification of the *Dictyoglomus* PP_i-PFK

The PP_i-PFK was purified to homogeneity as indicated by SDS-PAGE (Fig. 1), after elution from the red dye-120 column, and had a specific activity of 24.4 U mg^{-1} protein. The enzyme was not sensitive to oxygen and was purified 231 fold with a final yield of 2.4% of the activity in the cell-free extract (purification summarized in Table 1). A single band was also seen during IEF, which indicated that the pI of the enzyme was 4.4 (not shown). Based on the percent yield of activity and the quantity of enzyme recovery after the red dye-120 step, the PFK represents approximately 0.4% of the total soluble cell protein.

Subunit structure of the enzyme and thermostability

The subunit molecular mass of the PP_i-PFK was estimated to be 37 kDa by SDS-PAGE. Gel filtration showed that the native enzyme had a molecular weight of approximately 65 kDa (Fig. 2). Thus, the PP_i-PFK from *Dictyoglomus thermophilum* Rt46 B.1 is indicated to be a homodimer. The half-life for the enzyme was determined to be 10 min at 90°C and 150 min at 80°C.

Substrate specificities, cation effects, and kinetic properties

The enzyme had optimal activity in the forward reaction at pH 5.7–6.3 at 50°C. The pH optimum for the reverse reaction was 7.0–7.5. Optimal activity was obtained with 0.5–3.5 mM $MgCl_2$ (with a PP_i concentration of 1.0 mM; not shown). The apparent K_m and V_{max} values for the forward and reverse reactions, as determined by double-reciprocal plotting, are summarized in Table 2a. The apparent K_m values for both substrates for the reverse reaction were

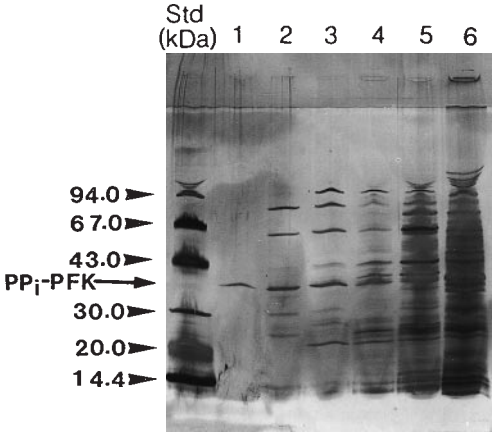

Fig. 1. SDS-PAGE gel (silver-stained) of fractions obtained during purification of the PP_i-PFK. *Lane 1* contained 0.3 μg purified *Dictyoglomus* PP_i-PFK and the remaining lanes contained (purification step, quantity in μg): *lane 2* (step 5, 1.0); *lane 3* (step 4, 1.0); *lane 4* (step 3, 1.0); *lane 5* (step 2, 4.0); and *lane 6* (step 1, 8.0). The standard (*Std*) contained the following molecular weight markers: phosphorylase b (molecular weight, 94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.0 kDa), and α-lactalbumin (14.4 kDa)

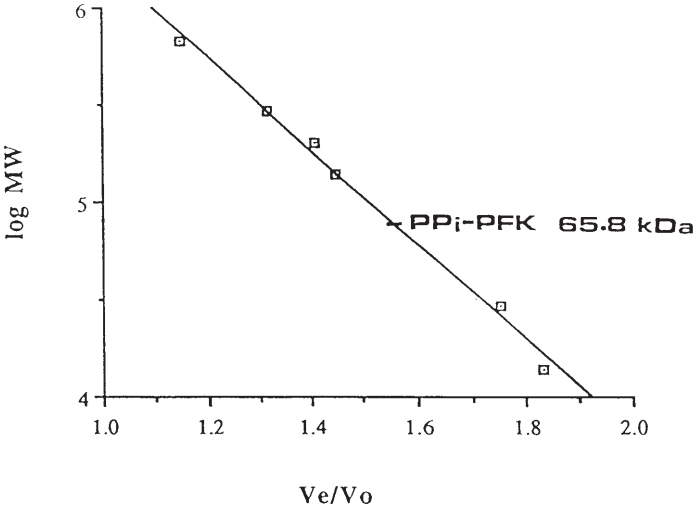

Fig. 2. Native molecular weight of *Dictyoglomus* PP_i-PFK. The standard proteins used for the calibration of the column were thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), lactate dehydrogenase (140 kDa), carbonic anhydrase (29 kDa), and α-lactate albumin (14 kDa), which were compared to the standard elution volume of purified PFK (25 μl). Blue dextran 2000 was used to determine the column void volume

Table 1. Purification of the PP_i-PFK from *Dictyoglomus thermophilum* Rt46B.1

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
(1) Cell-free extract	1374	13230	0.104	1	100
(2) Phenyl-Sepharose	955	927	1.03	10	70
(3) Q-Sepharose	730	633	1.10	11	53
(4) Mono P	103	58.7	1.75	17	7.5
(5) Gel filtration	72.8	30.3	2.4	23	5.3
(6) Red dye-120	32.9	1.35	24.4	231	2.4

Table 2. Biochemical characteristics of the PP_i-PFK from *Dictyoglomus thermophilum*

a. Summary of kinetic parameters:

Substrate	K_m (mM)	V_{max} (U mg^{-1})	V_{max}/K_m
F-6-P[a]	0.228	4.62	20.3
PP_i[b]	0.022	5.30	241
PPP_i[b]	0.220	2.86	13.1
F-1,6-P_2[c]	2.900	0.15	0.05
P_i[d]	4.300	0.60	0.14

b. Summary of cation effects on PP_i-PFK activity:[e]

Cation	mM	% activity
Mg^{2+}[f]	3.5	100
Mn^{2+}	0.1	54
Fe^{3+}	0.1	16
Se^{4+}	0.1	25
	0.01	38
Cu^{2+}	1.0	0
	0.1	8
	0.01	38
	0.001	80

[a] With 3 mM PP_i and 3.5 mM $MgCl_2$
[b] With 5 mM F-6-P and 3.5 mM $MgCl_2$
[c] With 20 mM P_i and 3.5 mM $MgCl_2$
[d] With 10 mM F-1,6-P_2 and 3.5 mM $MgCl_2$
[e] Experiments conducted using the following: 5 mM F-6-P, 1 mM PP_i, 3.5 mM $MgCl_2$, 0.2 mM NADH, 50 mM bis-Tris, pH 6.0, 50°C, with cations at the stated concentrations
[f] Control assay with $MgCl_2$

much higher than the substrates for the forward reaction and the V_{max} values much lower. For example, the K_m for F-1,6-P_2 was 23 fold higher than the K_m for F-6-P and the ratio of K_m (P_i) to K_m (PP_i) was 195 fold. In addition, the ratios of V_{max}/K_m for PP_i and F-6-P for the forward reaction were much higher than the V_{max}/K_m ratios for P_i and F-1,6-P_2 for the reverse reaction. The apparent K_m and V_{max} values for tripolyphosphate were an order of magnitude higher and approximately half that determined than those for PP_i, respectively.

In addition to the enzyme using PP_i as the phosphoryl donor, the enzyme could also utilize tripolyphosphate and polyphosphate ($n = 15 \pm 3$) at 82% and 75% of the control reaction rates, respectively (not shown). In contrast, no activity was detected with either ATP, ADP, AMP, TTP, TDP, GTP, GDP, CTP, CDP, UTP, UDP, phosphoenolpyruvate, phosphoarginine, or phosphocreatine as the phosphoryl donors or D-glucose-6-phosphate in place of F-6-P (not shown). The *Dictyoglomus* PP_i-PFK enzyme activity was not affected by the traditional allosteric effectors ATP, ADP, AMP, GDP, phosphoenolpyruvate, or citrate (all at 1.0 mM). In addition, the *Dictyoglomus* PP_i-PFK activity was not affected by either 0.1 or 1.0 mM fructose-2,6-diphosphate (not shown). The enzyme activity was strongly inhibited by Cu^{2+}, Se^{4+}, Mn^{2+}, and Fe^{3+} (Table 2b). For example, a concentration of only 10.0 μM of Cu^{2+} resulted in a decrease of 62% of PP_i-PFK activity, but in control reactions only a slight inhibition of the coupling enzyme system was found at a Cu^{2+} concentration of 100 μM. In contrast to the inhibitory effects of Cu^{2+}, the enzyme activity was partially activated by K^+ (10 mM, 140%), Na^+ (10 mM, 135%), Ca^{2+} (0.1 mM, 120%), Fe^{2+} (0.1 mM, 120%), and Co^{2+} (0.1 mM, 120%).

Determination of N-terminal sequence

The first 21 amino acid residues of the PP_i-PFK were SKMRIGVLTGGGDCPGLNPAI. The N-terminal sequence is 84% and 78% identical to the N-termini of the PP_i-PFKs from *Amycolatopsis methanolica* and *Thermoproteus tenax*, respectively, but only 58% identical to the PP_i-PFK of the primitive eukaryote, *Trichomonas vaginalis* (Altschul et al. 1990).

Discussion

We have presented here the first biochemical desciption of a PP_i-PFK from a hyperthermophilic bacterium, *Dictyoglomus thermophilum* strain Rt46 B.1. The properties of this thermostable enzyme can now be compared to other ATP- and PP_i-PFKs that have been characterized from other sources.

The *Dictyoglomus thermophilum* PP_i-PFK is indicated, by the SDS-PAGE (37 kDa) and gel filtration results (65 kDa), to be a homodimer. Homodimeric PP_i-PFKs have also been identified in the bacterium *Propionibacterium freundenreichii* (O'Brien et al. 1975), *Entamoeba histolytica* (Reeves et al. 1974), *Rhodospirillum rubrum* (Pfleiderer and Klemme 1979), *Acholeplasma laidlawii* (Pollack and Williams 1986), and in the anaerobic protozoa *Isotricha prostoma*, *Toxoplasma gondii*, and *Trichomonas vaginalis* (Mertens et al. 1989). The acidic pH optimum for the forward reaction and neutral to slightly alkaline optimum for the reverse reaction are similar to the PP_i-PFKs from some primitive eukaryal organisms, e.g., *Entamoeba histolytica* (Reeves et al. 1974), *Trichomonas* spp. (Mertens et al. 1989), *Tritrichomonas foetus* (Mertens et al. 1989), *Toxoplasma gondii* (Peng and Mansour 1982), *Eimeria tenella* (Denton et al. 1994), and *Naegleria fowleri* (Mertens et al. 1991, 1993). In contrast, the pH optima of PP_i-PFK from *P. freundenreichii* and higher plants are near neutral for the forward and the reverse reactions (Mertens 1991).

The lack of effect of traditional eukaryal or bacterial allosteric effectors on the *Dictyoglomus* enzyme, or by fructose-2,6-diphosphate, which is a potent glycolytic effector for most higher eukaryotes but not in either prokaryotes or most anaerobic protozoa, suggests that one of the major control points of the Embden–Meyerhof pathway is not operational (Mertens 1991). The lack of allosteric control is a general feature of PP_i-PFKs (Mertens 1991) and implies that the rate of the PP_i-PFK catalyzed reaction must instead be controlled by a combination of the level of activity of the enzyme, its kinetic properties, and the cellular concentrations of reactants and products. In support of the kinetic parameters having a significant effect on controlling the

glycolytic flux are the much lower apparent K_m values for the forward reaction, the higher V_{max} values compared to the reverse reaction, and the much higher V_{max}/K_m ratios seen with either PP_i or F-6-P for the forward reaction. These results taken together suggest that the glycolytic direction of the reaction is favored in this organism.

The *Dictyoglomus* PP_i-PFK has a requirement for Mg^{2+} ions for optimal activity and was partially activated by some monovalent cations, both of which are common biochemical attributes of PFKs (Uyeda 1979). The enzyme is sensitive to Se^{4+} and Fe^{3+} and, interestingly, extremely sensitive to Cu^{2+}. The extreme sensitivity of the enzyme to Cu^{2+} ions could have an important role in determining the ecological distribution of *D. thermophilum* strains. The organism must have cellular detoxifying mechanisms for maintaining the intracellular concentration of Cu^{2+} at extremely low levels.

The ability of the *D. thermophilum* PP_i-PFK to utilize pyrophosphate, tripolyphosphate, and polyphosphate is potentially relevant with regard to the origins of PFK activity. This is because pyrophosphate and tripolyphosphate are known to be formed under hydrothermal conditions (Yamagata et al. 1991) and are considered as relics of ancient metabolism (Kornberg 1995). These substrates may have provided a continuing energy source to drive metabolism during primeval conditions (Baltscheffsky 1996). In such a scenario, the earliest life forms would be expected to have possessed a PP_i-PFK activity (Morgan and Ronimus 1998). The reversible nature of the reaction with PP_i as phosphoryl donor with its $\Delta G^{\circ\prime}$ value of $-2.08\,kcal\,mol^{-1}$ ($8.7\,kJ\,mol^{-1}$) would be compatible with either a glycolytic or gluconeogenic origin of the Embden–Meyerhof pathway (Mertens 1991). In support of this scenario, Siebers et al. (1998) have recently inferred that the PP_i-PFKs from the hyperthermophilic crenarchaeon *Thermoproteus tenax* and the mesophilic bacterium *Amycolatopsis methanolica* represent a unique lineage of PFK, and possibly the most ancient form of the PFK enzyme. The PP_i-PFK from *D. thermophilum* Rt 46 B.1 is similar to the *T. tenax* enzyme in respect to its nonallosteric nature, subunit size, and N-terminal amino acid sequence.

It was an unexpected finding to discover a PP_i-PFK in *Dictyoglomus* because this organism branches at the base of the phylum Thermotogales (Love et al. 1993). Both *Thermotoga maritima* (Selig et al. 1997), and *Fervidobacterium nodosum*, which also branches within the Thermotogales (Love et al. 1993), possess an ATP-PFK activity (unpublished data). *Aquifex aeolicus*, the deepest branching thermophilic bacterium, has also been indicated by sequence analysis to contain an ATP-PFK (Deckert et al. 1998), and this enzyme clusters with the ATP-PFKs of *Thermus thermophilus* and *Bacillus stearothermophilus* (unpublished data). It will be interesting to see if the full sequence of the *Dictyoglomus* PP_i-PFK-encoding gene confirms a phylogenetic linkage with the *T. tenax* enzyme or whether it is more closely linked to the ATP-PFKs of *Aquifex*, *Thermotoga*, and *Fervidobacterium*. The presence of a PP_i-PFK activity in the deeply branching *D. thermophilum* lineage and the detailed properties of the enzyme are likely to play significant roles in the development of our understanding of the evolution of the PFKs and of the Embden–Meyerhof pathway itself.

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