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Thermotoga maritima Phosphofructokinases: Expression and Characterization of Two Unique Enzymes

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A pyrophosphate-dependent phosphofructokinase (PP_i-PFK) and an ATP-dependent phosphofructokinase (ATP-PFK) from *Thermotoga maritima* have been cloned and characterized. The PP_i-PFK is unique in that the K_m and V_{max} values indicate that polyphosphate is the preferred substrate over pyrophosphate; the enzyme in reality is a polyphosphate-dependent PFK. The ATP-PFK was not significantly affected by common allosteric effectors (e.g., phosphoenolpyruvate) but was strongly inhibited by PP_i and polyphosphate. The results suggest that the control of the Embden-Meyerhof pathway in this organism is likely to be modulated by pyrophosphate and/or polyphosphate.

The Embden-Meyerhof (EM), or glycolytic, pathway is nearly ubiquitous in all life forms, and enzymes of the reaction sequence are highly conserved. One of the key and definitive enzymes of the pathway is phosphofructokinase (PFK). In the majority of organisms, ATP is the phosphoryl donor for the enzyme and the reaction is a nonreversible step in the pathway. Due to its position, PFK is usually allosterically regulated by intracellular metabolites, e.g., phosphoenolpyruvate (PEP), GDP, and/or ADP (27). PFK subtypes utilizing pyrophosphate (PP_i) as the phosphoryl donor, where the reaction becomes more reversible and the enzyme is generally not subject to allosteric control mechanisms, have also been described (16, 18, 25).

Thermotoga maritima is a non-spore-forming, rod-shaped hyperthermophilic bacterium with an optimum growth temperature of 80°C and is phylogenetically classified in the order *Thermotogales*. The phylogeny of the small-subunit rRNA shows that this organism represents one of the deepest and most slowly evolving lineages of bacteria (12). *T. maritima* ferments various carbohydrates, including monosaccharides and polysaccharides, primarily via the EM pathway, and ATP-dependent PFK (ATP-PFK) activity in cell extracts has been reported (23, 24). The genome sequence of this organism indicated the presence of another PFK gene, and sequence comparison showed homology to PP_i-dependent PFK (PP_i-PFK) enzymes (17). If both genes code for functional enzymes, then *Thermotoga* would represent the unusual situation of an organism possessing two distinct PFK activities. Because of its phylogenetic position, the occurrence and origin of these genes are of importance with respect to the origins of the EM pathway. This paper describes the cloning, expression, and characterization of these enzymes, both of which exhibit unusual features.

T. maritima strain 3109 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH and grown in the medium described by Huber et al. (12). *Escherichia coli* DH5α and expression plasmid pPROEX HTb were obtained from Life Technologies. *E. coli* was grown at 30°C with vigorous aeration (200 rpm) in Luria-Bertani broth supplemented with ampicillin (100 μg ml^{-1}) when appropriate. The PFK assay was conducted essentially as described by Ding et al. (6). Preparation of genomic DNA and the alkaline lysis and cesium chloride gradient methods for large scale plasmid DNA purification followed standard procedures (21).

Construction of the PP_i-PFK and ATP-PFK expression clones. The open reading frames representing the full-length sequences of the PP_i- and ATP-PFK genes were amplified directly from genomic DNA from *T. maritima.* Primer design was based on the nucleotide sequences of the 5′and 3′ ends of the putative PFK genes (17). For the PP_i-PFK gene, the forward primer, corresponding to the N terminus, contained an upstream *Sfo*I site (in bold) and 5′-end spacer (5′-GGAA **GGC GCC** ATG GCT GAA AGA TTG GGG ATA CTC G-3′), and the reverse primer, corresponding to the C terminus, contained a flanking *Hin*dIII site (in bold) and a 5′-end spacer (5′-GCTA **AAG CTT** TAT GGA AGC TCT GTC GTA TGC CAG-3′). The primers for the ATP-PFK gene also contained *Sfo*I and *Hin*dIII sites, and their sequences were 5′-GGCT **GGC GCC** ATG AAG AAG ATA GCA GTA TAC-3′ and 5′-CCA T**AA GCT T**TA TGA AAG CAT ATG TGC TAT TTC-3′ for forward and reverse primers, respectively. Ampli*Taq* Gold DNA polymerase was used for PCR (Perkin Elmer). Both PCR products for the two genes (*pfp* and *pfk*) were of the sizes predicted from their nucleotide sequences, approximately 1,200 and 950 bp, respectively (17). These products were sequenced to confirm their identity (1) and then cloned into the expression vector after restriction digestion with *Sfo*I and *Hin*dIII, followed by ligation with T4 DNA ligase using standard protocols (21). The ligation mixture containing restriction enzyme-digested plasmid and PCR product was used to transform *E. coli* strain DH5α by electroporation, according to the manufacturer's instructions (Gene Pulser; Bio-Rad). Screening of the clones for those with inserts was carried out through alkaline lysis miniprep plasmid isolation (21) followed by restriction enzyme analysis.

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TABLE 1. Properties of the cloned *T. maritima* PP_i- and ATP-PFKs

Property	PP_i-PFK[a]	ATP-PFK[b]
pH optima		
Forward reaction	5.6–5.8	7.2–8.0
Reverse reaction	5.6–6.8	ND[c]
$MgCl_2$ optimum (mM)	0.5–7.0	1.0–10.0
Sp act (U mg^{-1})	203	432
Thermostability (half-life at 90°C)[d]	>5 h	>5 h
Apparent molecular mass (kDa)		
SDS-PAGE	48	38
Gel filtration	97	200
Phosphoryl donors (%)[e]	Poly-P (157), PPP_i (123), PP_i (100), ATP (0), ADP (0)	ATP (100), GTP (42), UTP (14), CTP (13), TTP (10), PP_i (0), ADP (0)
Cation specificity (%)[f]	Mg^{2+} (100) > Co^{2+} (49) > Mn^{2+} (40) > Ni^{2+} (38)	Mg^{2+} (100) > Mn^{2+} (90) > Fe^{2+} (34)
Sensitivity to cations (% of control activity)[g]		
1.0 μM Cu^{2+}	57	72
1.0 μM Zn^{2+}	56	72

[a] Experimental conditions were 1.0 mM PP_i, 3 mM F-6-P, 5 mM $MgCl_2$, 0.2 mM NADH, 175 mM KCl, and 30 mM bis-Tris (pH 5.8) at 50°C.
[b] Experimental conditions were 0.5 mM ATP, 3 mM F-6-P, 5 mM $MgCl_2$, 0.2 mM NADH, 175 mM KCl, and 30 mM Tris (pH 7.8) at 50°C.
[c] Not detected.
[d] 175 mM KCl, 0.02% Triton X-100, 0.05 mM dithiothreitol, 3 mM $MgCl_2$, and 50 mM phosphate buffer (pH 7.0).
[e] 0.1 mM phosphoryl donors. 3 mM F-6-P, 5 mM $MgCl_2$, 0.2 mM NADH, 175 mM KCl, and 30 mM bis-Tris (pH 5.8) at 50°C for PP_i-PFK; 30 mM Tris (pH 7.8) at 50°C for ATP-PFK.
[f] 0.1 mM cations, 3 mM F-6-P, 5 mM $MgCl_2$, 0.2 mM NADH, 175 mM KCl, and 30 mM bis-Tris (pH 5.8) for PP_i-PFK; 30 mM Tris (pH 7.8) at 50°C for ATP-PFK.
[g] Cu^{2+} or Zn^2 added in standard assay.

Expression, purification, and characterization of the recombinant PP_i- and ATP-PFKs. Flask cultures of the recombinant *E. coli* clones were grown at 30°C in 700 ml of Luria-Bertani broth plus 100 μg of ampicillin ml^{-1} and were induced with 1 mM isopropyl-β-D-thiogalactoside when the culture optical density at 600 nm reached approximately 0.6. After 5 h of induction, the cells were harvested by centrifugation and sonicated, and the cell lysate was incubated for 40 min at 80°C. Further purification of the enzymes from the supernatant was performed using a 3.0-ml column of nickel nitrilotriacetic acid resin and elution following the manufacturer's instructions (Life Technologies). Single bands were obtained for each of the nickel nitrilotriacetic acid resin-purified proteins on denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, indicating a high degree of purity. The estimated molecular weights for the PP_i- and ATP-PFK proteins from SDS-PAGE were approximately 48,000 and 38,000, respectively (Table 1), which is in close agreement with the molecular masses derived from the amino acid sequences (including the N-terminal histidine tag, which is approximately 2 kDa). The conceptual translation masses of the full-length open reading frames of the *pfp* and *pfk* genes are 46,403 Da and 34,447 Da for the PP_i- and ATP-PFKs, respectively. The recombinant PP_i-PFK had a molecular mass of 96 kDa as determined by its elution during gel filtration chromatography, which suggests that the active molecule exists as a homodimer. In contrast, the molecular mass of the ATP-PFK was 200 kDa, and thus a homotetramer is the most probable quaternary structure.

Both recombinant proteins showed enzyme activity specific for their expected phosphoryl donors; thus, the PP_i-PFK was active with PP_i but had no activity with ATP, and vice versa for the ATP-PFK. *Thermotoga* is thus confirmed as the only prokaryote reported which possesses two different functional phosphoryl donor subtypes of PFK. As expected, the enzymes were extremely thermostable, with half-lives for both being greater than 5 h at 90°C in phosphate buffer.

PFKs generally have a requirement for a low concentration (<100 mM) of either sodium or potassium ions for optimal activity (5, 20, 27). For the *Thermotoga* enzymes, the requirement for potassium ions and the stimulation of activity by their presence was more pronounced. The optimum concentration of K^+ for both enzymes was 175 mM, and activity for the ATP- and PP_i-PFKs fell 50 and 60%, respectively, when KCl was omitted from the reaction mixture. It is possible that this requirement reflects the marine environment from which the organism was isolated. Magnesium ions were required for optimal activities of both enzymes with Co^{2+}, Mn^{2+}, and Ni^{2+} being able to substitute for Mg^{2+} with the PP_i-PFK and Mn^{2+} and Fe^{2+} being able to substitute for Mg^{2+} with the ATP-PFK (Table 1). Both *Thermotoga* enzymes were extremely sensitive to Cu^{2+} and Zn^{2+} (Table 1). This sensitivity was also found within the *Dictyoglomus thermophilum* native and recombinant PP_i-PFKs (6, 7) and the archaeal *Desulfurococcus amylolyticus* ATP-PFK (5).

The *Thermotoga* PP_i-PFK was unique in that it exhibited higher activity with tripolyphosphate (PPP_i) and polyphosphate (poly-P) as phosphoryl donors than with PP_i as the donor, and the apparent K_m and V_{max} values (Table 2) indicate that the *Thermotoga* PP_i-PFK functions as a poly-P-dependent PFK. This is the first report of a PFK with such characteristics. The PP_i-PFK catalyzes a typically reversible reaction, but with the *Thermotoga* enzyme the pH optima for the forward and reverse reactions are unusually close; pH 5.6 to 5.8 for the forward reaction and pH 5.6 to 6.8 for the reverse reaction

TABLE 2. Kinetics of *Thermotoga* PP_i-PFK

Phosphoryl donor	K_m (mM)	V_{max} (U mg^{-1})	V_{max}/K_m
PP_i	0.067	203	3×10^3
PPP_i	0.010	249	2.5×10^4
Poly-P	0.0038	319	8.4×10^4

TABLE 3. Apparent ATP-PFK K_m values for ATP, F-6-P, and GTP

Phosphoryl donor	K_m (mM)	V_{max} (U mg^{-1})	V_{max}/K_m
ATP	0.009	432	4.8×10^4
F-6-P	0.437	464	1.0×10^3
GTP	1.36	294	2.1×10^2

(Table 1). In general, other PP_i-PFKs have a pH difference of up to one unit between the forward and reverse reactions. Similar to other PP_i-PFKs, the *Thermotoga* PP_i-PFK exhibited essentially no response to traditional allosteric effectors, and presumably the reaction direction and rate (due only to the PP_i-PFK) are dictated simply by the concentrations of intracellular metabolites and the level of the enzyme.

The *Thermotoga* ATP-PFK displayed the highest activity with ATP as the phosphoryl donor (Table 3) but had significant activity when this was replaced by GTP, UTP, CTP, and TTP. No activity was detected with either PP_i, PPP_i, poly-P, or ADP as the phosphoryl donor (Table 1). The pH optimum for the ATP-PFK was between 7.2 and 8.0 (which is more likely to reflect the intracellular pH of the organism). The ATP-PFK showed no significant response to the common allosteric regulators. Thus, activity was only slightly inhibited by citrate at 1.0 mM, and PEP concentrations up to 5 mM did not affect the normal hyperbolic kinetic curve for fructose 6-phosphate (F-6-P). The allosteric response of the ATP-PFKs from *E. coli* and *Bacillus stearothermophilus* is potentially controlled by a glutamic acid residue at position 187 (E^{187}) via the binding of PEP (2, 8, 22). Sequence alignment shows that the *Thermotoga* ATP-PFK also possesses an equivalent E^{187} residue, but the biochemical properties from this characterization suggest that PEP is not vital for regulating the *Thermotoga* enzyme and thus probably does not regulate glycolysis in this organism (7). ADP had opposing effects on ATP-PFK activity, as the enzyme was slightly activated at a low concentration of ADP (129% at 0.05 mM) and partially inhibited at higher concentrations (70% at 1.0 mM), but the magnitude of these effects does not reflect allosteric control.

Surprisingly, the *Thermotoga* ATP-PFK activity was strongly inhibited by PP_i, PPP_i, or poly-P ($n = 15 \pm 3$) at concentrations of less than 0.10 mM and under conditions in which chelation effects on available Mg^{2+} could be excluded (Table 4; Fig. 1). In particular, activity was strongly inhibited by both PP_i and poly-P at concentrations reported to be common in bacteria (10 to 100 μM) (14). Interestingly, the inhibition of ATP-PFK activity by PP_i could be partially alleviated by the presence of

TABLE 4. Effects of PP_i, PPP_i, poly-P, citrate, and PEP on the apparent K_m for F-6-P of the ATP-PFK

Effector	K_m (mM)	V_{max} (U mg^{-1})	V_{max}/K_m
0.01 mM PP_i	0.436	148	3.4×10^2
0.025 mM PP_i	0.749	136	1.8×10^2
0.05 mM PPP_i	0.983	256	2.6×10^2
0.05 mM poly-P	1.376	278	2.0×10^2
1.0 mM citrate	0.0765	224	2.9×10^2
5.0 mM PEP	0.315	260	8.2×10^2

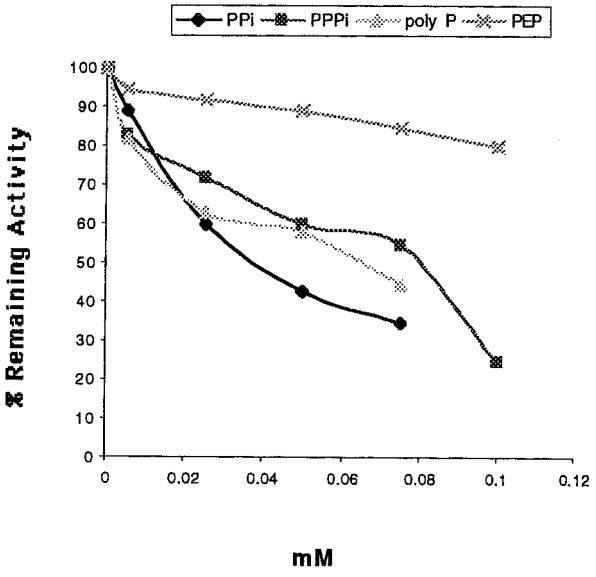

FIG. 1. Effects of PP_i, PPP_i, poly-P, and PEP on *Thermotoga* ATP-PFK activity.

nucleotide diphosphates, i.e., ADP, GDP, or TDP (Table 5). This type of allosteric control has not previously been reported, and it seems that PP_i and/or poly-P might replace, either partially or fully, the function of PEP and other potential modulators within this organism. Although nonallosteric ATP-PFKs have been identified in other organisms, including *D. amylolyticus* (5, 9), *Trypanosoma brucei* (15), and *Lactobacillus bulgaricus* (4), the responses of these enzymes to PP_i and poly-P have not been investigated.

Role of poly-P. Both *Thermotoga* enzymes have unique properties related to poly-P: it is a preferred substrate for the PP_i-PFK and an allosteric regulator for the ATP-PFK. Poly-P is a component of volcanic condensates and deep-oceanic hydrothermal vents, and it is ubiquitously distributed in all living organisms (13) and possibly played a role in the prebiotic evolution of metabolism (3, 29). Significantly, poly-P has been used as an alternate phosphoryl and/or energy source to ATP for other enzymes involved with glucose metabolism. For example, poly-P-dependent glucokinase activity has been observed in *Mycobacterium tuberculosis* (11) and *Propionibacterium freudenreichii* (26, 28), and a poly-P-fructokinase has been

TABLE 5. Effects of some compounds on the PP_i inhibition on *Thermotoga* ATP-PFK[a]

Effector	% Activity
None	100
0.1 mM PP_i	20
0.1 mM PP_i and 0.1 mM ADP	57
0.1 mM PP_i and 0.1 mM AMP	22
0.1 mM PP_i, 0.1 mM ADP, and 0.1 mM AMP	57
0.1 mM PP_i and 0.1 mM TDP	69
0.1 mM PP_i and 0.1 mM GDP	68
0.1 mM PP_i and 0.1 mM CDP	27
0.1 mM PP_i and 0.1 mM UDP	47

[a] Experimental conditions were 0.5 mM F-6-P, 0.25 mM ATP, 5 mM $MgCl_2$, 0.2 mM NADH, 175 mM KCl, and 30 mM Tris (pH 7.8) at 50°C.

found in *Mycobacterium phlei* (28). The poly-P-glucokinase from *P. freudenreichii* was particularly responsive to phosphoester chain length, with the apparent K_m declining from 4.3 μM to 0.2 nM for polymer lengths of 30 and 724 residues, respectively (28). The PP_i-PFK from *Thermotoga* demonstrated a similar, though less pronounced, effect, with a decline in K_m values from 67 to 3.8 μM as phosphoester chain length increased from 2 to 18. In contrast, the PP_i-PFKs from *D. thermophilum* and *Spirochaeta thermophila* favor the pyrophosphate substrate (7, 20).

The results presented here indicate that the control of the EM pathway in *Thermotoga* may be mediated by a quite different mechanism than that conventionally found, where the activity of ATP-PFK is allosterically controlled by either PEP, ADP, AMP, F-2,6-P_2, citrate, succinate, or a combination of these. For glycolysis to proceed utilizing the ATP-PFK, the PP_i and poly-P concentrations would have to remain low (<100 μM). If poly-P accumulated and/or the pH fell, then the ATP-PFK would be inhibited and the PP_i-PFK activity would predominate. Poly-P is regarded as ubiquitous in all tested organisms (13, 14) and is present at concentrations above that needed to inhibit the ATP-PFK. Interestingly, no gene encoding a poly-P kinase has been identified in the genome of *Thermotoga*, though in other organisms other enzymes have also been implicated in the synthesis of poly-P, e.g., adenylate kinase in *Acinetobacter johnsonii* (19) and an acetate kinase in *E. coli* (10). Possibly, the PP_i-PFK could produce poly-P by means of the reverse reaction at intracellular pH values between 6.0 and 7.0. The presence of ATP-PFK activity in cell extracts of *Thermotoga* has been reported (23, 24). We found both PP_i-PFK and ATP-PFK activities in cell extracts if the assay pH was adjusted to the optimum for each enzyme (results not shown), so the enzymes appear to be expressed simultaneously. The intracellular concentration of PP_i and poly-P and the internal pH of *Thermotoga* are unknown, but it will be important to determine these if the control of glycolysis in *Thermotoga* is to be understood. In summary, *Thermotoga* appears to be unique in that it contains the genes for two distinct PFKs and both genes can express functional enzymes. Both enzymes have unique properties, in particular, their responses to PP_i and poly-P, and it is likely that these metabolites may play a central role in the control of glucose metabolism in this organism.

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