Molecular and Biochemical Characterization of a Distinct Type of Fructose-1,6-Bisphosphatase from Pyrococcus furiosus

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Received 12 December 2001/Accepted 15 March 2002

The Pyrococcus furiosus fbpA gene was cloned and expressed in Escherichia coli, and the fructose-1,6-bisphosphatase produced was subsequently purified and characterized. The dimeric enzyme showed a preference for fructose-1,6-bisphosphate, with a \( K_m \) of 0.32 mM and a \( V_{\text{max}} \) of 12.2 U/mg. The \( P. \) furiosus fructose-1,6-bisphosphatase was strongly inhibited by \( \text{Li}^+ \) (50\% inhibitory concentration, 1 mM). Based on the presence of conserved sequence motifs and the substrate specificity of the \( P. \) furiosus fructose-1,6-bisphosphatase, we propose that this enzyme belongs to a new family, class IV fructose-1,6-bisphosphatase.

The hyperthermophilic archaeon Pyrococcus furiosus is capable of metabolizing sugar via an Embden-Meyerhof-like pathway. A combination of physiological, biochemical, and genetic studies has revealed that pyrococcal glycolysis differs from the regular Embden-Meyerhof pathway by its incorporation of new conversions, its novel enzymes, and its unique control (5). Compelling evidence of the deviation of pyrococcal glycolysis from the canonical glycolysis includes the recruitment of two unique ADP-dependent sugar kinases (10, 21) and the presence of a structurally distinct phosphoglucose isomerase (23) and a glyceraldehyde-3-phosphate dehydrogenase (22) in \( P. \) furiosus. fructose-1,6-bisphosphatase (FBPase) is an essential regulatory enzyme in the gluconeogenic pathway. It converts D-fructose-1,6-bisphosphate to classical FBPase (13). In its absence for fructose-1,6-bisphosphate, with a \( K_m \) of 0.32 mM and a \( V_{\text{max}} \) of 12.2 U/mg. The \( P. \) furiosus fructose-1,6-bisphosphatase was strongly inhibited by \( \text{Li}^+ \) (50\% inhibitory concentration, 1 mM). Based on the presence of conserved sequence motifs and the substrate specificity of the \( P. \) furiosus fructose-1,6-bisphosphatase, we propose that this enzyme belongs to a new family, class IV fructose-1,6-bisphosphatase.

In an attempt to complete the set of determined glycolytic and gluconeogenic enzymes in \( P. \) furiosus, we cloned and expressed the MJ0109 ortholog from \( P. \) furiosus (48\% identity on the amino acid level) in \( E. \) coli and investigated its ability to function as a thermotolerant FBPase.

Transcript analysis and cloning of \( fbpA \). An ortholog (\( fbpA \)) of MJ0109 (2) was identified in the \( P. \) furiosus genome database (http://www.genome.utah.edu). This ortholog was originally designated as an extragenic suppressor, \( suhB \). The start of the \( fbpA \) gene was predicted based on the presence and proper spacing of a potential Shine-Dalgarno sequence and on multiple alignment of the deduced amino acid sequence with those of related enzymes (Fig. 1). To test whether the \( fbpA \) gene was transcribed in \( P. \) furiosus, total RNA was isolated from a pyruvate-grown \( P. \) furiosus culture (40 mM) as described previously (25). The presence of the \( fbpA \) transcript was confirmed (data not shown) by using a reverse transcription-PCR system according to the instructions of the manufacturer (Promega) with 1 \( \mu \)g of \( P. \) furiosus RNA and the primers BG977 and BG978 (see below). Moreover, a recent genome-based microarray analysis of \( P. \) furiosus also revealed the expression of \( fbpA \) (designated \( suhB \)) (18).

The \( fbpA \) gene (765 bp) was PCR amplified from chromosomal DNA of \( P. \) furiosus as described previously (21) with the primers BG977 (\( 5'\)-GCCCGTCATGAACTTAAGTTCTG GAGGG [with the BspHI restriction site underlined], sense) and BG978 (\( 5'\)-GCCCGGATCCCTACTCCAGTAAAGCTTA AAAATTGTTT [with the BamHI restriction site underlined], antisense). The PCR product was digested with BspHI/BamHI and cloned into \( E. \) coli XL1-Blue by use of an \( NcoI\)/BamHI-digested pET24d vector according to established procedures and with 50 \( \mu \)g of kanamycin/ml for selection. Subsequently, the resulting plasmid, pLUW558, was transformed into \( E. \) coli BL21 (DE3).

Overexpression and purification of FBPase. An overnight culture of \( E. \) coli BL21 (DE3) harboring pLUW558 was used as a 1% inoculum in 0.5 liter of Luria-Bertani medium with 50 \( \mu \)g of kanamycin/ml. Gene expression was induced by adding 0.1 mM isopropyl-1-thio-\( \beta\)-D-galactopyranoside (IPTG) at an optical density at 600 nm of 0.5. Growth was allowed to continue for 10 h at 37°C, and cells were harvested by centrifugation (2,200 \( \times \) g for 20 min at 4°C) and resuspended in 10 ml of 50
mM Tris-HCl buffer, pH 8.0. The cells were disrupted by French press treatment (100 MPa), and cell debris was removed by centrifugation (10,000 g for 20 min at 4°C). The resulting cell extract was heat treated for 30 min at 80°C, and the precipitated proteins were removed by centrifugation (10,000 g for 30 min at 4°C). The heat-stable cell extract was filtered through a 0.45-μm-pore-size filter and applied to a MonoQ HR 5/5 column (1 ml; Amersham Pharmacia Biotech) equilibrated with 50 mM Tris-HCl buffer, pH 8.0. The FBPase activity eluted at 0.37 M NaCl in a linear gradient of 0.0 to 1.0 M NaCl. Active fractions were pooled and concentrated 20-fold to a final volume of 100 μl by use of a filter with a 10-kDa cutoff (Microsep; Pall Filtron). The concentrated pool was loaded on a Superdex 200 HR 10/30 gel filtration column (24 ml; Amersham Pharmacia Biotech) equilibrated with 50 mM Tris-HCl buffer, pH 7.8, containing 100 mM NaCl. The elution pattern (not shown) suggested that the active configuration was a dimer (66.8 kDa) of two identical 33-kDa subunits, which is in good agreement with the results of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (data not shown). The calculated size of the subunit was slightly smaller, i.e., 27.9 kDa. The purified enzyme was desalted in 50 mM Tris-HCl buffer, pH 8.0, by use of a filter with a 10-kDa cutoff (Microsep; Pall Filtron). From 2.7 g of cell paste consisting of E. coli BL21(DE3) containing pLUW558, a total of 27.7 mg of FBPase was purified to 95% purity, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). To ensure that the detected activity corresponded to the P. furiosus FBPase, the N-terminal sequence of the purified enzyme was determined by the Edman degradation method to be Met-Lys-Leu-Lys-Phe-Trp-Arg-Glu-Val-Ala-Ile-Asp-Ile-Ile-Ser-Asp-Phe-Glu-Thr-Thr-Ile-Met-Pro-Phe, revealing that the obtained amino acid sequence exactly matched the N-terminal sequence of the translated \textit{fbpA} from \textit{P. furiosus} (Fig. 1). This indicates that the \textit{P. furiosus} FBPase had been produced and purified successfully.

**FIG. 1.** Multiple sequence alignment of the deduced amino acid sequence of the \textit{P. furiosus} FBPase with the sequences of its FBPase IV homologs and of I-1-Pases and FBPases from eukarya and bacteria. Abbreviations (accession numbers are given in parentheses): H.s. IMP, \textit{Homo sapiens} IMP 1-1-Pase 1 (P29218); E.c. IMP, \textit{E. coli} SuhB I-1-Pase (P22783); T.m. FBPIV, \textit{T. maritima} TM1415 FBPase (O33832); A.f. FBPIV, \textit{A. fulgidus} AF2372 FBPase (NP_071195); M.j. FBPIV, \textit{M. jannaschii} MJ0109 FBPase (Q57573); E.c. FBP1, \textit{E. coli} FBPase (P09200); S.s. FBPass, \textit{Sus scrofa} FBPase (P00636). Gaps introduced by the alignment are indicated by dashes. Completely conserved regions are indicated by black boxes. Highly conserved hydrophobic bars above the alignments. The FBPase motif is indicated with a horizontal gray bar under the alignment. The IMP motifs are indicated with horizontal black bars above the alignments. IMP 1 motif, [FWV]-x (0, 1)-[LIVM]-D-P-[LIVM]-D-[SG]-[ST]-x (2)-[FY]-x-[HKRNSTY]; inositol monophosphatase family signature 1 (PS00629). IMP 2 motif, [WV]-D-x-[AC]-[GSA]-[GSAPV]-x-[LIVACP]-[LIV]-[LIVAC]-x (3)-[GH]-[GA]; inositol monophosphatase family signature 2 (PS00630). FBPase motif, [AG]-[RK]-[LI]-x (1, 2)-[LIV]-[FY]-E-x (2)-P-[LIVM]-[GSA] (PS00124) (http://www.expasy.ch/prosite). Asterisks denote residues involved in the Li⁺ binding site (24).
Acid) buffer, pH 7.4, and 10 mM MgCl₂. The vials were submerged in an oil bath at temperatures varying from 20 to 120°C and preheated for 2 min, and the enzyme reaction was initiated by the injection of 15 mM fructose-1,6-bisphosphate. At different times up to 15 min, the reaction was stopped by transferring the vials to a mixture of ice and ethanol. Aliquots were taken, and the amount of fructose-6-phosphate formed was determined spectrophotometrically by measuring the reduction of NADP⁺ (340 nm) at room temperature in an assay with glucose-6-phosphate isomerase (EC 5.3.1.9) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49), both from Saccharomyces cerevisiae. A linear increase in fructose-6-phosphate production over time was observed, indicating that no P. furiosus FBPase was inactivated during incubation. The P. furiosus FBPase showed maximal activity at approximately 100°C (data not shown).

In accordance with first-order inactivation kinetics (data not shown), the enzyme (18 µg/ml) lost 50% of its activity after incubation for 2 h at 100°C in 50 mM Tris-HCl buffer, pH 8.0. For the determination of the melting temperature, P. furiosus FBPase was dialyzed extensively against a 100 mM sodium phosphate buffer, pH 8.0, and diluted to 0.3 mg/ml in dialysis buffer. After being degassed for 10 min, the samples were analyzed against the dialysis buffer in a differential scanning microcalorimeter (VP-DSC; MicroCal) between 50 and 125°C at 0.5°C/min. Enzyme scans were corrected with a buffer-buffer baseline, and data were analyzed with the MicroCal Origin 5.0 SR2 software package. For FBPase, an apparent melting temperature of 107.5°C was determined (data not shown), which is in good agreement with the inactivation kinetics.

Catalytic properties. The kinetic parameters of the P. furiosus FBPase were determined discontinuously at 85°C by varying the concentration of fructose-1,6-bisphosphate (between 0.005 and 5 mM) and by measuring the release of inorganic phosphate at room temperature as described previously (8). The 0.2-ml assay mixture contained 50 mM Tris-HCl buffer (pH 8.0; room temperature), 10 mM MgCl₂, and 0.4 µg of purified FBPase. At this temperature, the Kₘ and Vₘₐₓ of the P. furiosus FBPase with fructose-1,6-bisphosphate were 0.32 ± 0.03 mM and 12.2 ± 0.1 U/mg, respectively, resulting in a catalytic efficiency (kₗₜ/Kₘ) of 17.7 s⁻¹ mM⁻¹. The determined affinity of the purified FBPase for fructose-1,6-bisphosphate is in good agreement with the previously determined Kₘ of 0.5 mM (75°C) in a P. furiosus extract (16). The kinetic parameters of the purified FBPase determined at 50°C were as follows: a Kₘ of 0.31 ± 0.06 mM, a Vₘₐₓ of 0.72 ± 0.04 U/mg, and a catalytic efficiency of 1.12 s⁻¹ mM⁻¹. Thus, the P. furiosus FBPase clearly is a thermoactive enzyme with affinities for fructose-1,6-bisphosphate at 50 and 85°C that are similar.

The specific activities of the P. furiosus FBPase for fructose-1,6-bisphosphate and related substrates were determined at 85°C in the standard assay that measures the release of inorganic phosphate. The 1-ml assay mixture contained 50 mM Tris-HCl buffer (pH 8.0; room temperature), 2.5 mM substrate, 10 mM MgCl₂, and 0.02 mg of purified FBPase. At fructose-1,6-bisphosphate concentrations above 10 mM, the enzyme was subjected to substrate inhibition (data not shown). The highest activity was obtained with fructose-1,6-bisphosphate (12.2 U/mg). In addition, myo-inositol-1-phosphate, glucose-1-phosphate, and glycerol-2-phosphate could also be dephosphorylated by the enzyme, although the activities for these substrates were relatively low (1.7 to 7.5%) (Table 1). The recently described I-1-Pase/FBPase from M. jannaschii (MJI0109) also dephosphorylates these substrates but with a higher relative activity (42 to 61%) (20) (Table 1). The P. furiosus FBPase appeared to be a rather specific phosphatase, since fructose-1-phosphate, fructose-6-phosphate, glucose-6-phosphate, phosphoenolpyruvate (PEP), 5'-AMP, 5'-ADP, and 5'-ATP could not be used as substrates by the P. furiosus FBPase.

Effectors of FBPase. The effect of inhibitors on the activity of the P. furiosus FBPase was investigated by adding cations and metabolites (0 to 100 mM) to the standard enzyme assay mixture (85°C) (Table 2). The enzyme has an absolute requirement for Mg²⁺ (data not shown). The inhibition characteristics of the P. furiosus FBPase clearly differ from those of characterized eukaryal and bacterial FBPases as well as from those of the other presently characterized archaeal I-1-Pase/FBPase homologs. FBPase I from E. coli is very sensitive to AMP and PEP (1). FBPase II from E. coli is strongly inhibited by ATP and ADP, whereas AMP has no effect on the enzyme activity. Furthermore, FBPase II activity is enhanced in the presence of PEP (6). PEP also affects FBPase III activity, i.e., inhibition by

### Table 1. Substrate specificity of P. furiosus FBPase compared to that of M. jannaschii MJI0109

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. furiosus FBPase</strong></td>
<td><strong>M. jannaschii MJI0109</strong></td>
</tr>
<tr>
<td>Fructose-1,6-bisphosphate</td>
<td>100</td>
</tr>
<tr>
<td>Inositol-1-phosphate</td>
<td>7.5</td>
</tr>
<tr>
<td>Glucose-1-phosphate</td>
<td>1.7</td>
</tr>
<tr>
<td>Glucose-1-phosphate</td>
<td>2.8</td>
</tr>
<tr>
<td><strong>M. jannaschii MJI0109</strong></td>
<td></td>
</tr>
<tr>
<td>Inositol-1-phosphate</td>
<td>100</td>
</tr>
<tr>
<td>Glucose-1-phosphate</td>
<td>64</td>
</tr>
<tr>
<td>Glucose-1-phosphate</td>
<td>49</td>
</tr>
<tr>
<td>Glucose-1-phosphate</td>
<td>42</td>
</tr>
</tbody>
</table>

* Assays for P. furiosus FBPase were performed at 85°C as described in the text. The data from assays of MJI0109 performed at 85°C were obtained from Stec et al. (20). An activity of 100% corresponds to 12.2 and 15.2 U/mg for P. furiosus FBPase and MJI0109, respectively. Fructose-1-phosphate, fructose-6-phosphate, glucose-6-phosphate, PEP, 5'-AMP, 5'-ADP, and 5'-ATP could not be used as substrates by the P. furiosus FBPase.
AMP is reduced when PEP is present (7). The *P. furiosus* FBPase was inhibited by ADP and ATP (and to some extent by AMP), but PEP (up to 100 mM) did not influence FBPase activity at all. Therefore, PEP presumably is not an important metabolite in the regulation of FBPase in *P. furiosus*. In addition, glucose-6-phosphate significantly reduced *P. furiosus* FBPase activity in vitro (Table 2).

Li⁺ is generally a strong inhibitor of FBPase activity (Ki ~ 0.3 mM) (24). Under the test conditions we used, Li⁺ significantly reduced the *P. furiosus* FBPase activity (50% inhibitory concentration [IC₅₀], 1 mM) (Table 2), whereas the addition of Na⁺ and K⁺ produced no effect. Previously, it was shown that I-1-Pases are also strongly inhibited by Li⁺ (IC₅₀ ~ 0.3 mM) (12). These enzymes have a structural fold similar to that of FBPases (27), and both are members of the sugar phosphatase superfamily (http://scop.mrc-lmb.cam.ac.uk/scop). Inhibition of mammalian I-1-Pase by Li⁺ is of particular interest, since this enzyme is expressed in brain tissue and forms the main target in medical treatment of manic-depression (14). The presence of a tetrameric structure of this enzyme is of particular interest, since it contains both I-1-Pase and FBPase domains (Fig. 1) (9). Minor variations will probably distinguish the inhibitory effect of Li⁺ on the I-1-Pase from that on FBPase (9).

**Classification of FBPases.** Recently, a new classification of bacterial FBPases into three groups (FBPase I, FBPase II, and FBPase III) has been proposed (6). Eukaryal FBPases are orthologous to the bacterial FBPase I enzymes, since both contain typical FBPase domains (http://www.expasy.ch) and display no I-1-Pase activity (20). The typical FBPase domain is absent in the bacterial FBPase II and FBPase III enzymes (Table 3), suggesting that they are phylogenetically unrelated to FBPase I enzymes. Remarkably, typical I-1-Pase domains (IMP 1 and IMP 2) are also present in the eukaryal FBPase and the bacterial FBPase I enzymes (http://www.expasy.ch). Bacterial and eukaryal I-1-Pases contain two specific domains (IMP 1 and IMP 2) and, together with the eukaryal FBPase and bacterial FBPase I enzymes, belong to the sugar phosphatase superfamily (http://scop.mrc-lmb.cam.ac.uk/scop). Comparison of the primary structure of the *P. furiosus* FBPase with the FBPase and IMP family signatures revealed that this enzyme contains both I-1-Pase domains (IMP 1 and IMP 2) and, together with the eukaryal FBPase and bacterial FBPase I enzymes, belong to the sugar phosphatase superfamily (http://scop.mrc-lmb.cam.ac.uk/scop). Inhibition of mammalian I-1-Pase by Li⁺ is of particular interest, since this enzyme is expressed in brain tissue and forms the main target in medical treatment of manic-depression (14). The presence of a tetrameric structure of this enzyme is of particular interest, since it contains both I-1-Pase and FBPase domains (Fig. 1) (9). Minor variations will probably distinguish the inhibitory effect of Li⁺ on the I-1-Pase from that on FBPase (9).

**Nucleotide sequence accession number.** The *P. furiosus* fbpA gene was deposited in the *GenBank* database under the accession number AF453319.

### Table 2. Inhibitors of *P. furiosus* FBPase activity

<table>
<thead>
<tr>
<th>Effector</th>
<th>IC₅₀ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li⁺</td>
<td>1</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>&lt;5</td>
</tr>
<tr>
<td>AMP</td>
<td>30</td>
</tr>
<tr>
<td>ADP</td>
<td>30</td>
</tr>
<tr>
<td>ATP</td>
<td>4</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>4</td>
</tr>
<tr>
<td>Fructose-6-phosphate</td>
<td>25</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>60</td>
</tr>
</tbody>
</table>

* Enzyme assays were performed at 85°C as described in the test (10 mM fructose-1,6-bisphosphate). IC₅₀, the concentration of the effector when activity at 50% is reduced.

### Table 3. Classification of phosphatases

<table>
<thead>
<tr>
<th>Phosphatase class</th>
<th>Taxonomic range</th>
<th>Subunit size (kDa)</th>
<th>Oligomerization</th>
<th>Fold type</th>
<th>Sequence motif(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBPase I</td>
<td>Eukarya, bacteria</td>
<td>~38</td>
<td>Tetramer</td>
<td>Sugar phosphatase</td>
<td>FBPase, IMP 1</td>
</tr>
<tr>
<td>FBPase II</td>
<td>Bacteria</td>
<td>~36</td>
<td>Dimer</td>
<td>Unknown</td>
<td>None</td>
</tr>
<tr>
<td>FBPase III</td>
<td>Bacteria</td>
<td>~76</td>
<td>Tetramer</td>
<td>Unknown</td>
<td>None</td>
</tr>
<tr>
<td>FBPase IV</td>
<td>Archaea, HT bacteria</td>
<td>~28</td>
<td>Dimer²</td>
<td>Sugar phosphatase</td>
<td>IMP 1, IMP 2</td>
</tr>
<tr>
<td>I-1-Pase</td>
<td>Eukarya, bacteria</td>
<td>~30</td>
<td>Dimer</td>
<td>Sugar phosphatase</td>
<td>IMP 1, IMP 2</td>
</tr>
</tbody>
</table>

* FBPase IV enzymes are at least present in the euryarchaea *P. furiosus* (AF453319), *Pyrococcus hontokoshii* (PH1897), *Pyrococcus abyssi* (PAB0189), *Methanococcus jannaschii* (MJ0109), *Archeoglobus fulgidus* (AF2372), *Methanosarcina barkeri* (MB1918), and *Methanobacterium thermoautotrophicum* (MTH871) and the hyperthermophilic HT bacteria *Thermotoga maritima* (TM1415) and *Aquifex aeolicus* (AA1983).

**Characteristics**

- FBPase I enzymes are present in all Eukarya, Bacteria, and Archaea.
- FBPase II enzymes are present in all Bacteria.
- FBPase III enzymes are present in all Bacteria.
- FBPase IV enzymes are present in Archaea. 

**Table 3. Classification of phosphatases**

- **FBPase I**
  - Eukarya, bacteria
  - Subunit size: ~38 kDa
  - Oligomerization: Tetramer
  - Fold type: Sugar phosphatase
  - Sequence motif(s): FBPase, IMP 1

- **FBPase II**
  - Bacteria
  - Subunit size: ~36 kDa
  - Oligomerization: Dimer
  - Fold type: Unknown
  - Sequence motif(s): None

- **FBPase III**
  - Bacteria
  - Subunit size: ~76 kDa
  - Oligomerization: Tetramer
  - Fold type: Unknown
  - Sequence motif(s): None

- **FBPase IV**
  - Archaea, HT bacteria
  - Subunit size: ~28 kDa
  - Oligomerization: Dimer²
  - Fold type: Sugar phosphatase
  - Sequence motif(s): IMP 1, IMP 2

- **I-1-Pase**
  - Eukarya, bacteria
  - Subunit size: ~30 kDa
  - Oligomerization: Dimer
  - Fold type: Sugar phosphatase
  - Sequence motif(s): IMP 1, IMP 2

* Enzyme assays were performed at 85°C as described in the test (10 mM fructose-1,6-bisphosphate). IC₅₀, the concentration of the effector when activity at 50% is reduced.

**Characteristics**

- FBPase I enzymes are present in all Eukarya, Bacteria, and Archaea.
- FBPase II enzymes are present in all Bacteria.
- FBPase III enzymes are present in all Bacteria.
- FBPase IV enzymes are present in Archaea.
- I-1-Pase enzymes are present in all Eukarya, bacteria.
nucleotide and amino acid sequence data reported in this study have been submitted to GenBank under accession no. AF453319.

We thank L. Kluskens (Wageningen University) for assistance during the differential scanning calorimetry measurements and Stefan Wolff (Essen University, Essen, Germany) for providing myo-inositol-1-phosphate.

This work was supported by the Earth and Life Sciences foundation (ALW), which is subsidized by The Netherlands Organization for Scientific Research (NWO).

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