A Novel Candidate for the True Fructose-1,6-bisphosphatase in Archaea*

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Fructose-1,6-bisphosphatase (FBPase) is one of the key enzymes of the gluconeogenic pathway. Although enzyme activity had been detected in Archaea, the corresponding gene had not been identified until a presumably inositol monophosphatase gene from Methanococcus jannaschii was found to encode a protein with both inositol monophosphatase and FBPase activities. Here we display that a gene from the hyperthermophilic archaeon, Thermococcus kodakaraensis KOD1, which does not correspond to the inositol monophosphatase gene from M. jannaschii, displays high FBPase activity. The FBPase from strain KOD1 was partially purified, its N-terminal amino acid sequence was determined, and the gene (Tk-fbp) was cloned. Tk-fbp encoded a protein of 375 amino acid residues with a molecular mass of 41,658 Da. The recombinant Tk-Fbp was purified and characterized. Tk-Fbp catalyzed the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate following Michaelis-Menten kinetics with a $K_m$ value of 100 $\mu$M toward fructose 1,6-bisphosphate, and a $k_{cat}$ value of 17 s$^{-1}$ subunit$^{-1}$ at 95 °C. Unlike the inositol monophosphatase from M. jannaschii, Tk-Fbp displayed strict substrate specificity for fructose 1,6-bisphosphate. Activity was enhanced by Mg$^{2+}$ and dithioerythritol, and was slightly inhibited by fructose 2,6-bisphosphate. AMP did not inhibit the enzyme activity. We examined whether expression of Tk-fbp was regulated at the transcriptional level. High levels of Tk-fbp transcripts were detected in cells grown on pyruvate or amino acids, whereas no transcription was detected when starch was present in the medium. Orthologous genes corresponding to Tk-fbp with high similarity are present in all the complete genome sequences of thermophilic Archaea, including M. jannaschii, Pyrococcus furiosus, Sulfolobus solfataricus, and Archaeoglobus fulgidus, but are yet to be assigned any function. Taking into account the high FBPase activity of the protein, the strict substrate specificity, and its sugar-repressed gene expression, we propose that Tk-Fbp may represent the bona fide FBPase in Archaea. Although most enzymes are shared by the two pathways, fructose-6-phosphate kinase catalyzes the unidirectional phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate in glycolysis. Fructose-1,6-bisphosphatase (n-fructose 1,6-bisphosphate 1-phosphohydrolase, FBPase) catalyzes the reverse reaction in gluconeogenesis, the hydrolysis of fructose 1,6-bisphosphate (Fru-1,6-P$_2$) to fructose 6-phosphate (Fru-6-P) and inorganic phosphate (1). Therefore FBPase is regarded as one of the key enzymes in gluconeogenesis (2).

FBPases have been characterized from bacteria (3–5), yeast (6, 7), as well as higher eukaryotes (8–11). The regulation of FBPase gene expression has been extensively studied in yeast, and is regarded as a typical example of glucose repression/derepression (12). In Saccharomyces cerevisiae, gene transcription is repressed by the MIG1 repressor in the presence of glucose, and is derepressed in the absence of glucose via the SNF1/CAT8 regulation pathway (13). Besides the regulation at the transcriptional level, FBPase is also known to be an allosteric enzyme (14). FBPases from bacteria, yeast, and mammals have been reported to be inhibited allosterically by AMP (15). Addition of AMP causes the enzyme to cooperatively shift from the fully active R-state (relaxed) to the inactive T-state (tense) (16). Fructose 2,6-bisphosphate has also been found to inhibit FBPase activity as a substrate analog (17). The amino acid sequences of mammalian enzymes are 85% identical to each other, and are similar to the Class I FBPases in bacteria (3). In Escherichia coli, two FBPases have been identified. The Class I enzyme encoded by the fbp gene (Ec-Fbp) has been long recognized as the sole FBPase in E. coli (18). However, a second FBPase encoded by the glpX gene (Ec-GlpX) has recently been identified (4). Ec-GlpX does not display structural similarity with Ec-Fbp, and has been classified as a Class II enzyme. A very divergent Class III FBPase has also been identified in Bacillus subtilis (5). In E. coli, disruption of the fbp gene led to a mutant that grew as well as the wild type strain on glucose or fructose, but could not grow on glycerol or other gluconeogenic substrates. In contrast, the glpX disruptant strain did not display a particular phenotype. In the case of E. coli, the Class I Ec-Fbp is presumed to be the major enzyme involved in gluconeogenesis (4).

In Archaea, the identification of FBPase has attracted much attention. This is because of the fact that although FBPase activity had been detected in cell extracts of several Archaea (19–22), orthologous genes with structural similarity to previously reported FBPases are not present on their genomes. This was resolved to some extent by the finding that the MJ0109

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The nucleotide sequence for the Tk-fbp gene reported in this paper has been submitted to the DDBJ/GenBankTM/EBI Data Bank with accession number AB081839.

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1 The abbreviations used are: FBPase, l-fructose 1,6-bisphosphate 1-phosphohydrolase; Fru-6-P, fructose 6-phosphate; Fru-1,6-P$_2$, fructose 1,6-bisphosphate; MES, 4-morpholineethanesulfonic acid.
Novel Archaeal FBPase

Thermococcus kodakarensis KOD1 is a hyperthermophilic archaeon isolated from Kodakara Island, Kagoshima, Japan (23). The strain can grow with amino acids as a carbon and energy source and sulfur as the terminal electron acceptor. The cells can also assimilate starch or pyruvate, providing a good tool for studying gluconeogenesis and its regulation in Archaea. Here we report the FBPase from T. kodakarensis KOD1, a structurally distinct enzyme from previously identified FBPases, including the MJ0109 gene product from M. jannaschii.

**Experimental Procedures**

**Bacterial Strains, Plasmids, and Bacteriophages—** T. kodakarensis KOD1 was isolated from a soliflucaric hot spring at a wharf in Kodakara Island, Kagoshima, Japan (23). E. coli strain DH5α was used for subcloning of the gene fragments and DNA manipulations. E. coli strain BL21(DE3) (Novagen, Madison, WI) was used as a host and pET-5c vector (Novagen) was used for gene expression.

**DNA Manipulation—** Restriction enzymes and DNA polymerase were purchased from Toyobo (Osaka, Japan) and Takara Shuzo (Kyoto, Japan). Genomic, plasmid and phage DNAs were isolated using Qiagen genomic, plasmid, and phage DNA isolation kits, respectively (Qiagen, Hilden, Germany). DNA ligations were performed using the DNA ligation kit (Toyobo). The QIAEX gel extraction kit (Qiagen) was used to recover DNA fragments from agarose gels.

**Partial Purification of FBPase from KOD1 Cells—** T. kodakarensis KOD1 cells were cultivated in a medium containing pyruvate as a carbon source. After overnight cultivation, cells were harvested, resuspended in 50 mM potassium phosphate buffer (pH 7.0), and disrupted by sonication in ice water. All purification steps were performed at room temperature unless mentioned otherwise. Membrane and cytosolic fractions from the cell lysate were separated by ultracentrifugation at 110,000 × g for 70 min at 4 °C. The cytosolic fraction exhibiting the FBPase activity was loaded on an anion exchange column (RESOURCE Q, Amersham Biosciences AB, Uppsala, Sweden) equilibrated with 50 mM potassium phosphate buffer (pH 7.0). Fractions with FBPase activity were pooled and dialyzed against 50 mM potassium phosphate buffer (pH 7.0) for 4 days.

**Purification of Recombinant Tk-Fbp—** Cells were harvested at centrifugation at 6,000 × g for 10 min at 4 °C and washed with 50 mM potassium phosphate buffer (pH 7.0). The cell pellet was resuspended in the same buffer and the cells were then disrupted by sonication in ice water. Soluble and insoluble fractions were separated by centrifugation (15,000 × g for 30 min at 4 °C). The soluble fraction containing the recombinant Tk-Fbp was incubated at 85 °C for 20 min and centrifuged at 15,000 × g for 30 min at 4 °C to remove heat-labile proteins from the host E. coli. The supernatant carrying Tk-Fbp was purified to homogeneity with the same methodology described above for the native Tk-Fbp. The purity of the protein was examined by SDS-PAGE. Apparent molecular mass of the purified protein was calculated by gel filtration on a Superdex 200 HR 10/30 column (Amersham Biosciences). DNA and amino acid sequence analyses and data base homology search were performed using the Basic Local Alignment Search Tool (BLAST) program. Open reading frame search and molecular mass calculations were performed using DNASIS software (Hitachi Software, Yokohama, Japan). Multiple alignment and phylogenetic analysis was performed using the Clustal W program provided by the DNA Data Bank of Japan (DDBJ).

**Expression of Fbp Gene in E. coli—** The Tk-fbp gene was amplified by PCR and a BamHI site was introduced in the 3'-flanking region of the gene. The DNA fragment was inserted into the pET-5c expression vector (Novagen) at NcoI and BamHI sites and designated pET-fbp.

**Enzyme Activity Assay—** To detect FBPase activity during protein purification, a spectrophotometric coupled enzyme assay was employed to measure the enzyme activity. FBPase activity was coupled with phosphoglucomerase isomerase and NADP-dependent glucose-6-phosphate dehydrogenase, and NADPH formation was measured. Assay mixture (1 ml) contained: 100 mM Tris-HCl buffer (pH 8.0), 0.4 mM NADP+, 20 mM MgCl2, 20 mM dithioerythritol, 0.5 units of phosphoglucomerase isomerase, 0.5 units of glucose-6-phosphate dehydrogenase (Sigma), and 50 μl of the protein sample. The reaction was initiated by adding 2 mM fructose 1,6-bisphosphate. The values obtained when glucose-6-phosphate dehydrogenase was omitted from the reaction mixture were subtracted in each measurement.

**To determine the effects of temperature on enzyme activity, a reaction mixture containing 100 mM Tris-HCl buffer (pH 8.0), 20 mM MgCl2,
The asterisks shown on the mM of the following buffers: citrate buffer (pH 4.5 to 6.5), MES buffer of the various metal ions effect on the enzyme activity, the first reaction mixture to bring the pH of the reaction mixture to 8.0. For examination reaction, 100 °C for 3 min. Generation of NADPH was monitored at 340 nm. The FBPase reaction was initiated with the addition of enzyme and fructose 1,6-bisphosphate and was incubated for 1 min. Product formation was proportional to incubation time under the reaction was carried out in a reaction volume of 100 ml containing 20 mM fructose 1,6-bisphosphate, and 12 μg of purified Tk-Fbp were incubated at 50 °C. Substrates other than fructose 1,6-bisphosphate, such as fructose 2,6-bisphosphate, fructose 6-phosphate, fructose 1-phosphate, glucose 6-phosphate, and glucose 1-phosphate were also incubated under the same conditions. Samples, after incubation for the desired period of time, were kept on ice for 10 min. The reaction mixture was centrifuged at 15,000 g and the supernatant was analyzed by high performance liquid chromatography with a Shodex Asahipak NH2P-50 4E column (Shodex, Tokyo, Japan). Sodium phosphate buffer (300 mM) at pH 4.4 was used as an eluent at a flow rate of 1 ml/min. Column temperature was set at 40 °C and the product was detected with a refractive index detector. RNA Isolation and Northern Blot Analysis—for isolation of RNA from strain KOD1, cells were harvested at the early log phase when A660 was ~0.1. RNA was isolated using the RNeasy Midi Kit (Qiagen). For Northern blot analysis, 15 μg of total RNA was denatured by heat treatment at 65 °C for 15 min, separated by 1% agarose gel electrophoresis, and transferred to a nylon membrane (Hybond TM-N; Amerham Biosciences) by capillary blotting. Digoxigenin labeling of DNA fragments, hybridization, and washing of the membranes were performed according to the instructions of the manufacturer (Roche Molecular Biochemicals). A DNA fragment corresponding to the entire Tk-fbp coding region was used as a probe. A 1.5-kilobase pair region within the coding region of the DNA ligase gene from strain KOD1 (24) was also used as a probe. RESULTS FBPase Activity in KOD1 Cells—T. kodakaraensis KOD1 cells were grown on pyruvate (1%) in the presence of 0.5% yeast extract and 0.5% tryptone. Under these growth conditions, we detected FBPase activity in the cell extracts with a specific activity of 0.4 units/mg at 95 °C. Partial Purification and N-terminal Amino Acid Sequence of FBPase—We partially purified the FBPase from the cell extracts of pyruvate-grown cells. FBPase was purified 13-fold by molecular masses of 25 and 42 kDa (Fig. 1). During the purification procedure, intensities of the 42-kDa protein on SDS-PAGE corresponded well to the levels of FBPase activity in each fraction. The 25- and 42-kDa proteins were both subjected to N-terminal amino acid sequencing. The sequence of the 25-kDa protein (V1G1EKPEVEVKTT) showed high similarity to probable peroxiredoxin proteins from various Archaea species. On the other hand, the N-terminal amino acid se-
sequence of the 42-kDa protein (AVGDKITISVIKADI) exhibited high similarity with hypothetical proteins with no assigned function from *Pyrococcus furiosus* (AE010183), *Pyrococcus abyssi* (F75039), and *Pyrococcus horikoshii* (H71123).

Cloning of the Tk-fbp Gene—Among the 15 N-terminal amino acid residues of the 42-kDa protein, 13 residues were identical to those of the hypothetical proteins from *P. furiosus*, *P. abyssi*, and *P. horikoshii*. Therefore, two oligonucleotides were designed: one from the N-terminal amino acid sequence of the 42-kDa protein, and the other based on a conserved C-terminal region of the hypothetical proteins from the *Pyrococcus* strains mentioned above. PCR with the two primers and genomic DNA of KOD1 as a template led to specific amplification of a DNA fragment with the expected length of 11011 kilobase pair. The entire gene was then isolated from the genomic library of strain KOD1 using the 1-kilobase pair DNA fragment as a probe. DNA sequence analysis identified an open reading frame consisting of 1125 bp encoding a protein of 375 amino acids with a calculated molecular mass of 41,658 Da. The N-terminal sequence deduced from the open reading frame was identical to the N-terminal sequence of the 42-kDa protein from strain KOD1. No other open reading frames were found in the immediate flanking regions of the open reading frame. A putative ribosomal binding site (5'-GGTGG) was identified 6 nucleotides upstream from the initiation codon along with a putative TATA-like element (TATAA, A-Box) 24 nucleotides upstream of the ribosomal binding site. A transcriptional termination signal (poly-(TC)) was also found downstream of the stop codon TGA. As we found that the gene encoded a protein with FBPase activity (see below), we named the gene *Tk-fbp*.

Amino Acid Sequence of Tk-Fbp—The deduced amino acid sequence of Tk-Fbp displayed high similarity to hypothetical proteins of unknown function from various Archaea strains and the bacterium *Aquifex aeolicus*. These included the hypothetical proteins from the *Pyrococcus* species with similar N-terminal amino acid sequences. The Tk-Fbp sequence did not show similarity with previously reported Class I, Class II, or Class III FBPases. All orthologue gene products that were found in the genome data bases are listed in Table I and a representative alignment of the Archaea sequences is shown in Fig. 2. Six regions relatively conserved among Class I FBPases from mammals, plants, fungi, and bacteria have been identified (6). However, sequences with notable similarity to these regions were not found in the Tk-Fbp orthologues.

Production and Purification of Recombinant Tk-Fbp—To characterize the protein product of the *Tk-fbp* gene, and to

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**TABLE II**

| Step                  | Volume (ml) | Total protein (mg) | Activity (units) | Specific activity (units/mg) | Yield (%) | Purification fold |
|----------------------|-------------|--------------------|-----------------|-------------------------------|-----------|------------------|
| Cell-free extract    | 40          | 190                | 361             | 1.9                           | 100       | 1                |
| Heat-treatment       | 39          | 26                 | 336             | 13                            | 93        | 1                |
| Resource Q           | 18          | 16                 | 288             | 18                            | 79        | 1                |
| Mono Q               | 15          | 11                 | 220             | 20                            | 60        | 1                |
| Resource ISO         | 13          | 9                  | 207             | 23                            | 57        | 1                |
| Superdex 200         | 11          | 8                  | 192             | 24                            | 53        | 1                |

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**Fig. 3.** SDS-PAGE of purified recombinant Tk-Fbp. Lane M, molecular mass markers; lane 1, soluble fraction after centrifugation at 15,000 × g; lane 2, soluble fraction after heat treatment at 85 °C for 20 min; lane 3, eluate after ion exchange chromatography with Resource Q and Mono Q; lane 4, eluate after hydrophobic chromatography; lane 5, purified Tk-Fbp after gel-filtration chromatography.

**Fig. 4.** Effect of metal cations and reducing agents on the FBPase activity of Tk-Fbp. A, effect of various metal cations. A chloride salt of each metal cation was used and the activity was examined at 95 °C. Symbols used were: Mg²⁺, closed circles; Mn²⁺, closed triangles; Zn²⁺, open circles; Ca²⁺, open squares; Ni²⁺, open triangles. B, effect of Mg²⁺ (Mg), dithioerythritol (DTE), dithiothreitol (DTT), and 2-mercaptoethanol (2-ME). All components were added at a final concentration of 20 mM. Activity was measured as described under “Experimental Procedures.”
determine whether the enzyme was a \textit{bona fide} FBPase, we expressed the gene in \textit{E. coli}. When \textit{E. coli} BL21(DE3) cells harboring the expression plasmids were grown at 37°C and induced with 1 mM isopropyl-1-thio-D-galactopyranoside for 1 min, the following buffers were used: citrate buffer (open squares), MES buffer (closed triangles), and Tris buffer (closed circles). In a linked assay coupled with phosphoglucoisomerase and glucose-6-phosphate dehydrogenase, Tk-Fbp displayed significant FBPase activity in the linked assay, and no activity was observed when any component of the reaction mixture was omitted. Particularly, we could not detect activity when phosphoglucoisomerase was removed from the reaction mixture, indicating that Tk-Fbp harbored only FBPase activity and not an additional phosphoglucoisomerase activity. We examined the effects of divalent metal cations on the enzyme activity. Addition of EDTA did not lead to a decrease in enzyme activity. Addition of Mg \textsuperscript{2+} significantly enhanced enzyme activity up to concentrations of 20 mM (>5-fold) (Fig. 4A). Addition of 1 mM Mn \textsuperscript{2+} led to a 4-fold increase in activity, but higher concentrations led to a decrease in this effect. Zn \textsuperscript{2+} also enhanced activity at 1 mM, whereas higher concentrations displayed an inhibitory effect. Ca \textsuperscript{2+} and Ni \textsuperscript{2+} had no notable effects on activity (Fig. 4A). We observed that dithioerythritol and, to a lesser extent, other reducing agents such as dithiothreitol and 2-mercaptoethanol stimulated enzyme activity at concentrations of 20 mM (Fig. 4B). One possibility may be that the reducing agents prevent inactivation of the enzyme by oxygen. We did observe a slight oxygen sensitivity in the enzyme, as we found a small decrease in activity when the purified enzyme solution was subjected to bubbling with air (−6% decrease/min bubbling). A combination of Mg \textsuperscript{2+} and dithioerythritol resulted in a 9-fold increase in enzymatic activity (Fig. 4B).

We examined the effect of pH and temperature on the FBPase activity of Tk-Fbp in the presence of 20 mM Mg \textsuperscript{2+} and 20 mM dithioerythritol. Tk-Fbp displayed maximal activity at pH 8.0 (Fig. 5A). The enzyme showed a nearly linear increase in activity between 37 and 95°C, with a −6-fold increase between these temperatures (Fig. 5B). At 95°C and pH 8.0, Tk-Fbp displayed a specific activity of 24 units/mg. Kinetic analysis was also carried out, and Tk-Fbp catalyzed the reaction following Michaelis-Menten kinetics with a $K_{m}$ value of 100 mM toward fructose 1,6-bisphosphate, and a $V_{max}$ value of 17 s\textsuperscript{-1} subunit\textsuperscript{-1} at 95°C. In a linked assay with fructose-1,6-bisphosphate aldolase and glycerol-3-phosphate dehydrogenase, Tk-Fbp did not exhibit activity for the reverse reaction. Thermosensitivity of the recombinant protein was monitored in the presence of Mg \textsuperscript{2+} and the protein was found highly stable even...
at 100 °C. The enzyme displayed a half-life of ~150 min in boiling water (data not shown).

Detection of Substrate and Product with High Performance Liquid Chromatography—To further confirm the FBPase activity of Tk-Fbp, we examined the production of Fru-6-P from Fru-1,6-P₂. Analysis with high performance liquid chromatography was carried out using d-Fru-6-P and d-Fru-1,6-P₂ as standards. Under our measurement conditions, d-Fru-6-P eluted at a retention volume of 4.22 ml, whereas the retention volume for d-Fru-1,6-P₂ was 17.63 ml. When purified Tk-Fbp was added in the reaction mixture we could detect the specific production of d-Fru-6-P from Fru-1,6-P₂ (data not shown). The peak corresponding to d-Fru-6-P increased with longer incubation periods of the reaction mixture, and the substrate peak decreased. An important observation was that when fructose 2,6-bisphosphate, fructose 6-phosphate, fructose 1-phosphate, glucose 6-phosphate, and glucose 1-phosphate were incubated with Tk-Fbp, substrate levels did not change. The result indicates that the enzyme was specific for fructose 1,6-bisphosphate.

Transcriptional Regulation of Tk-fbp Gene—As mentioned above, FBPase is necessary when cells require the synthesis of sugars from gluconeogenic substrates such as pyruvate (2). To examine the regulation of gene expression of Tk-fbp, KOD1 cells were grown independently on pyruvate and starch. Two probes were constructed for Northern blot analysis, one corresponding to the Tk-fbp gene, and the other corresponding to the DNA ligase gene from strain KOD1 (24, 25) as a control. The mRNA of the Tk-fbp gene was clearly detected from the RNA of cells grown on pyruvate (Fig. 6). In contrast, a positive signal could not be detected with the RNA of cells grown on starch. When cells were grown on amino acids, a condition that requires gluconeogenesis, Tk-fbp transcripts could also be detected. When pyruvate and starch were both present in the medium, only a very faint signal could be observed. Under all conditions, the signals of mRNA for the DNA ligase gene were visible irrespective of the carbon source. This result provides direct evidence that the transcription of the Tk-fbp gene was regulated at the transcription level and under the control of glucose repression. We also measured FBPase activity in cells grown on pyruvate or starch. We found that starch-grown cells displayed a specific activity of 0.03 units/mg, ~8% of the activity detected in pyruvate-grown cells (0.4 units/mg).

DISCUSSION

Complete genome sequences have contributed enormously in identifying the presence or absence of particular genes in various microorganisms (26, 27). However, this advantage heavily relies on the assumption that proteins with similar function will display similarity in primary structure. The archaeal FBPases have represented an intriguing exception to this assumption. Although FBPase activity had been detected in P. furiosus (20) and Methanobacterium thermoautotrophicum (19), no orthologous genes of previously identified FBPases were present on Archaea genomes. A most interesting and valuable finding was that the MJ0109 gene product from M. jannaschii harbored an unexpected FBPase activity in addition to its expected inositol monophosphatase activity. The report also mentions that orthologous genes from A. fulgidus and Thermotoga maritima also encode a protein with FBPase activity (21).

In this study, we have identified and characterized a novel FBPase, Tk-Fbp, from the hyperthermophilic archaeon, T. kodakaraensis KOD1. The structure is distinct to the structures of all previously identified FBPases, including the MJ0109 gene product from M. jannaschii. The FBPase activity has been detected by both an enzyme linked assay and direct observation of substrate and product, leaving no doubt that Tk-Fbp harbors FBPase activity. Tk-Fbp displayed a $K_m$ value of 100 μM toward fructose 1,6-bisphosphate, and a $k_{cat}$ value of 17 s⁻¹ subunit⁻¹ at 95 °C. The $K_m$ value was slightly higher than that observed for the MJ0109 gene product (38 μM). The $k_{cat}$ value of Tk-Fbp at 85 °C was ~2-fold higher than the M. jannaschii enzyme (7 s⁻¹ subunit⁻¹ at 85 °C). The $k_{cat}$ value of Tk-Fbp at 37 °C (2.9 s⁻¹ subunit⁻¹) was ~20% of that of the FBPase from E. coli (14.6 s⁻¹ subunit⁻¹). Tk-Fbp did not display catalytic activity for the reverse reaction, indicating that it is not the protein responsible for 6-phosphofructokinase activity in strain KOD1. In support, phosphofructokinases have been identified and characterized in various Archaea including P. furiosus (28), M. jannaschii (29), and Aeropyrum pernix (30, 31), and these proteins do not correspond to the Tk-Fbp orthologues mentioned in this study.

The results of this study strongly indicate that Tk-Fbp is the major FBPase in T. kodakaraensis KOD1. Besides the high activity of the purified enzyme mentioned above, we could not detect FBPase activity in fractions other than those containing Tk-Fbp during partial purification from pyruvate-grown KOD1 cells. Furthermore, the enzyme displayed high substrate specificity toward fructose 1,6-bisphosphate, unlike the MJ0109 gene product from M. jannaschii (21). No activity could be observed with fructose 2,6-bisphosphate, fructose 6-phosphate, fructose 1-phosphate, glucose 6-phosphate, and glucose 1-phosphate. Finally, the gene expression was strictly regulated in a manner that perfectly agreed with its presumed physiological role. Gene transcription was repressed in the presence of starch, regardless of the presence or absence of pyruvate. Derepression was also observed in cells grown on either amino acids or pyruvate, indicating that the regulation was not because of induction by a certain carbon source, but a typical example of glucose repression/derepression. These findings lead us to conclude that, at least in the case of T. kodakaraensis KOD1, Tk-Fbp is the “missing” archaeal FBPase.

At present, it is difficult to determine whether the Tk-Fbp orthologues represent the missing FBPases in other Archaea strains. However, it should be noted that among the complete microbial genome sequences, all thermophilic Archaea harbored a highly similar gene on their chromosomes (Table I). In addition, a Tk-fbp orthologue was also present in the hyperthermophilic bacterium A. aeolicus. This at least denies that Tk-fbp itself is not a unique gene for T. kodakaraensis KOD1. Additionally, as most of these genes display 75–97% similarity to Tk-fbp, it is highly likely that their gene products harbor FBPase activity. Gene disruption studies and biochemical characterization of Tk-Fbp orthologues in other Archaea strains will be an attractive subject of future research on gluconeogenesis in Archaea.

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