Archaeal Fructose-1,6-bisphosphate Aldolases Constitute a New Family of Archaeal Type I Aldolase*

Received for publication, April 18, 2001, and in revised form, May 30, 2001
Published, JBC Papers in Press, May 31, 2001, DOI 10.1074/jbc.M103447200

Bettina Siebers‡§, Henner Brinkmann‡, Christine Dörr‡, Britta Tjaden‡, Hauke Lilie, John van der Oost**, and Corné H. Verhees**

From the ‡Department of Microbiology, Universität Essen, 45117 Essen, the §Institute of Evolutionary Biology, Department of Biology, Universität Konstanz, 78547 Konstanz, the **Institute of Biotechnology, Department of Biochemistry and Biotechnology, Martin-Luther-Universität Halle, 06120 Halle (Saale), Germany, and the Laboratory of Microbiology, Wageningen University, NL-6703 CT Wageningen, The Netherlands

Fructose-1,6-bisphosphate (FBP) aldolase activity has been detected previously in several Archaea. However, no obvious orthologs of the bacterial and eucaryal Class I and II FBP aldolases have yet been identified in sequenced archaeal genomes. Based on a recently described novel type of bacterial aldolase, we report on the identification and molecular characterization of the first archaeal FBP aldolases. We have analyzed the FBP aldolases of two hyperthermophilic Archaea, the facultatively heterotrophic Crenarchaeon Thermoproteus tenax and the obligately heterotrophic Euryarchaeon Pyrococcus furiosus. For enzymatic studies the fba genes of T. tenax and P. furiosus were expressed in Escherichia coli. The recombinant FBP aldolases show preferred substrate specificity for FBP in the catalytic direction and exhibit metal-independent Class I FBP aldolase activity via a Schiff-base mechanism. Transcript analyses reveal that the expression of both archaeal genes is induced during sugar fermentation. Remarkably, the fbp gene of T. tenax is co-transcribed with the ppf gene that codes for the reversible PP-dependent phosphofructokinase. As revealed by phylogenetic analyses, orthologs of the T. tenax and P. furiosus enzyme appear to be present in almost all sequenced archaeal genomes, as well as in some bacterial genomes, strongly suggesting that this new enzyme family represents the typical archaeal FBP aldolase. Because this new family shows no significant sequence similarity to classical Class I and II enzymes, a new name is proposed, archaeal type Class I FBP aldolases (FBP aldolase Class IA).

Fructose-1,6-bisphosphate (FBP) aldolase (EC 4.1.2.13) catalyzes the reversible aldol condensation of glyceraldehyde 3-phosphate (GAP) and dihydroxyacacetone phosphate (DHAP) yielding FBP. The enzyme fulfills an amphibolic function being involved in catabolic (glycolysis) as well as anabolic pathways (gluconeogenesis and Calvin cycle). In spite of this central function in carbohydrate metabolism, up to now no archaeal genes coding for the respective enzyme activities have been analyzed.

Two distinct classes of FBP aldolases occur in nature, which differ in their enzymatic mechanisms (1–4). Class I FBP aldolases form a Schiff-base intermediate between the carbonyl substrate (FBP and DHAP) and the e-amino group of the active site lysine residue and are inactivated by borohydride (NaBH₄), whereas Class II FBP aldolases depend on divalent metal ions to stabilize the carbanion intermediate and are, therefore, inhibited by EDTA. Class II enzymes of bacterial and eucaryal origin generally form dimers with a subunit molecular mass of ~40 kDa, whereas the Class Ipendants are heterogeneous. Eucaryal aldolases are homomeric tetramers with a subunit molecular mass of ~40 kDa, and for bacterial enzymes oligomeric arrangements from monomer to decamer and subunit molecular masses of 27–40 kDa have been described (5, 6).

Sequence comparisons of Class I and II FBP aldolases revealed no detectable sequence homology, suggesting convergent evolution (4, 5, 7–11). The latter is supported by comparisons of available crystal structures of rabbit muscle Class I and Escherichia coli Class II FBP aldolases indicating that even though both classes adopt a common folding topology (5) trisose-phosphate isomerase (TIM)-barrel fold) and catalyze identical reactions, they share no conserved catalytic residues, and the location of their active sites is distinct (12). However, more recent analysis combining sequence, structure, and functional information indicates that many of the (βα)₈ TIM barrel superfamilies, such as aldolases, TIMs, and enolases, share a common evolutionary origin (ancestral βα barrel), although they adopt a wide range of enzymatic functions (13, 14).

The distribution of FBP aldolases during evolution is complex and still puzzling. Class II aldolases seem to be confined to more simple organisms such as bacteria and a few unicellular eucaryotes (fungi, including yeast), whereas Class I FBP aldolases are present in higher forms of life (animals, higher plants, ferns, and mosses), and only a few bacteria possess a Class I enzyme, sometimes in addition to a Class II enzyme. Earlier branching protists studied so far show a marked diversity of harboring Class I and/or Class II enzymes (for review see Refs. 5 and 10).

Recently, Thomson et al. (6) described a new type of FBP aldolase in E. coli, which belongs to Class I aldolases according to its Schiff-base mechanism but differs significantly from the
Archaeal Type Class I Fructose-1,6-bisphosphate Aldolases

other members of this class by its low sequence similarity. The *E. coli* Class I FBP aldolase was originally mis-annotated in the *E. coli* genome as dehydrin (DhnA, *dhnA* gene) due to its overall identity (13–20%) to dehydrins in plants, which are stress proteins that are induced in response to dehydration (6).

Although Class I and Class II FBP aldolase activities have been demonstrated in Archaea (15–21), no genes encoding classical Class I or II enzymes have been identified in any of the sequenced archaeal genomes suggesting that Archaea possess novel types of aldolases that are either absent or not yet recognized as such in Bacteria and Eucarya. The latter is supported by initial database searches of Galperin et al. (22) who identified gene homologs of the unusual Class I FBP aldolase gene (*dhnA*) of *E. coli* in the sequenced archaeal genomes. However, none of this archaeal gene product was examined with respect to its enzymatic function. In order to prove that DhnA homologs in the two major archaeal kingdoms code for FBP aldolases, we expressed the *dhnA* gene homologs of the crenarchaeote *Thermoproteus tenax* and the euryarchaeote *Pyrococcus furiosus* in *E. coli*, and we analyzed the function of their gene products. The two hyperthermophiles differ from each other not only with respect to phylogeny but also with respect to physiology. *T. tenax* is a facultative chemooorganotroph (23, 24), and *P. furiosus* is an obligate chemooorganotroph (25). *T. tenax* uses two different pathways for carbohydrate catabolism, i.e. a modified, non-phosphorylative Entner-Doudoroff pathway and a variant of the reversible Embden-Meyerhof-Parnas pathway (19, 26). The latter is characterized by a PPi-dependent phosphofructokinase (PPi-PFK) (27), two different glyceraldehyde-3-phosphate dehydrogenases (28, 29), and a pyruvate kinase with reduced allosteric potency (30). *P. furiosus* possesses one catabolic pathway, a variant of the Embden-Meyerhof-Parnas pathway that differs significantly from the *T. tenax* variant (21) and involves an ADP-dependent glucokinase (31), an ADP-dependent PFK (32), a canonic glyceraldehyde-3-phosphate dehydrogenase, and a ferredoxin-dependent glyceraldehyde-3-phosphate oxidoreductase (33, 34).

**Experimental Procedures**

**Chemicals and Plasmids—**DL-GAP was prepared from monobarium salts of the diethyl acetyl, according to the manufacturer’s instructions (Sigma). All other chemicals and enzymes were purchased from Sigma, Megazyme Biotechnologies Diagnostics GmbH in analytical grade. For heterologous expression the vectors pET-15b and pET-24d (Novagen) and for generating antisense mRNA the vector pSPT 19 (Roche Diagnostics GmbH) were used.

**Strains and Growth Conditions—**Mass cultures of *T. tenax* Kra1 (DSM 2078) were grown as described previously (19). *P. furiosus* (DSM 3638) was grown in CDM medium as described previously (35) with the only exception that yeast extract was omitted and substituted by the individual amino acids (0.25 mM final concentration). Maltose (10 mM) or pyruvate (40 mM) was added as the primary carbon source. *E. coli* strains DH5α (Life Technologies, Inc.), XL1Blue (Stratagene), BL21(DE3), and BL21(DE3)pLysS (Novagen) for cloning and expression studies were grown under standard conditions (36) following the instructions of the manufacturer.

**Enzyme Assay—**The FBP aldolase activity was determined in catalytic direction (FBP cleavage) at 50 °C in a coupled assay with glyceral-3-phosphate dehydrogenase (EC 1.1.1.12) and triose-phosphate isomerase (TIM, EC 5.3.1.1) of rabbit muscle as auxiliary enzymes. For the *T. tenax* enzyme the assay (total volume 1 ml) was performed in 100 mM Tris/HCl (pH 7.0, 50 °C) in the presence of 0.4 mM NADH, 5 mM FBP, 4 units of glyceral-3-phosphate dehydrogenase, and 20 units of TIM. Enzymatic activities were measured by monitoring the increase in absorption at 366 nm (εmax = 3.36 mM⁻¹ cm⁻¹). The assay mixture (1-ml volume) for the *P. furiosus* FBP aldolase contained 50 mM Tris/HCl (pH 7.0, 50 °C), 0.2 mM NADH, 2.5 mM FBP, 4 units of glyceral-3-phosphate dehydrogenase, and 11 units of TIM. The absorbance was followed at 340 nm (ε = 6.3 mM⁻¹ cm⁻¹).

Reactions were started by addition of the substrate FBP, and the enzyme concentrations ranged from 2 to 40 μg of protein/ml test volume. To determine the substrate specificity of the FBP aldolases, the standard enzyme assay was used substituting FBP by other substrates, such as fructose 1-phosphate (Fru-1-P). For effector studies citrate was added to an end concentration of 10 mM in the presence of half-saturating concentrations of FBP. The maximum of product concentrations were added to the mixture. Protein concentration was measured according to the method of Bradford (37) using the Bio-Rad Protein-Assay (Bio-Rad) with BSA as standard.

**Active Site Labeling—**To investigate the involvement of a Schiff-base mechanism in the FBP aldolase catalysis of *T. tenax* (0.09 mg of protein/ml assay (total volume 1 ml) was performed in 100 mM Tris/HCl (pH 7.0, 70 °C) in the presence of 5 mM NAD⁺, 5 mM FBP, and 5 units of NAD⁺-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9) (28) of *T. tenax* as auxiliary enzyme. The assay (total volume 1 ml) was performed in 100 mM Tris/HCl (pH 7.0, 70 °C) in the presence of 5 mM NAD⁺, 5 mM FBP, and 5 units of NAD⁺-dependent glyceraldehyde-3-phosphate dehydrogenase. The increase in absorption was measured at 366 nm (εmax = 3.51 mM⁻¹ cm⁻¹). The *T. tenax* enzyme (EMBL accession number AJ310483) was identified by sequencing the genomic clone (5.2-kb HindIII fragment) harboring the *pfp* gene (27). The *P. furiosus* gene (GenBank™ accession number AF368256, NCBI) was identified in the *P. furiosus* data base (www.genome.utah.edu).

**Expression of the FBP Aldolases in E. coli—**For expression of the *T. tenax* FBP aldolase, the coding region was cloned into pET-15b via two new restriction sites (NcoI and BamHI) introduced by PCR mutagenesis with the primers FBPA-f (GCTCAAGGATCCATGCGAAG, sense) and FBPA-rev (CCCCCGTCAAGGATCCATGCGAAG, antisense) and FBPAeva (CCCCCGTCAAGGATCCATGCGAAG, sense) and BG688 (GGCGCGGATCCATGCGAAG, antisense). The introduced mutations are shown in boldface, and introduced NcoI and BamHI restriction sites are underlined. The sequence of both expression clones was confirmed by sequencing both strands. Expression of the *T. tenax* enzyme in *E. coli* BL21(DE3)pLysS and of the *P. furiosus* enzyme in BL21(DE3) was performed following the instructions of the manufacturer (Novagen).

**Site-directed Mutagenesis of the P. furiosus FBP Aldolase—**The active site mutation was introduced in the *P. furiosus* *fbp* gene using *Pfu* polymerase in the PCR-based overlap extension method (38): BG749 (GGCGCGGATCCATGCGAAG, sense), BG750 (TTAATCATGCGAAG, antisense), and BG688 (GGCGCGGATCCATGCGAAG, antisense). The introduced mutations are shown in boldface, and introduced NcoI and BamHI restriction sites are underlined. The sequence of both expression clones was confirmed by sequencing both strands. Expression of the *T. tenax* enzyme in *E. coli* BL21(DE3)pLysS and of the *P. furiosus* enzyme in BL21(DE3) was performed following the instructions of the manufacturer (Novagen).

**Purification of Recombinant FBP aldolases of T. tenax and P. furiosus—**For purification of the recombinant *T. tenax* enzyme, 10 g of *E. coli* cells were resuspended in 20 ml of 100 mM HEPES/KOH (pH 7.5) containing 300 mM 2-mercaptoethanol and passed three times through a French press cell at 150 megapascals. After centrifugation (20,000 × g, 45 min, 4 °C), the crude extract was heat-precipitated (90 °C, 30 min), centrifuged again, and dialyzed overnight against 50 mM HEPES/KOH (pH 7.5) containing 5 mM dithiothreitol (2-liters volume, 4 °C). The dialyzed fraction was applied to Q-Sepharose fast-flow (Amersham Pharmacia Biotech) equilibrated in the same buffer and eluted with a linear salt gradient of 0–500 mM KCl. Fractions containing the homogeneous enzyme solution were pooled.

**For the purification of the recombinant FBP aldolase from *P. furiosus* E. coli cells were resuspended in 10 ml of 100 mM HEPES/KOH (pH 7.5) containing 300 mM 2-mercaptoethanol and passed three times through a French press cell at 150 megapascals, and cell debris was removed by centrifugation (10,000 × g, 20 min, 4 °C). After heat precipitation (70 °C, 30 min) and centrifugation, the supernatant was filtered through a 0.45-μm filter and loaded onto a Mono Q HR 5/5 column (Amersham Pharmacia Biotech) equilibrated in 50 mM Tris/HCl (pH 7.8). Proteins were eluted by a linear salt gradient of 0–1000 mM NaCl. Active fractions were pooled,

for details see [19].

The *P. furiosus* enzyme (27) is based on significant sequence similarity to the recently described *E. coli* Class I FBP aldolase (DhnA, GenBank™ accession number P71295). The *fbp* gene of *T. tenax* (EMBL accession number AJ310483) was identified by sequencing the genomic clone (5.2-kb HindIII fragment) harboring the *fbp* gene (27). The *P. furiosus* gene (GenBank™ accession number AF368256, NCBI) was identified in the *P. furiosus* data base (www.genome.utah.edu).
concentrated by microfiltration (Centricon 30, Amicon), and applied to a Superdex 200 prep grade column (Amersham Pharmacia Biotech), equilibrated in 50 mM Tris/HCl (pH 7.8), 100 mM NaCl. Fractions containing the homogeneous enzyme were pooled.

**Analytical Ultracentrifugation of the T. tenax FBP Aldolase—**Sedimentation equilibrium and analytical ultracentrifugation analyses were conducted using an analytical ultracentrifuge Optima X-LA (Beckman Instruments, Palo Alto, CA) equipped with double sector cells and an AnTi 50 rotor. The protein was dissolved in 50 mM HEPES/KOH (pH 7.5) containing 1 mM KCl and 2 mM dithiothreitol at a concentration of 0.48 mg of protein/ml. Sedimentation velocity experiments were performed at 30,000 rpm (20 °C) and the data were analyzed according to the sedimentation time derivative method (39). Sedimentation equilibrium was analyzed at 6,000 rpm (20 °C) using the software provided by Beckman Instruments.

**Northern Blot Analyses of the T. tenax fba Transcript—**Preparation of total RNA from auto- and heterotrophically grown T. tenax cells and Northern blot analyses were performed as described before (30). Digoxigenin-labeled antisense mRNA of FBP aldolase and PP-PFK were obtained by in vitro transcription from the T7 promoter of vector pSPT 19 (Roche Diagnostics GmbH). A part of the coding region of FBP aldolase (502 bp) and the coding region of PP-PFK (1011 bp) was cloned into the EcoRI and BamHI restriction sites of the vector by PCR mutagenesis using the primer sets CGAGGGGAGGCCGATGGGATC (sense) and GAAGGCTTCTGGGATCCCGCCCGCCGCCGCCGAGAAGGATAG (antisense) for FBP aldolase and GCTGCGCCGCCTTCCTGAAATGATCAGAATAG (sense) and CTAGGACAGGGATCGCGGCTTTAGC (antisense) for PP-PFK. The introduced mutations are shown in boldface, and the EcoRI and BamHI restriction sites are underlined.

**Primer Extension Analyses—**Primer extension analyses for T. tenax were performed as described previously (30). To map the transcription start site of the fba-pfpx transcript the 5'-32P-labeled antisense oligonucleotide (5'-CCGTGAATTCGCCGTAG-3', position 72-89 of the fba gene) was used as primer for cDNA synthesis. For P. furiosus total RNA was isolated from maltose and pyruvate grown cells as described previously (40), and the transcription start was determined with a fluorescein detection system (Promega) according to the instructions of the manufacturer with the following modifications. Hybridization of total RNA (15 μg) and oligonucleotide (5 pmol) was performed for 10 min at 68 °C before allowing to cool to room temperature. The reaction (20 μl final volume) was started by addition of dNTPs (1 mM), MgCl₂ (15 mM), RNasin (20 units), and Avian Myeloblastosis Virus reverse transcriptase (22.5 units). After incubation for 30 min at 45 °C, the reaction volume was diluted to 50 μl with 10 mM Tris/HCl (pH 8.5), 1 μl of RNase A (5 mg/ml) was added, and the sample was incubated for 10 min at 37 °C. cDNA was precipitated with ethanol and dissolved in 3 μl of loading buffer, and 1 μl was applied to a sequencing gel in parallel with the sequencing reactions obtained with the same oligonucleotide.

**Sequence Retrieval and Phylogenetic Analyses—**Protein sequences were extracted from GenBank34 and the TIGR microbial database using BLAST and first aligned with ClustalW (41); this alignment was manually refined using the MUST program package (42). Regions of uncertain alignment and partial sequences were omitted from the analyses leaving a total of 27 sequences and 172 amino acid positions.

**RESULTS**

**Nucleotide Sequence of the fba Genes of T. tenax and P. furiosus—**Both fba genes were identified due to their sequence similarity with the recently characterized Class I FBP aldolase from E. coli (DhnA, dhnA gene) (6). The T. tenax enzyme was identified by sequence analysis of the genomic clone comprising the pfpx gene (5.2-kb HindIII fragment), which revealed an additional open reading frame of 792 bp (Fig. 1) preceding the pfpx gene (1014 bp) (27). This open reading frame codes for a polypeptide of 263 amino acid residues with a calculated molecular mass of 28.7 kDa and showed high overall similarity (26% identity, blast data base search) to the Class I FBP aldolase (DhnA) of E. coli (6). Strikingly, the coding regions of both T. tenax genes fba and pfpx overlap by 1 bp with the A of the start codon (ATG) of the pfpx gene being the last nucleotide of the triplet encoding the C-terminal valine (GTA) of the fba gene (Fig. 1). The fba gene of P. furiosus (846 bp) was identified in the P. furiosus data base by similarity of the translated 31.1-kDa polypeptide (282 amino acid residues) to E. coli DhnA (26% identity, blast data base search). Contrary to T. tenax, the P. furiosus fba gene is separated from the next neighbored downstream open reading frame with similarity to agmatinase (speB gene) by 61 nucleotides and therefore is presumably not organized in an operon structure (Fig. 1).

**Expression of the fba Genes from T. tenax and P. furiosus in E. coli and Purification of Recombinant FBP Aldolases—**The fba gene products of T. tenax and P. furiosus were expressed in E. coli, and their FBP aldolase activity was confirmed for both enzymes using a coupled enzyme assay. For further biochemical studies both recombinant enzymes were purified. From 10 g wet cells of recombinant E. coli, 14 mg of homogeneous T. tenax FBP aldolase with a specific activity of 0.23 units/mg protein (50 °C) and from 3 g wet cells of recombinant E. coli 5 mg of homogeneous P. furiosus protein with a specific activity of 0.58 units/mg (50 °C) were recovered, respectively.

**Enzymatic Properties of the Recombinant FBP Aldolases of T. tenax and P. furiosus—**The purified, recombinant FBP aldolases of T. tenax and P. furiosus exhibit Michaelis-Menten kinetics for FBP in the catabolic (aldol cleavage) direction. The K_m and V_max values for FBP were 9 μM and 0.23 units/mg for T. tenax and 3.6 μM and 0.61 units/mg for P. furiosus and as such were comparable to the E. coli Class I FBP aldolase (DhnA) (Table I) (6). Like the E. coli enzyme both archaean FBP aldolases showed additional activity with Fructose-1-P, although the much higher K_m value for Fructose-1-P (T. tenax 498-fold, P. furiosus 1650-fold) of all three enzymes strongly suggests that FBP is the physiological substrate (Table I). As shown for the FBP aldolase of T. tenax other sugar phosphates such as fructose-6-phosphate, glucose-6-phosphate, fructose 2,6-bisphosphate, and 6-phosphogluconate (concentration range of 5–10 mM) do not serve as substrates in the catabolic direction.

Both archaean FBP aldolases, however, like the E. coli enzyme, were activated in presence of saturating concentrations of citrate (10 mM) by a factor of 2.2 and 2.4, respectively (Table I).

The involvement of a Schiff-base mechanism in the FBP aldolase reaction was examined for the T. tenax enzyme by treating the enzyme with sodium borohydride in the presence and absence of the substrates GAP, DHAP, and FBP. The significant reduction of the specific activity in the presence of the carbonyl substrates DHAP (38% residual activity) and FBP (29% residual activity) as compared with the presence of GAP (80% residual activity) and the control, after NaBH₄ treatment (100% activity, 0.8 units/mg protein, 70 °C), accounts for the formation of a Schiff-base in the enzyme reaction. In accordance with these results, a lysine residue is conserved at position 177 in the T. tenax sequence (Fig. 4), which corresponds to the active site Lys-237 (falsely marked as Lys-236) in the E. coli Class I FBP aldolase (DhnA) (6). Finally, the observation that neither metal ions such as Mn²⁺, Mg²⁺, Zn²⁺, Ca²⁺, and Fe²⁺ (concentrations tested, 0.1 and 1 mM) nor EDTA (concent-
trations tested, 0.1, 1, and 10 mM) affects the enzyme activity supports the biochemical classification of the T. tenax enzyme as a Class I aldolase. As shown in Fig. 4 also the P. furiosus FBP aldolase exhibits the active site lysine residue (position 191), and the assumed involvement of a Schiff-base mechanism was supported by site-directed mutagenesis of the active site lysine to alanine (K191A) resulting in a virtually inactive mutant enzyme (not shown).

**Molecular Mass**—The homogeneous FBP aldolases from T. tenax and P. furiosus revealed similar subunit sizes in SDS-polyacrylamide gel electrophoresis of ~30 and 33 kDa, respectively, thus being in good agreement with the calculated molecular mass of 28.7 and 31.1 kDa. However, differences between the two enzymes are obvious concerning their oligomeric state under native conditions (Table I). Gel filtration experiments revealed for the recombinant P. furiosus enzyme an apparently uniform oligomer with a molecular mass of 272 kDa (representing presumably octamers), whereas for the T. tenax FBP aldolase two different oligomeric forms were identified. As shown by repeated chromatography of the separated oligomers, both forms are convertible to one another. Sedimentation velocity experiments revealed two distinct oligomers with apparent sedimentation coefficients of 9.34 S and 14.5 S indicating a slow equilibration reaction between the two forms of the T. tenax FBP aldolase. For the smaller association form an apparent molecular mass of 237–245 kDa was determined by sedimentation equilibrium centrifugation suggesting a stoichiometry of eight monomers per oligomer.

**Transcript Analyses**—To determine if the expression of FBP aldolase of T. tenax and P. furiosus is controlled at transcriptional levels, we examined the effect of the carbon source on fbp transcription. Since the juxtaposition of the fba and pfp gene in T. tenax suggests an operon organization-specific antisense mRNA probes for the pfp and fba gene were used to test for the formation of co-transcripts (Fig. 2). Northern blot experiments were performed with total RNA from autotrophically (in the presence of CO₂ and H₂) and heterotrophically (in the presence of glucose) grown T. tenax cells. They revealed a strong hybridization signal for both probes at 1.9 kb and two additional, weaker, probe-specific signals at 1.2 kb for the pfp probe and 0.8 kb for the fba probe, thus indicating the presence of bicistrionic as well as monocistronic messages. The signals of both probes were much stronger with mRNA from heterotrophically compared with autotrophically grown cells (Fig. 2). Densitometric quantification of slot blot analysis using the pfp probe and different concentrations of total RNA (10–0.625 μg) from auto- or heterotrophically grown cells revealed a 6-fold higher transcript abundance in the latter (data not shown). Also in P. furiosus cells grown on maltose or pyruvate the transcript level of the fba gene varied similarly (dot blot analysis, data not shown). Like in T. tenax conditions favoring the catabolic direction (growth on maltose) induce a higher transcript amount (2–3-fold increase) as compared with anabolic conditions (growth on pyruvate).

For a more accurate assignment of the promoter region in T. tenax and P. furiosus, the transcription starts of the fba-pfp mRNA and the fba mRNA, respectively, were determined by primer extension analyses. For the T. tenax fbp-pfp operon an antisense oligonucleotide binding at positions 72–89 of the fba gene was used. As shown in Fig. 3A transcription is initiated at the adenosine (A) immediately in front of the start codon (ATG) of the fba gene (position +1). A similar proximity of transcription and translation start site was already observed for the pyk gene, coding for the pyruvate kinase of T. tenax (30) and corresponds with the observation that some Archaea contain a high portion of mRNAs lacking Shine-Dalgano sequences in front of their coding genes (49, 50). In accordance with the Northern analyses the amount of copy DNA in the primer extension studies was by a factor 4.5–7.1 higher in hetero- than in autotrophically grown T. tenax cells. The transcription start of the P. furiosus fba mRNA (Fig. 3B) was initiated at the guanosine 10 bp upstream of the ATG start codon (position +1), and in contrast to T. tenax a putative RBS was identified.

![Gene expression and transcription](Fig. 1. Genomic organization and flanking regions of the P. furiosus fba gene and the T. tenax fba-pfp operon. Arrows represent the open reading frames and their orientation. The enlargement shows the overlapping regions of the fba and pfp gene in T. tenax, and the respective protein sequence is shown in bold letters. The fba stop codon is marked by an asterisk, and the ATG start codon of the pfp gene is underlined.)

**Table I**

Comparative analysis of archael type Class I FBP aldolases

| Characteristics | Crenarchaea T. tenax | Euryarchaea P. furiosus | Bacteria E. coli (6) |
|-----------------|----------------------|------------------------|---------------------|
| Molecular mass of native enzyme (kDa) | 241 (small form) | 272 | 340 |
| Molecular mass of subunit size (kDa) | 28.7 | 31.1 | 38.0 |
| Oligomeric structure | 8 (small form) | 8 | 8–10 |
| Active site | Lys-177 | Lys-191 | Lys-237 |
| Activation by citrate (10 mM) | 2.2× | 2.4× | 14.6× |
| K<sub>m</sub> FBP (mM) | 0.009 | 0.0036 | 0.02 |
| V<sub>max</sub> FBP (units/mg) | 0.23 | 0.61 | 0.34 |
| k<sub>cat</sub>K<sub>m</sub> (mM<sup>−1</sup>min<sup>−1</sup>) | 734.4 | 5275 | 646 |
| K<sub>m</sub> Fru-1-P (mM) | 4.48 | 0.71 | 33 |
| V<sub>max</sub> Fru-1-P (units/mg) | 0.3 | 0.75 | 0.18 |
| k<sub>cat</sub>K<sub>m</sub> (mM<sup>−1</sup>min<sup>−1</sup>) | 1.89 | 32.8 | 0.21 |
Inspection of the 5'-flanking regions (Fig. 3C) revealed for the fba genes of *T. tenax* and *P. furiosus* AT-rich regions 20–30 nucleotides upstream of their transcription start sites, which correspond well with the archaeal promoter consensus sequences (51–53). In *T. tenax* the TATA box (creshaalconsensus sequence (C/T)/TTTTAAA) is centered around position -25/-26, and 2 bp (-30 GA -31) upstream of the TATA box is the putative transcription factor B recognition element (BRB site, consensus sequence (A/G)(N)(T)AA/(T)). A putative ribosome-binding site (RBS, GGAGG) seems to be absent. In *P. furiosus* a putative RBS (GGTGA) is identified at position +1 to +5, and the TATA box is positioned around -24/-25, and 2 bp upstream is the putative purine-rich BRE site (54).

**Phylogenetic Analyses—**Data bank searches with the fba genes of *T. tenax* and *P. furiosus* revealed sequences with apparent similarity to the Class I FBP aldolases of *E. coli* (DhnA) in some bacterial and all archaeal genomes, with the only exception being *Thermoplasma acidophilum*. Whereas most of the genomes analyzed contain only a single dnaA-like gene, *Archaeoglobus fulgidus*, *Methanococcus jannaschii*, *Halobacterium* sp. NRC-1, and *E. coli* possess two paralogous genes (22). This new FBP aldolase family represents a divergent group with sequence similarities as low as about 20% identity (based on the 172-amino acid core region used for the phylogenetic analyses) between the different groups. Nevertheless, despite this substantial divergence, the universal conservation of the active site lysine (Lys-177, *T. tenax*; Lys-191, *P. furiosus*; and Lys-237, *E. coli* DhnA) and an additional conserved sequence motif preceding the active site lysine (positions 171–176 *T. tenax*) as well as three further conserved regions, ranging from positions 20–27, 98–109, and 199–204 (numbering of *T. tenax* fba gene), characterize them unequivocally as homologs of *E. coli* class I FBP aldolase (DhnA) (Fig. 4).

Strikingly, DhnA homologs do not display significant overall similarity with the members of the classical Class I and Class II FBP aldolases as deduced from automated sequence comparison programs (e.g., Blast search). However, by closer inspection, sequence signatures could be identified resembling the active site region (position 177, *T. tenax*) and the phosphate-binding motif (position 203–204, *T. tenax*) for some members of the (αβ)₅ TIM barrel superfamilies (13), strongly suggesting that this new family of Class I FBP aldolases is at least distantly related to classical Class I FBP aldolases. Moreover, secondary structure predictions (47, 48) performed with the aldolase sequences of *T. tenax*, *P. furiosus*, and *Sulfolobus solfataricus* not only identified these enzymes as (αβ)₅ barrel proteins but also locate the functionally important residues at equivalent positions to the ones found in classical Class I FBP aldolases as well as in other enzymes of the (αβ)₅ TIM barrel superfamilies (active site lysine in β₅, phosphate binding region at the end of β₅; Fig. 4) (13). From the high conservation of these key residues we further conclude that the new type of Class I FBP aldolase generally functions as a Schiff-base aldolase acting on phosphorylated substrates.

To analyze the phylogenetic relationships between the various DhnA homologs of Bacteria and Archaea, we aligned 27 sequences of 23 different species and selected a sequence fragment of 172 amino acid residues (Fig. 4) for construction of phylogenetic trees (Fig. 5). The phylogenetic analyses include the three mostly used methods (maximum likelihood, maximum parsimony, and distance-based neighbor joining) and resulted in a complex tree topology with at least 7 deeply rooting branches. Two of them bear exclusively bacterial (branch 1B and 4B) or archaeal sequences (branch 2 and 3) and three compose both archaeal and bacterial sequences (branch 1A, 1C, and 4A).

**DISCUSSION**

**Aldolases of *T. tenax* and *P. furiosus*, Members of a New Type of Class I FBP Aldolase—**The FBP aldolases of *T. tenax* and *P. furiosus* resemble specifically the Class I FBP aldolase of *E. coli* (DhnA) not only on sequence level but also in regard to biochemical properties. In common with *E. coli* Class I FBP aldolase (DhnA), catalysis of both archaeal enzymes proceeds via a Schiff-base mechanism. The archaeal enzymes, like the *E. coli* enzyme, exhibit (i) additional enzyme activity with Fru-1-P, albeit at a much higher *Kₘ* value than for FBP, and (ii) maximal turnover rates that are stimulated by citrate (Table I). Finally, also with respect to quaternary structure both archaeal aldolases show specific resemblance to the *E. coli* enzyme of *E. coli* (DhnA). All three enzymes tend to form higher oligomerization states representing octa-/decamers or even higher oligomers, whereas the members of the classical Class I and II FBP aldolases form mostly tetramers or dimers, respectively. Thus, structural features and mode of enzyme mechanism classify the FBP aldolases of *T. tenax* and *P. furiosus* as members of a new type of Class I FBP aldolase, distinct from classical Class I enzymes, which consists of homologs in almost all Archaea and some Bacteria.

**Transcription of the fba Genes of *T. tenax* and *P. furiosus*, Integration of the FBP Aldolases in the Physiological Framework—**The PP₁-PFK (27) and the FBP aldolase catalyze reversible reactions of successive steps in the variant of the Embden-Meyerhof-Parnas pathway of *T. tenax*, and as such both enzymes fulfill equivalent functions in the anabolic as well as catabolic direction of the pathway. Therefore, the co-transcription of the fba and pfp gene gives rise to the coordinated expression of both enzymes in *T. tenax*. On the contrary, in most organisms using pathways characterized by a unidirectional working PFK, either dependent of ATP or like in *P. furiosus* of ADP (21, 32), a linkage of FBP aldolase and PFK coding genes does not seem to be meaningful. Sometimes FBP aldolases are co-transcribed with genes coding for other reversible enzymes of glycolysis (e.g., glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase) or of the Calvin cycle (e.g., ribulose bisphosphate carboxylase/oxygenase and phosphoribulokinase) as shown for classical Class II FBP aldolases (5, 55, 56). Because FBP aldolase is an essential constituent of glycolysis as well as gluconeogenesis, it is remarkable that the fba expression in both organisms *T. tenax*
and \( P. \) furiosus is significantly higher under catabolic than under anabolic growth conditions (\( T. \) tenax, glucose/CO\(_2\); \( P. \) furiosus, maltose/pyruvate). An explanation might be that the higher transcript level under catabolic conditions is caused by the necessity of higher carbon flux rates through the pathway for energy conservation than is required for biosynthesis.

**A New Family of Aldolases, the Archaeal Type Class I FBP Aldolases**—Despite functional similarity with the classical Class I FBP aldolases, the new family of Class I aldolases differs significantly at sequence level. These non-significant average sequence similarities as well as the absence of certain DhnA-typical motifs in classical Class I enzymes characterize this new family of Class I FBP aldolases as a very divergent, new type in addition to classical Class I aldolases. However, both types of Class I FBP aldolases share, beside the predicted similar secondary structure arrangement, basic common sequence features in regions flanking the active site lysine or engaged in phosphate binding (13, 57).

Strikingly, all completed archaeal genomes contain at least one homolog of this new type of Class I FBP aldolases, with the only exception of \( T. \) acidophilum, which is supposed to use only the non-phosphorylative Entner-Doudoroff pathway for carbohydrate metabolism (58, 59). In contrast to Archaea, only in about 50% of completely sequenced bacterial genomes DhnA-related open reading frames have been identified, and no eucaryal homolog has been assigned yet. At the moment we do not know whether this new type of Class I FBP aldolases is the only enzyme responsible for aldolase activity in Archaea. Reports of metal-dependent Class II aldolase enzyme activity in Haloarchaea (e.g. Halobacterium halobium) (16) suggest that additional enzymes might be present, which have not been identified yet in the sequenced genomes, due to their low sequence similarity to known Class I and II aldolases. Because of this so far obviously exclusive occurrence of this new type of aldolase, together with the absence of classical Class I and II aldolases, we propose to classify this new family as archaeal type Class I FBP aldolases (Class IA) to oppose them to classical Class I aldolases only found in Eucarya and Bacteria.

**Phylogenetic Implications**—The phylogenetic tree (Fig. 5) is composed of seven deeply branching lineages each bearing members of one or both prokaryotic domains, whose relationships among each other are rather poorly resolved. The presence of Class IA FBP aldolases from Bacteria and Archaea, from Euryarchaeota and Crenarchaeota (e.g. aldolases of Euryarchaeota in branch 1A, 2, 3, 4A; enzymes of Crenarchaeota in branch 2 and 3), or even from one organism (e.g. enzymes of \( E. \) coli in branch 1B and 4B) in at least two different deeply rooting main branches suggests that early gene duplication events confer mainly to the characteristic topology of the tree. Probably an early, first gene duplication event (before the separation of Archaea and Bacteria) has led to a segregation into two main paralogous lineages (1A, B, C, 2, and 3, 4A, B). Subsequent gene duplication events in each of the two main lineages combined with differential loss might have created four lineages exhibiting only Archaea (branch 2 and 3) or Bac-
**FIG. 4. Multiple sequence alignment of archael type Class I FBP aldolases.** Boldface letters indicate amino acid residues used in the phylogenetic analyses. The predicted secondary structure of the *T. tenax* enzyme is shown above the sequences (47, 48). Conserved sequence motifs are shaded. The predicted phosphate-binding motif of many TIM barrel proteins is indicated by (P) and the catalytic lysine residue (Lys-237) determined for the *E. coli* Class I FBP aldolase (DhnA) (6) and the *P. furiosus* enzyme (this study) by an asterisk. The abbreviations used are as follows (accession numbers are in parentheses; for bigger nucleotide sequences with multiple open reading frames, first the protein and then the nucleotide accession numbers are given): Aa, *Aquifex aeolicus* (O67506, AE000745); Dv, *Desulfovibrio vulgaris* (TIGR); Mt, *Methanobacterium thermoautotrophicum* (O26679, AE000840); Af, *Archaeoglobus fulgidus* ((1) NP068949, AE001090 and (2) NP069068, AE001099); Mj, *Methanococcus jannaschii* ((1) Q57843, U67492 and (2) Q58980, U67598); Hs, *Halobacterium salinarum* ((1) AAG18889, AE004991 and (2) AAG19176, AE005000); Bt, *Bacillus thuringiensis* (AE001081; AF067248); Db, *Deinococcus barrenensis* (AE001086; AF067249); Ce, *C. elegans* (NP001069, AF067250); Bc, *B. caldarius* (AE001092; AF067251); A. archaica (AE001093; AF067252); A. fulgidus (AE001094; AF067253); A. onnurina (AE001095; AF067254); H. marismortui (AE001096; AF067255); and *H. marismortui* (AE001097; AF067256).
bacteria and Archaea (branch 1A-C and branch 4A, B). This scenario seems to be supported by the fact that the separation of the main branches is in all cases clearly older than the corresponding speciation events in the bacterial or archaeal lineages (e.g. divergence of Euryarchaeota and Crenarchaeota, branch 2 and 3) and in two cases even older than the divergence of Bacteria and Archaea (branch 1A-C and branch 4A, B). An example of a more recent gene duplication is found in branch 1A leading to the isoenzymes of *A. fulgidus*.

Phylogenetic analyses identified at least 4 lineages in Archaea and 2 lineages in Bacteria. The only limited presence of the archaeal type Class I FBP aldolase even in Bacteria might be explained by the assumption of differential loss of the coding genes in some bacterial lineages. Several independent lateral gene transfers from Archaea to Bacteria seem to be unlikely since the branching order of archaeal enzymes (branch 1A and 2) as well as of bacterial enzymes (branch 1B and 4B) fits quite well with the archaeal and bacterial radiation. However, lateral gene transfer events, most likely from Archaea to Bacteria, may be responsible for the surprising occurrence of the aldolases of *Aquilux aerolica* and *Desulfovibrio vulgaris* in branch 1A as well as of *Thiobacillus ferrooxidans* in branch 4A.

Surprisingly, the FBP aldolase of *Treponema denticola* shows no affinity to the bacterial branch 1B but is rather closely related to the enzyme of the Crenarchaeote *S. solfataricus*, with which it forms a highly supported monophyletic group. This close association may perhaps also be due to a lateral gene transfer event. If the *T. denticola* sequence is omitted from the tree calculation, the *S. solfataricus* sequence is placed at the basis of branch 1A (albeit with a low bootstrap support of 50), thus complementing the archaeal lineage by a crenarchaeal member.

In summary, the archaeal type Class I aldolase genes seem to be descendants of an ancient lineage separated very early...

---

**Fig. 5.** Phylogenetic tree of archaeal type Class I FBP aldolases. The numbers at the nodes are bootstrap proportions according to Maximum likelihood (ML), Neighbor joining (NJ), and Maximum parsimony (MP). Only values greater 30% are shown. Archaeal members are indicated by boldface letters, and the biochemical characterized enzymes are underlined. The accession numbers are given in the alignment of Fig. 4.
Archaeal Type Class I Fructose-1,6-bisphosphate Aldolases

from the gene lineages of the classical Class I and Class II aldolases, probably already in the common ancestor of extant life. The evolution of the new family seems to be mainly the result of non-lateral orthologous evolution including a complex mixture of gene duplications with subsequent differential loss and probably some late lateral gene transfer elements. Surprisingly, up to now no homologs of that gene family have been identified in Eucarya, questioning whether they never possessed these genes or secondarily lost them.

Acknowledgments—We thank T. Knura, N. Meijer, and I. Revet for technical assistance; K. Michalke for computer assistance; and R. Hensel for critically reading the manuscript and for continuous support.

REFERENCES

1. Rutter, W. J. (1964) *Fed. Proc.* 23, 1248–1257
2. Lehner, H. G., and Rutter, W. J. (1969) *Biochemistry* 8, 109–121
3. Alefounder, P. R., Baldwin, S. A., Perham, R. N., and Shorts, N. J. (1989) *J. Biochem. (Tokyo)* 257, 529–534
4. Fothergill-Gilmore, L. A., and Michels, P. A. M. (1993) *Biophys. Mol. Biol.* 59, 105–235
5. Plaumann, M., Pelzer-Reith, B., Martin, W. F., and Schnarrenberger, C. (1997) *Trends Biochem. Sci.* 22, 511–513
6. Thomson, G. J., Howlett, G. J., Ashcroft, A. E., and Berry, A. (1998) *J. Biol. Chem.* 273, 430–438
7. Kengen, S. W. M., Luesink, E. J., Stams, A. J. M., and de Vos, W. M. (1993) *Nucleic Acids Res.* 21, 331, 7351–7367
8. Rutter, W. J. (1964) *Fed. Proc.* 23, 1248–1257
9. Higuchi, R., Krummel, B., and Saiki, R. K. (1988) *Nucleic Acids Res.* 16, 2559–2566
10. Swofford, D. L. (1999) *PAUP*, version 2.3, Institute of Statistical Mathematics, Tokyo
11. Bray, D. A., and Vesham, L. (1999) *Extremophiles* 4, 175–179
12. Bell, S. D., and Jackson, S. P. (2001) *Proteins* 55, 55–72
13. Condon, I., Ciammarucchi, A., Benelli, D., Ruggero, D., and Lendoi, P. (1999) *mol. Biol. Microbiol.* 34, 377–384
14. Thirumaran, R., Sensen, C. W., Garrett, R. A., and Clausen, I. G. (2000) *Extremophiles* 4, 175–179
15. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
16. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
17. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
18. Bell, S. D., and Jackson, S. P. (2001) *Curr. Opin. Microbiol.* 4, 208–213
19. van der Oost, J., Ciammarucchi, A., Benelli, D., Ruggero, D., and Lendoi, P. (1999) *Mol. Biol. Microbiol.* 34, 377–384
20. Tolstrup, N., Sensen, C. W., Garrett, R. A., and Clausen, I. G. (2000) *Extremophiles* 4, 175–179
21. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
22. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
23. Tolstrup, N., Sensen, C. W., Garrett, R. A., and Clausen, I. G. (2000) *Extremophiles* 4, 175–179
24. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
25. Tolstrup, N., Sensen, C. W., Garrett, R. A., and Clausen, I. G. (2000) *Extremophiles* 4, 175–179
26. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
27. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
28. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
29. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
30. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
31. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
32. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
33. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
34. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
35. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
36. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
37. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
38. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
39. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
40. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
41. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
42. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
43. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
44. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
45. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
46. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
47. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
48. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
49. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
50. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
51. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
52. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
53. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
54. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
55. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
56. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
57. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
58. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
59. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
60. Soppa, J. (1999) *Mol. Microbiol.* 31, 1295–1305
Archaeal Fructose-1,6-bisphosphate Aldolases Constitute a New Family of Archaeal Type Class I Aldolase
Bettina Siebers, Henner Brinkmann, Christine Dörr, Britta Tjaden, Hauke Lilie, John van der Oost and Corné H. Verhees

J. Biol. Chem. 2001, 276:28710-28718.
doi: 10.1074/jbc.M103447200 originally published online May 31, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103447200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 56 references, 15 of which can be accessed free at http://www.jbc.org/content/276/31/28710.full.html#ref-list-1