FRUCTOSE-6-PHOSPHATE ALDOLASE IS A NOVEL CLASS I ALDOLASE FROM
ESCHERICHIA COLI AND IS RELATED TO A NOVEL GROUP OF BACTERIAL
TRANSALDOLASES

Melanie Schürmann and Georg A. Sprenger*

Institut für Biotechnologie 1, Forschungszentrum Jülich GmbH, P.O. Box 1913, D-52425 Jülich, Germany.

Running title: fructose-6-phosphate aldolase from Escherichia coli

Subdivision: Enzyme catalysis and regulation

* corresponding author to whom all correspondence should be directed

Tel: 0049-2461-616205
Fax: 0049-2461-612710
e-mail: g.sprenger@fz-juelich.de
Abbreviations:

FBP = fructose-1,6-bisphosphate
Fru-6-P = D-fructose-6-phosphate
FSA = fructose-6-phosphate aldolase
FBP aldolase = fructose-1,6-bisphosphate aldolase

Footnotes:

Footnote 1: Isomura and co-workers, EMBL database entry ECD188

Footnote 2: we established that our DNA sequence of the *B. subtilis ywjH* gene is in conflict with the deposited sequence in the databanks; the corrected derived YwjH peptide sequence is therefore longer at its C-terminus.

Footnote 3: M. Schürmann and G. A. Sprenger, manuscript in preparation.

Footnote 4: M. Schürmann, S. Thorell, G.Schneider, Y. Lindqvist and G.A. Sprenger, unpublished observations
SUMMARY

We have cloned an open reading frame (ORF) from the Escherichia coli K-12 chromosome which had been assumed earlier to be a transaldolase or a transaldolase-related protein, termed MipB. Here we show that instead a novel enzyme activity, fructose-6-phosphate aldolase, is encoded by this ORF which is the first report of an enzyme that catalyzes an aldol cleavage of fructose-6-phosphate from any organism. We propose the name FSA (mnemonic for fructose six phosphate aldolase; gene fsa). The recombinant protein was purified to apparent homogeneity by anion exchange and gel permeation chromatography with a yield of 40 mg of protein from 1 liter of culture. Using electrospray tandem mass spectroscopy, a molecular mass of 22,998 per subunit was determined. From gel filtration a size of 257,000 (+/- 20,000) was calculated. The enzyme most likely forms either a decamer or dodecamer of identical subunits. The purified enzyme displayed a $V_{\text{max}}$ of 7 U mg$^{-1}$ of protein for fructose-6-phosphate cleavage (at 30°C, pH 8.5 in 50 mM glycylglycine buffer). For the aldolization reaction a $V_{\text{max}}$ of 45 U mg$^{-1}$ of protein was found; $K_m$ values for the substrates were: 9 mM for fructose-6-phosphate, 35 mM for dihydroxyacetone, and 0.8 mM for glyceraldehyde3-phosphate. FSA did not utilize fructose, fructose-1-phosphate, fructose-1,6-bisphosphate, or dihydroxyacetone phosphate. FSA is not inhibited by EDTA which points to a metal-independent mode of action. The lysine-85 residue is essential for its action as its exchange to arginine (K85R) resulted in complete loss of activity in line with the assumption that the reaction mechanism involves a Schiff base formation through this lysine residue (class I aldolase). Another fsa-related gene, talC of E. coli, was shown to also encode fructose-6-phosphate aldolase activity and not a transaldolase as proposed earlier.
INTRODUCTION

Aldolases are lyases which typically catalyze a stereoselective addition of a keto donor on an aldehyde acceptor molecule (1). Aldol condensation and cleavage reactions play crucial roles in the central sugar metabolic pathways of all organisms. For instance in glycolysis, fructose-1,6-bisphosphate is reversibly cleaved into the trioses dihydroxyacetone-phosphate and glyceraldehyde 3-phosphate, whereas in gluconeogenesis, the bisphosphate is formed through action of aldolase (fructose-1,6-bisphosphate or FBP aldolase, EC 4.1.2.13). FBP aldolases and other aldolases can be broadly divided into two groups according to their reaction mechanisms. Class I aldolases are characterized by a covalent intermediate, which is a protonated Schiff base formed between a lysine residue and the carbonyl carbon of the substrate (2-4). Class II aldolases have an absolute requirement for a divalent metal ion which stabilizes the reaction intermediates by polarization of the substrate carbonyl (5). Class I and II aldolases vary in other criteria such as subunit structure, pH profile, and substrate affinity. They share little if any sequence homology and are apparently of different evolutionary origins (2). Class II aldolases prevail in bacteria, in fungi and algae (4). Class I FBP aldolases are mainly distributed in higher eukaryotes including animals, plants, protozoa and algae; they generally are tetramers (4). Bacterial class I FBP aldolases are known from Staphylococcus carnosus (6), Escherichia coli (7), or from the archaeon Halobacterium vallismortis (4,8). They either form monomers (S. carnosus; 6), or homodecamers (H. vallismortis; 8). Recently, a class I aldolase (dhnA; 7) has been described for Escherichia coli in addition to the well-known class II FBP aldolase of glycolysis (9).

Microbial FBP aldolases are known to split fructose-1,6-bisphosphate only. In higher eukaryotes, fructose-1-phosphate is a lesser substrate of aldolase (2,10) while fructose-6-phosphate is either an inhibitor of FBP aldolase (11) or a very weak substrate (less than 0.01% relative activity compared with FBP); however, no aldol formation from dihydroxyacetone and glyceraldehyde-3-P was reported (12); muscle and plant chloroplast FBP aldolases are reported to split
sedoheptulose-1,7-bisphosphate (13,14). To our best knowledge, no aldol cleavage of fructose-6-phosphate has been reported so far from any organism (1).

Transaldolases (EC 2.2.1.2) are class I aldolases which serve in transfer reactions in the pentose phosphate cycle. Transaldolases use fructose-6-P as donor and transfer a dihydroxyacetone group to acceptor compounds as erythrose-4-P or glyceraldehyde-3-P (3,15-18). As a side reaction, formation of fructose-6-P from dihydroxyacetone and glyceraldehyde-3-P is known but the corresponding aldol cleavage reaction has not been documented (3). Recently, a group of gene sequences presumably encoding transaldolase-like proteins (19) have been reported as outcome of total genome analyses of various Eu- and Archaebacteria. We have cloned two of these sequences (mipB, talC) from the genome of *Escherichia coli* K-12. During the course of characterization of the gene products, however, we noticed that the corresponding proteins did not act as transaldolases. Instead, they perform a novel reaction, cleavage or formation of fructose-6-phosphate:

\[
\begin{align*}
\text{CH}_2\text{OH} & \quad \text{CH}_2\text{OH} \\
\text{C}=\text{O} & \quad \text{C}=\text{O} \\
\text{HO}-\text{C}-\text{H} & \quad + \quad \text{H}-\text{C}=\text{O} \\
\text{H}-\text{C}-\text{OH} & \quad \text{CH}_2\text{OH} \\
\text{H}-\text{C}-\text{OH} & \quad \text{CH}_2\text{OPO}_3^{2-} \\
& \quad \text{CH}_2\text{OPO}_3^{2-}
\end{align*}
\]

Fructose-6-phosphate \quad \text{Dihydroxyacetone} + \text{Glyceraldehyde 3-phosphate}

Here we present results in the characterization of fructose-6-phosphate aldolase encoded by the gene *fsa* (formerly termed *mipB*).
**EXPERIMENTAL PROCEDURES**

**Materials**

Sugar phosphates, antibiotics and other fine chemicals were purchased from Sigma (Deisenhofen, Germany) if not indicated otherwise. Aldehydes and erythrose were from Fluka (Neu-Ulm, Germany). Auxiliary enzymes (triose phosphate isomerase/glycerol 3-phosphate dehydrogenase, phosphoglucone isomerase, glucose-6-phosphate dehydrogenase), restriction endonucleases, Taq DNA polymerase, T4 DNA ligase, and were from Boehringer Mannheim, Germany. SDS was from Serva (Heidelberg, Germany), acrylamide/ bisacrylamide was from Roth (Karlsruhe, Germany), chromatographic standards (Combithek) were from Boehringer, Q sepharose HP was from Pharmacia, Freiburg, Germany. Glycylglycine, NADH and NADP(H) were purchased from Biomol (Hamburg, Germany). Bacterial media were from Difco.

**Bacterial strains and growth conditions**

The bacterial strains and plasmids used in this study are listed in Table 1. The strains were grown under aeration at 37°C in LB medium (20) with appropriate antibiotics added. Ampicillin was used in a concentration of 100 mg/l.

**DNA techniques**

Chromosomal DNA of *E. coli* strain MC4100 (21) was prepared and used as template for oligonucleotide-directed DNA amplification (22). Standard techniques for cloning (20) and transformation (23) were applied. The *E. coli* mipB gene was amplified by polymerase chain reaction using primers MipB5 (5´ GATGTGCGTCGACTGTTCAGAGAGTTTTCCC 3´) and MipB3 (5´ GAGGCTGCAGAACGTCCGGTTAAATCGACG 3´) corresponding to bps 862,865 to 862,896 (5´end), and 863,497 to 863,527 bps (3´end), respectively, of the sequence deposited at EMBL/ GenBank (Isomura and co-workers, accession number ECD188; 24); the underlined sequences denote the engineered restriction sites for *Sal*I and *Pst*I, respectively. 20 pmols of each primer were used with template chromosomal DNA (500 ng). The resulting 0.7
kb PCR fragment was purified, cleaved with PstI plus SalI, and ligated with pUC18 which had been opened likewise. Strain JM109 was used for transformations; resulting clones were checked for their integrity by restriction analyses and DNA sequencing using an automatic non-radioactive system (LI-COR, MWG Biotech, Ebersberg, Germany). Site-directed mutagenesis was carried out using the Chameleon Double-Stranded Site-directed Mutagenesis Kit of Stratagene. Mutagenesis primers were 5´ GGCGGTCACCGGAACGCGCACCACGATATCCGC 3´ and 5´ CATCATTGGAAAACGCTCTTCGGGGCG 3´. Databank searches were done using the NCBI Blast server with the program of Altschul et al. (25). Preliminary sequence data were, a.o., obtained from The Institute for Genomic Research website at http://www.tigr.org.

**Purification of the new enzyme from a recombinant strain**

FSA (formerly: MipB) aldolase from recombinant strain JM109/pUC18/fsa was purified by the following procedure; all operations were carried out at 4°C in glycylglycine buffer (50 mM; pH 8.0; 1 mM dithiothreitol): A single colony was inoculated into 50 ml of LB + Amp and incubated overnight at 37°C with shaking. This culture served as starter for the main culture which was performed in 3 2-liter Erlenmeyer flasks (400 ml of LB+Amp medium each) with shaking at 37°C. Cells were collected by centrifugation (yield of 24 g wet weight). After washing with glycylglycine buffer, pellets were broken by ultrasonic treatment (Branson Sonifier, Danbury, CT, USA) for 8 x 30 sec at 40 W under cooling in an ethanol/ice-bath. After centrifugation at 20,000 x g, the supernatant was used as cell-free extract. Cell-free extract was dissolved in 240 ml of buffer and directly applied onto a Q-Sepharose HP anion exchange column (XK 26/20; 26 x 200 mm). At a flow rate of 1 ml /min, FSA was eluted in a linear NaCl gradient at a concentration of 352-380 mM NaCl. Active fractions were pooled, diluted fourfold with buffer and passed over a gel filtration column (Superdex G-200, Pharmacia). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in the presence of 1% SDS on 12 % vertical polyacrylamide gels using the buffer system of Lämmli (26). Gels
were run at room temperature in a BioRad MiniProteanII chamber with a LKB 2297 Macrodrive 5 power supply at a constant voltage of 100 V. For native polyacrylamide gel electrophoresis, gradient gels were run for 6 hours with a constant voltage of 125 V. Protein bands were visualized by staining with Coomassie Brilliant Blue G250. By using different reference marker proteins, the subunit mass of the FSA was calculated from a plot of the log of the molecular mass versus the relative mobility on SDS-polyacrylamide gels. Purified FSA was blotted onto PVDF membranes (Immobilon-P from Millipore) in a semi-dry blot apparatus and stained with amidoblack. The protein band was cut out and subjected to N-terminal sequenation. Electrospray tandem mass spectroscopy was carried out as described (27) using a Q-TOF (Micromass, Manchester England).

**Aldolase Assays**

Two different assays for fructose-6-phosphate aldolase activity were used (all at 30°C in a Shimadzu UV160A spectrophotometer with a thermostatted cuvette holder at a wavelength of 340 nm):

I) Cleavage of fructose-6-phosphate (Fru-6-P, 50 mM) was followed using the auxiliary enzymes triosephosphate isomerase and glycerol-3-phosphate dehydrogenase to detect formation of D-glyceraldehyde 3-phosphate. The oxidation of NADH (0.5 mM) was monitored and 1 µmole of NADH oxidized was set equivalent to 1 µmole of Fru-6-P cleaved. Enzyme activities are given in U (µmol/min). The standard buffer was glycylglycine (50 mM, pH 8.5) including 1 mM DTT in a total volume of 1 ml.

II) Using the same buffer system as in I), the formation of Fru-6-P from glyceraldehyde 3-phosphate and dihydroxyacetone (3 and 50mM, respectively) was monitored by the combined enzymes phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. The reduction of NADP (0.5 mM) was followed. A prereaction of glyceraldehyde 3-phosphate with the auxiliary enzymes and NADP was run until no further NADPH formation occurred. Influence of possible
inhibitors of aldolase activity was measured by aldolase assays I and II. Glycerol was added at different concentrations up to 230 mM; inorganic phosphate was added up to 5 mM and EDTA was added at 10 mM. Transaldolase activity was determined as described earlier (16). A dye-binding method (28) was used to estimate the concentration of protein in solution.
RESULTS

Cloning of the *fsa (mipB)* gene and expression of the plasmid-encoded aldolase

During a databank search for transaldolase-like proteins in the genome of *E. coli* K-12 strain MG1655 (24; accession number U00096) we found two open reading frames (ORFs) which showed a degree of identical amino acid residues in the range of 25% to the derived peptide sequence of *talB* (Tab. 2; 16). One of the putative ORFs (“talC”) had been classified earlier by the group of Saier as a transaldolase, albeit without experimental evidence (19). The other (*mipB*) was originally proposed as a transaldolase-like protein (Footnote 1; 29).

In our efforts to understand the transaldolase activities of *E. coli* (16,17,30), we amplified the *mipB*-containing region with a PCR method (22) using chromosomal DNA of strain MC4100 as template and by using specific primers with engineered unique restriction sites (see Fig. 1 and Experimental procedures for details). The amplification product (about 700 bp of DNA) was cloned into the expression vector pUC18. In crude extracts from strains carrying the gene on high copy number vectors, an extra protein band at 24,000 Da (+/- 1,000) appeared on SDS-PAGE. This protein band could be further augmented by addition of the inducer IPTG to recombinant cells in the exponential phase and was estimated to constitute up to 10% of the total soluble protein content of the crude extract (Fig. 2); thus a rapid and high-yield enzyme purification could be undertaken. The purification strategy using recombinant strain JM109/pUC18/fsa is described in the Experimental procedures. A total of about 40 mg of pure enzyme was obtained from 1 litre of culture, with an overall yield of 38% corresponding to a purification factor of 5.2 (Tab. 3). The degree of purity was monitored with polyacrylamide gel electrophoresis (26).

Contrary to our expectation that *mipB* encoded a new transaldolase species, no such activity was enriched concomitantly with the new protein species. Instead, we noticed that a fructose-6-phosphate-cleaving activity was present and further enriched by subsequent steps of
protein purification. In the homogeneous state, a fructose-6-phosphate aldolase activity (at 30°C in glycyglycine buffer, pH 8.5) of 7 U per mg of protein was found (Tab. 4). No fructose 1,6-bisphosphate aldolase or transaldolase activity could be detected in the gel filtration fractions (data not shown). As a literature search did not reveal evidence for a previous description of a fructose-6-phosphate aldolase from any organism, we like to term the novel activity as fructose-6-phosphate aldolase. Furthermore, we propose to rename the corresponding gene (formerly mipB) as fsa (mnemonic for fructose six phosphate aldolase); the enzyme is abbreviated as FSA.

To verify that the novel enzyme activity was the true product of the fsa (mipB) gene, the purified protein was subjected to SDS-PAGE, blotted onto a PVDF membrane and stained with amidoblack. The first 10 amino acid residues were determined by an automated Edman degradation and analysed by reversed phase high performance liquid chromatography. The sequence was determined as:

\[ H_2N-(\text{Met})-\text{Glu-Leu-Tyr-Leu-Asp-Thr-Ser-Asp-Val}. \]

The formyl-methionine was cleaved off in a portion of the sample. The N-terminal amino acid sequence was in full agreement with the sequence submitted by Isomura and co-workers (EMBL entry ECD188; SwissProt entry P78,055).

**Properties of the novel aldolase**

Examination of the comparative SDS gel electrophoretic mobility of the novel *E. coli* recombinant aldolase with a number of known reference proteins indicated a subunit mass for the purified protein of 24,000 ± 1,000 (Fig. 2). Using a Q-TOF electrospray tandem mass spectrometer the molecular mass of FSA was determined to 22,998 (Fig 3). This was in excellent agreement with the mass calculated from the deduced protein sequence (including the initial f-Met) of 22,997 Da. (SwissProt entry P78,055). The molecular mass of native *E. coli* recombinant aldolase was judged by gel filtration with reference proteins of known molecular
masses ranging from 12 to 400 kDa. Active aldolase was eluted at a volume of 152 ml buffer. In a logarithmic plot of elution volume versus molecular mass an average mass of 257,000 +/- 20,000 Da was calculated. This points to either a decameric or dodecameric structure of *E. coli* Fru-6-P aldolase, consisting of ten or twelve identical subunits, respectively.

The influence of different buffer substances, pH and temperature on the activity of the enzyme as well as the storage stability were analysed using enzyme assay I (see Experimental Procedures). The auxiliary enzymes were first checked for activity under the different reaction conditions and were added to the reaction mixture in excess. As buffer substances, Tris, glycylglycine, Hepes, Imidazole, CHAS, or phosphate were used. Of these, glycylglycine (50mM) was the best buffer compound. Optimal activity was found around pH 8.5, with a broad range of activity in buffers from pH 6.0 to 12.0.

FSA displayed a broad temperature optimum and was active in the range from 20-75°C. While no significant loss of activity was detected after 600 hours of incubation at 45°C (in glycylglycine buffer, pH 8.0), the respective half-lives of the enzyme were 200 h at 55°C, 30 h at 65°C, and 16 h at 75°C. A significant loss in activity was found in Tris buffers at concentrations higher than 10 mM pointing to a reaction of Tris with the enzyme. The purified protein could be stored frozen at -20°C in the presence of 1 mM DTT with a loss of activity of about 20-40%. At 4°C in glycylglycine buffer, the loss of activity was 20 % per month. Alternatively, the enzyme could be lyophilized and stored at -20°C for several months.

FSA was inhibited by glycerol, inorganic phosphate, and arabinose-5-phosphate, but not by EDTA (at 10mM). Rapid loss of activity was seen if kept in contact with glycerol (see Fig. 4a). After 10 min of incubation in the presence of 20% glycerol, a decrease of more than 70% of enzyme activity was found. This inhibition was fully reversible (by dilution or removal through ultrafiltration) and appeared to be of the uncompetitive type. Inorganic phosphate was a
competitive inhibitor with an apparent $K_i$ value of 0.22 mM (see Fig. 4b). Arabinose-5-phosphate was a competitive inhibitor ($K_i$ of 0.07 mM; data not shown).

**Kinetic studies on aldolase substrates**

The kinetic constants $K_m$ and $V_{\text{max}}$ were determined in 50mM glycylglycine buffer, at pH 8.5 and 30°C. The cleavage of fructose-6-phosphate was monitored by enzyme assay I (see Experimental Procedures). When aldolase activities with different donor and acceptor compounds were compared, the $V_{\text{max}}$ values of the standard reaction with Fru-6-P were determined each time as a control and were set 100%. No cleavage products were obtained from fructose, fructose-1-phosphate, glucose-6-phosphate, sedoheptulose-1,7-bisphosphate, xylulose-5-phosphate, ribulose-5-phosphate, and fructose-1,6-bisphosphate (up to 100 mM final concentrations). Neither were these compounds inhibitors of the standard reactions at concentrations up to 20 mM (data not shown).

Aldol forming activity of FSA (dihydroxyacetone as donor, glyceraldehyde-3-P as standard acceptor) was followed by measuring NADPH formation in the presence of phosphoglucone-isomerase and glucose-6-phosphate dehydrogenase (assay II). Aldol formation took place at a faster rate than the cleavage reaction ($V_{\text{max}}$ was calculated to be at 45 U/mg). Using HPLC measurements we checked whether other donor compounds are used by FSA. Dihydroxyacetone served as standard donor compound for comparison. Hydroxyacetone (acetol) served as donor but at reduced rates, erythrose and glycolaldehyde were weak acceptors (data not shown). DHAP did not serve as donor compound, nor was D-glyceraldehyde used as acceptor (i.e. no fructose was formed).

**Occurrence of FSA homologs in other organisms**

Databank searches with total genome sequences from various eu- and archaebacterial microorganisms revealed sequences with apparent homology to FSA. Databank searches were
done using the NCBI Blast server (25). Preliminary sequence data were obtained, a.o., from The Institute for Genomic Research website at http://www.tigr.org. In *E. coli*, another sequence is present (**talC**, see above) which shared 68% identical (79% similar) residues with FSA. *fsa*-related genes with prominent similarity were only found in prokaryotic genomes such as in *Clostridium beijerinckii* (31), as well as in the total genomes of *Yersinia pestis*, *Bacillus subtilis*, *Bacillus stearothermophilus*, in the extreme thermophilic eubacteria *Aquifex aeolicus* and *Thermotoga maritima*, and in the archaeabacterium *Methanococcus jannaschii*. Fig. 5 shows an alignment of sequences with the highest similarity to FSA. *Bona fide* transaldolases (transaldolases A and B from *E. coli*, the two isozymes from *S. cerevisiae*, or the human transaldolase) showed less pronounced similarity to FSA and are therefore excluded from Fig. 5. All sequences in Fig. 5 have in common that no function has been experimentally assigned to them. They are in a size range of about 23 to 24 kDa (average of about 220 amino acid residues) per subunit. 24 of these residues are invariantly present in all 15 sequences of the alignment. As FSA was not inhibited by EDTA, it was likely that this novel aldolase does not belong to class II (metal-dependent aldolases) and instead is a new member of class I aldolases. Therefore, a reactive lysine residue should be prominent. Indeed, among the 24 invariant residues of the alignment in Fig. 5, only one lysine residue appeared (at position 85 of FSA). To test whether this conserved lysyl-residue indeed fulfills a function in enzyme activity, we changed the Lys-85 residue to an arginine residue by site-directed mutagenesis (see Fig.1 and *Experimental procedures* for details). The K85R mutein was expressed at good quantity and was purified through the same procedure as wild-type FSA. The K85R mutein nearly lacked enzyme activity (less than 0.03 U/mg of protein), both for cleavage of fructose-6-phosphate or its formation. We propose that FSA is therefore likely to be a class I aldolase with a reactive lysine residue (Lys-85).
Are all FSA homologs also fructose-6-phosphate aldolases?

As the talC gene from *E. coli* showed striking similarity to the fsa gene, we tested whether it also encoded an aldolase activity. Recombinant strains of *E. coli* carrying a high-copy number plasmid with the PCR-amplified talC-gene, indeed showed fructose-6-phosphate aldolase activity in the crude extracts. The purified protein lacked transaldolase activity and is thus the second example of a fructose-6-phosphate aldolase (although with reduced specific activities when compared with FSA; data not shown). In order to find whether other related proteins included in Fig. 5 display transaldolase or the novel fructose 6-P aldolase activities, we cloned the corresponding genes from the Gram-positive bacterium *Bacillus subtilis* (where no transaldolase gene had been functionally assigned so far) and from the hyperthermophilic bacterium *Thermotoga maritima*. TM0295 was amplified as a 680 bp PstI-SalI fragment and ywjH as a 790 bp PstI-SalI fragment (Tab.1). Both genes were amplified by PCR [footnote 2], cloned into suitable expression vectors and were transformed in *E. coli* strain JM109. Both genes led to formation of extra protein bands visible in SDS-PAGE (subunit size ca. 24 kDa). Crude extracts from the recombinant strains showed elevated transaldolase activities but no fructose-6-P aldolase activity (Footnote 3). To our best knowledge, this is the first proof for a transaldolase gene and enzyme function in *Bacillus subtilis* as well as in *Thermotoga maritima*.
DISCUSSION

We have cloned an open reading frame from the *E. coli* chromosome which had been assumed earlier to be a transaldolase or a transaldolase-related protein (19; Footnote 1). Here we show that this gene encodes a novel enzyme activity, fructose-6-phosphate aldolase. This activity was found in cell-free extracts of *fsa*-recombinant *E. coli* strains and could be purified to apparent homogeneity with a yield of about 40 mg (38 % of initial total activity). We propose the gene name *fsa* (mnemonic for fructose-6(six)-phosphate aldolase) instead of *mipB* (whose true function was unknown sofar). Enzyme purification was accelerated by the availability of the cloned gene from this organism on a high-copy number vector and was enhanced by adding IPTG to derepress an IPTG-responsive promoter, leading to elevated activities already in the crude extracts. Evidence for the purity of the recombinant protein was provided a) by visual inspection of Coomassie-stained SDS-PAGE, b) by the unanimous determination of the N-terminal amino acid residues, and c) by electrospray tandem mass spectrometry. The preparation was suitable for crystallization (Footnote 4), underlining the purity of the preparation. The enzyme most likely forms either a decamer or dodecamer of identical subunits with a *M*$_r$ of 22,998.

FSA is not inhibited by EDTA which points to a metal-independent mode of action. The lysine-85 residue is essential for its action as its exchange to arginine (K85R) resulted in complete loss of activity; this could be best interpreted if the reaction mechanism involves a Schiff base formation through this lysine residue. This we take for evidence that FSA is a class I aldolase.

To our knowledge, this is the first report on a genuine fructose-6-phosphate aldolase from any source. As we show here, the gene *talC* of *E. coli*, also encodes a fructose-6-phosphate aldolase and not a transaldolase as proposed earlier (19). The gene product shows a high degree of similarity with FSA. Two other genes (from *B. subtilis* and *T. maritima*) with high similarity
to *fsa* were cloned but were shown to encode true transaldolase functions. From our data it becomes obvious that these clearly homologous sequences do not encode same functions. Both new transaldolase genes are members of a novel class of transaldolases as they show limited similarity to classical transaldolases from man, yeast, or *E. coli* (average size about 35 kDa; 16) or from plants and cyanobacteria (average size about 42 kDa; 36). In this context it may be of interest that muscle FBP aldolase, when truncated at the C-terminus by treatment with carboxypeptidase, displays a distinct transaldolase activity, e.g., transfer of the enzyme-bound DHAP to an aldehyde (32). Thus, the limits between the two enzyme activities (aldolase vs. transaldolase) may be shifted by exchange of amino acid residues.

The substrate specificity of the *E. coli* FSA appeared to be narrow with fructose-6-phosphate being the only substrate for aldol cleavage from all tested compounds which were at our hands. Although we cannot exclude the possibility that another sugar phosphate is the cognate substrate of this novel aldolase, we wish to emphasize that the common building block fructose-6-phosphate, has not been reported to be a substrate for aldolase to our best knowledge. We do not yet know the true physiological function of FSA in *E. coli*. Using FSA-specific polyclonal antibodies, we were unable to detect immunologically active material against FSA in crude extracts of *E. coli* (grown either in LB or defined mineral salts media with various carbon sources; data not shown). It needs to be established under which circumstances *fsa* and *talC* are transcribed (if at all) and to which amounts. Experiments to elucidate the structure and function of FSA are underway.

We were not able to determine the reaction equilibrium constants due to the rapid chemical degradation of one of the cleavage products, glyceraldehyde 3-phosphate (data not shown). However, we estimated a standard free energy change of reaction $\Delta G^\circ$ of +32 kJ mole$^{-1}$, which is about 10 kJ mole$^{-1}$ more endergonic than the fructose-bisphosphate cleavage reaction (33). If the subsequent reactions cannot compensate for this strongly endergonic reaction it is not likely that
the cleavage reaction contributes much to the \textit{in vivo} function of the FSA enzyme, and the aldol condensation reaction might prevail in the cell. However, phosphorylation of one cleavage product, dihydroxyacetone, by an ATP-dependent kinase or by PEP-dependent phosphorylation through a PTS might help the cells to circumvent this activation problem. As well, a NADH-dependent glycerol dehydrogenase could withdraw DHA from the reaction. A glycerol dehydrogenase is known from \textit{E. coli} and, in this context, it is of interest that the encoding \textit{gldA} gene (34,35) lies immediately downstream (overlapping for 28 bp in the 3’ region) of the \textit{talC} gene of \textit{E. coli} which encodes this second fructose 6-P aldolase. This chromosomal location indicates that both \textit{talC} and \textit{gldA} are part of an operon and may serve in a metabolic pathway which handles dihydroxyacetone. No such glycerol dehydrogenase gene, however, is found adjacent to the \textit{fsa} (formerly \textit{mipB}) gene in the chromosome. The function of both new aldolases remains to be unveiled.

\textbf{Acknowledgements} – We thank William J. Griffiths at the Protein Analysis Center, Department of Medical Biochemistry and Biophysics Karolinska Institute, Stockholm for carrying out the mass spectroscopy. We thank Rainer Kappes from our institute for chromosomal DNA of \textit{Bacillus subtilis}, Wolfgang Liebl (University of Göttingen, Germany) for the kind donation of \textit{T. maritima} chromosomal DNA, Gunter Schneider for critically reading the manuscript, and Hermann Sahm for his continuous support. This work was supported by a grant of the Deutsche Forschungsgemeinschaft through SFB380/B21.
REFERENCES

1) Machajewski, T.D., and Wong C-H. (2000) *Angew. Chemie Intl. Ed.* **39**, 1352-1375.

2) Rutter, W.J. (1964) *Fed. Proc.* **23**, 1248-1257.

3) Horecker, B.L., Tsolas, O., and Lai, C.Y. (1972) in *The Enzymes* (Boyer, P.D., Ed.) 3rd ed., Vol. 7, pp 213-258, Academic Press, New York.

4) Marsh, J.J., and Lebherz, H.G. (1992) *Trends Biochem. Sci.* **17**, 110-113.

5) Mildvan, A.S., Kobes, R.D., and Rutter, W.J. (1971) *Biochemistry* **10**, 1191-1204.

6) Witke, C., and Götz, F. (1993) *J. Bacteriol.* **175**, 7495-7499.

7) Thomson, G.J., Howlett, G.J., Ashcroft, A.E., and Berry, A. (1998) *Biochem. J.* **331**, 437-445.

8) Krishnan, G., and Altekar, W. (1991) Eur. J. Biochem. **195**, 343-350.

9) Alefounder, P.R., Baldwin, S.A., Perham, R.N., and Short, N.J. (1989) *Biochem. J.* **257**, 529-534.

10) Gefflaut, T., Blonski, C., Perie, J., and Willson, M. (1995) *Prog. Biophys. Molec. Biol.* **63**, 301-340.

11) Crans, D.C., Sudhakar, K., and Zamborelli, T.J. (1992) *Biochemistry* **31**, 6812-21.

12) Richards, O.C., and Rutter, W.J. (1961) *J. Biol. Chem.* **236**, 3185-3192.

13) Horecker, B.L., Smyrniotis, P.Z., Hiatt, H.H., and Marks, P.A. (1955) *J. Biol. Chem.* **212**, 827-836.

14) Flechner, A., Gross, W., Martin, W.F., and Schnarrenberger, C. (1999) *FEBS Lett.* **447**, 200-202.

15) Bonsignore, A., Pontremoli, S., Grazi, E., and Mangiarotti, M. (1959) *Biochem. Biophys. Res. Commun.* **1**, 79-82.

16) Sprenger, G.A., Schörken, U., Sprenger, G., and Sahm, H. (1995) *J. Bacteriol.* **177**, 5930-36.
17) Jia, J., Huang, W., Schörken, U., Sahm, H., Sprenger, G.A., Lindqvist, Y., and G. Schneider (1996) *Structure* 4, 715-724.

18) Jia, J., Schörken, U., Lindqvist, Y., Sprenger, G.A., and Schneider, G. (1997) *Protein Sci.* 6, 119-124.

19) Reizer, J., Reizer, A., and Saier, M.H. (1995) *Microbiology* 141, 961-971.

20) Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

21) Casadaban, M. J. (1976) *J. Mol. Biol.* 104, 541-555.

22) Mullis, K.B., and Faloona, F.A. (1987) *Meth. Enzymol.* 155, 335-350.

23) Hanahan, D. (1983) *J. Mol. Biol.* 166, 557-580.

24) Blattner, F.R., Plunkett,G. III, Bloch,C.A., Perna,N.T., Burland,V., Riley,M., Collado-Vides,J., Glasner,J.D., Rode,C.K., Mayhew,G.F., Gregor,J., Davis,N.W., Kirkpatrick,H.A., Goeden,M.A., Rose,D.J., Mau,B. and Shao,Y. (1997) *Science* 277, 1453-1474.

25) Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) *Nucl. Acids Res.* 25, 3389-3402.

26) Lämmli, U.K. (1970) *Nature* 227, 680-685.

27) Rai, D.K., Alvelius, G., Landin, B., and Griffiths, W.J. (2000) *Rapid Commun. Mass Spectrom.* 14, 1184-1194.

28) Bradford, M.M. (1976) *Anal. Biochem.* 72, 248-254.

29) Thorell, S., Gergely jr., P., Banki, K., Perl, A., and Schneider, G. (2000) *FEBS Lett.* 475, 205-208.

30) Schörken, U., Jia, J., Sahm, H., Sprenger, G.A., and Schneider, G. (1998) *FEBS Lett.* 441, 247-250.

31) Tangney, M., Brehm, J.K., Minton, N.P., and W.J. Mitchell (1998) *Appl. Environm. Microbiol.* 64, 1612-1619.
32) Rose, I.A., O’Connell, E.L., and Mehler, A.H. (1965) J. Biol. Chem. 240, 1758-1765.

33) Kröger, A. (1999) in The Biology of the Prokaryotes (Lengeler, J.W., Schlegel, H.G., Drews, G. eds.). Thieme Verlag, Stuttgart; Blackwell Science Inc., Oxford.

34) Sprenger, G.A., Hammer, B.A., Johnson, E.A., and Lin, E.C.C. (1989) J. Gen. Microbiol. 135, 1255-1262.

35) Truniger, V. and Boos, W. (1994) J. Bacteriol. 176, 1796-1800.

36) Köhler, U., Cerff, R., and Brinkmann, H. (1996) Plant Mol. Biol. 30, 213-218.

37) Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene 33, 103-119.

38) Vieira, J., and Messing, J. (1982) Gene 19, 259-268.
Legends to the Figures

Figure 1: Features of the cloned *fsa* gene from *E. coli*.

PCR primer sites MipB5 and MipB3 (including the engineered *SalI* and *PstI* restriction sites) are denoted as well as the site of site-directed mutagenesis of the critical Lys-85 residue. The putative ribosome binding site is underlined.

Figure 2: SDS-PAGE analysis of the *E. coli* aldolase purification

The gel was run as described in Experimental Procedures with the following reference marker proteins in lane D: phosphorylase b, 97,400 Da; bovine serum albumin, 66,200 Da; fructose-bisphosphate aldolase, 39,200 Da; triosephosphate isomerase, 26,600 Da; and trypsin inhibitor, 21,500 Da. In the lanes A to C, samples of the purification steps were applied, FSA appears in all lanes at a molecular mass of 24,000 Da. Lane C: crude extract after ultrasonication and centrifugation; in lane B: after chromatography on Q-Sepharose HP. Lane A: after gel filtration on Superdex G-200 column.

Figure 3: Electrospray tandem mass spectroscopy of FSA

5 µl of purified FSA solution at a concentration of 10 mg/ml was used according to Experimental Procedures.

Figure 4: Inhibitory effects of glycerol and inorganic phosphate

a) Inhibitory effect of glycerol at various inhibitor concentrations, shown as a double-reciprocal Lineweaver-Burk plot. ●, without inhibitor; ■, 20 mM glycerol; ▲, 59 mM glycerol; ×, 118 mM glycerol; ●, 230 mM glycerol

b) Inhibition by inorganic phosphate at final concentrations 0.5 mM (■); 1 mM (▲) and 5 mM (×) compared without phosphate (●). Fru-6-P was added in concentrations up to 50 mM.
**Figure 5:** Alignment of *E. coli* FSA with related derived protein sequences

Residues in bold face (red) are conserved in at least 50% of the sequences. # denotes residues which are conserved throughout all sequences. The * above residue 85 denotes the putative reactive lysyl-residue of the novel aldolase. Abbreviations are: Ecofsa = *E. coli* fructose-6-phosphate aldolase FSA (MipB); Ypestis = *Yersinia pestis* (unfinished genome; SANGER-Institute); EcotalC = *E. coli* "transaldolase C"; Styphi = *Salmonella typhi* (unfinished genome; SANGER); Smutans = *Streptococcus mutans* (unfinished genome, OU-AGCT); Entfaec = *Enterococcus faecalis* (TIGR); Clobeij = *Clostridium beijerinckii* (gut-cluster gene; 31); BsatalC = *B. subtilis* "transaldolase C" accession no. AL009126; Bstearo = *Bacillus stearothermophilus* (unfinished genome; OU-ACGT); Rhodoca = *Rhodobacter capsulatus* (ORF M3.gl379 start: 347190, end: 346540, unfinished genome, TITAN); Deinora = *Deinococcus radiodurans* R1, accession nos. AE000513 and AE001825.; Caucrees = *Caulobacter crescentus* (TIGR); Methja = *Methanococcus jannaschii*; L77117; Aquiaeo = *Aquifex aeolicus*; acc.no. AE000657; Thermot = *Thermotoga maritima* accession no. AE000512.
Table 1: Strains and plasmids used in this study
All strains are derived from *E. coli* K-12.

| Strain designation | Relevant genotype/marker | Reference/origin |
|--------------------|--------------------------|------------------|
| MC4100             | $F^- araD 139 \Delta(\argF-loa)U169$ | (21) |
|                    | $rpsL150 \text{ relA1 deoC1 ptsF25}$ | |
| JM109              | $recA \text{ hsdR relA thi} \Delta(lac-proAB)$ | (37) |
|                    | $/F^+ \text{ traD proAB}^+ \text{ lacI}^f \text{ lacZ} \Delta M15$ | |

| Plasmids: | Relevant markers | Reference/origin |
|-----------|------------------|------------------|
| pUC18     | $bla$ (Ap-resistance) | (38) |
| pUC19     | $bla$ (Ap-resistance) | (38) |
| pBLKS     | $bla$ (Ap-resistance) | Stratagene |
| pUC18*fsa | pUC18 with 740 bp $PstI-SalI$-f$sa$-fragment | this study |
| pUC18*talC| pUC18 with 730 bp $PstI-SalI$-talC-fragment | this study |
| pUC19TM0295| pUC19 with 680 bp $PstI-SalI$-TM0295-fragment | this study |
|           | from *Thermotoga maritima* | |
| pBLKSywjH | pBLKS with 790 bp $PstI-SalI$-yw$jH$-fragment | this study |
|           | from *Bacillus subtilis* | |
Table 2: Sequence relationships of transaldolases and FSA-related proteins

| Protein          | Size [kDa] | Similarity to FSA (%) (identity) | Similarity to TalB (identity) |
|------------------|-----------|---------------------------------|------------------------------|
| FSA              | 24        | 100                             | 46 (24)                      |
| TalC             | 24        | 79 (68)                         | 50 (29)                      |
| TM0295 (T. maritima) | 24        | 55 (29)                         | 54 (34)                      |
| YwjH (B. subtilis) | 23        | 54 (30)                         | 41 (27)                      |
| OrfX (Cl. beijerinckii) | 24        | 58 (36)                         | 47 (26)                      |
**Table 3: Purification scheme for E. coli fructose-6-phosphate aldolase**

| Sample                | Purification factor | Yield | Total activity [U] | Total protein content [mg] | Specific activity aldolization [U/mg] |
|-----------------------|---------------------|-------|--------------------|----------------------------|--------------------------------------|
| Cell-free extract     | 1.0                 | 100   | 3400               | 540                        | 6.4                                  |
| Q-Sepharose HP        | 2.4                 | 49    | 1680               | 110                        | 15.3                                 |
| Gel filtration        | 5.2                 | 38    | 1320               | 40                         | 33.0                                 |
**Table 4:** Kinetics of fructose-6-P aldolase FSA

| Substrate                      | $K_M$ [mM] | $V_{max}$ [U/mg] |
|--------------------------------|------------|------------------|
| Fructose-6-phosphate           | 9          | 7                |
| Dihydroxyacetone               | 35         | 45               |
| Glyceraldehyde 3-phosphate     | 0.8        | 45               |
663 bps, 220 amino acid residues
Fig. 4

a) Glycerol: uncompetitive inhibition type

b) Phosphate: competitive inhibition type:
