THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 14, Issue of April 6, pp. 11055–11061, 2001

Printed in U.S.A.

Fructose-6-phosphate Aldolase Is a Novel Class I Aldolase from *Escherichia coli* and Is Related to a Novel Group of Bacterial Transaldolases*

Received for publication, September 4, 2000, and in revised form, November 22, 2000
Published, JBC Papers in Press, December 18, 2000, DOI 10.1074/jbc.M008061200

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We have cloned an open reading frame from the *Escherichia coli* K-12 chromosome that 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 open reading frame, 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 (for fructose-six phosphate aldolase; gene name *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. By using electrospray tandem mass spectroscopy, a molecular weight 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_{max} of 7 units 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_{max} of 45 units 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 glyceraldehyde 3-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 *Escherichia coli*, was shown to also encode fructose-6-phosphate aldolase activity and not a transaldolase as proposed earlier.

Aldolases are lyases that 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 triose dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, whereas in gluconeogenesis, the bisphosphate is formed through action of aldolase (fructose-1,6-bisphosphate or FBP aldolase,[1] 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 that 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*; see Ref. 6) or homodecamers (*H. vallismortis*; see Ref. 8). Recently, a class I aldolase (*dhnA*; see Ref. 7) has been described for *E. 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), whereas 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 that 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) has been reported as outcome of total genome analyses of various Eubacteria and Archaebacteria. We have cloned two of these sequences (*mipB* and *talC*) from the genome of *E. coli* K-12.

* This work was supported by Grant SFB380/B21 of the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: FBP aldolase, fructose-1,6-bisphosphate aldolase; FBP, fructose 1,6-bisphosphate; Fru-6-P, D-fructose 6-phosphate; FSA, fructose-6-phosphate aldolase; ORFs, open reading frames; bp, base pair; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

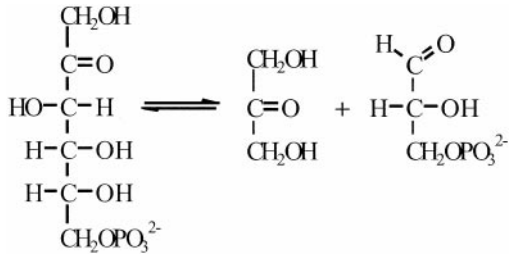
TABLE I
Strains and plasmids used in this study

All strains are derived from *E. coli* K-12.

Strain designation	Relevant genotype/marker	Ref./origin
MC4100	F$^-$ *araD* 139 Δ(*argF-lac*)U169 *rpsL*150 *rel*A1 *deo*C1 *pts*F25	21
JM109	*recA hsdR relA thi* Δ(*lac-proAB*)/F′ *traD proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*	37

Plasmids	Relevant markers	Ref./origin
pUC18	*bla* (ampicillin resistance)	38
pUC19	*bla* (ampicillin resistance)	38
pBLKS	*bla* (ampicillin resistance)	Stratagene
pUC18*fsa*	pUC18 with 740-bp *Pst*I-*Sal*I *fsa* fragment	This study
pUC18*talC*	pUC18 with 730-bp *Pst*I-*Sal*I *talC* fragment	This study
pUC19*TM0295*	pUC19 with 680-bp *Pst*I-*Sal*I *TM0295* fragment from *T. maritima*	This study
pBLKS*ywjH*	pBLKS with 790-bp *Pst*I-*Sal*I *ywjH* fragment from *B. subtilis*	This study

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 as shown in Reaction 1.



REACTION 1

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 unless indicated otherwise. Aldehydes and erythrose were from Fluka (Neu-Ulm, Germany). Auxiliary enzymes (triose-phosphate isomerase/glycerol-3-phosphate dehydrogenase, phosphoglucose isomerase, and glucose-6-phosphate dehydrogenase), restriction endonucleases, *Taq* DNA polymerase and T4 DNA ligase, were from Roche Molecular Biochemicals. SDS was from Serva (Heidelberg, Germany); acrylamide/bisacrylamide was from Roth (Karlsruhe, Germany); chromatographic standards (Combithek) were from Roche Molecular Biochemicals; and Q-Sepharose HP was from Amersham Pharmacia Biotech. 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 I. 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/liter.

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 base pairs 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 coworkers,[2] GenBank™ accession number ECD188; see Ref. 24); the underlined sequences denote the engineered restriction sites for *Sal*I and *Pst*I, respectively. 20 pmol of each primer were used with template chromosomal DNA (500 ng). The resulting 0.7-kilobase pair PCR fragment was purified, cleaved with *Pst*I plus *Sal*I, 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 nonradioactive system (LI-COR, MWG Biotech, Ebersberg, Germany). Site-directed mutagenesis was carried out using the Chameleon Double-stranded Site-directed Mutagenesis kit from Stratagene. Mutagenesis primers were 5′ GGCGGTCACCGGAACGCGCACCACGATATCCGC 3′ and 5′ CATCATTGGAAAACGCTCTTCGGGGCG 3′. Data bank searches were done using the NCBI Blast server with the program of Altschul *et al.* (25). Preliminary sequence data were obtained from The Institute for Genomic Research.

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 + ampicillin and incubated overnight at 37 °C with shaking. This culture served as starter for the main culture that was performed in three 2-liter Erlenmeyer flasks (400 ml of LB + ampicillin 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) eight times for 30 s at 40 watts under cooling in an ethanol/ice bath. After centrifugation at 20,000 × *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 × 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 4-fold with buffer, and passed over a gel filtration column (Superdex G-200, Amersham Pharmacia Biotech). SDS-polyacrylamide gel electrophoresis (PAGE) was carried out in the presence of 1% SDS on 12% vertical polyacrylamide gels using the buffer system of Laemmli (26). Gels were run at room temperature in a Bio-Rad 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 h with a constant voltage of 125 V. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. 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 polyvinylidene difluoride membranes (Immobilon-P from Millipore) in a semi-dry blot apparatus and stained with Amido Black. 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, UK).

Aldolase Assays—Two different assays for fructose-6-phosphate aldolase activity were used (all at 30 °C in a Shimadzu UV160A spectrophotometer with a thermostated 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 triose-phosphate isomerase and glycerol-

[2] M. Isomura, T. Oqino, and T. Mizuno, personal communication.

TABLE II
Sequence relationships of transaldolases and FSA-related proteins

Protein	Size	Similarity to FSA (identity)	Similarity to TalB (identity)
	kDa	*%*	
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)

3-phosphate dehydrogenase to detect formation of D-glyceraldehyde 3-phosphate. The oxidation of NADH (0.5 mM) was monitored and 1 μmol of NADH oxidized was set equivalent to 1 μmol of Fru-6-P cleaved. Enzyme activities are given in units (μmol/min). The standard buffer was glycylglycine (50 mM, pH 8.5) including 1 mM dithiothreitol in a total volume of 1 ml.

(ii) By using the same buffer system as in i, the formation of Fru-6-P from glyceraldehyde 3-phosphate and dihydroxyacetone (3 and 50 mM, 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 data bank search for transaldolase-like proteins in the genome of *E. coli* K-12 strain MG1655 (Ref. 24; GenBank™ accession number U00096), we found two open reading frames (ORFs) that showed a degree of identical amino acid residues in the range of 25% to the derived peptide sequence of *talB* (Table II; see Ref. 16). One of the putative ORFs ("*talC*") had been classified earlier by Saier and co-workers (19) as a transaldolase, albeit without experimental evidence. The other (*mipB*) was originally proposed as a transaldolase-like protein (29).[2]

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 (± 1000) 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 under "Experimental Procedures." A total of about 40 mg of pure enzyme was obtained from 1 liter of culture, with an overall yield of 38% corresponding to a purification factor of 5.2 (Table III). 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

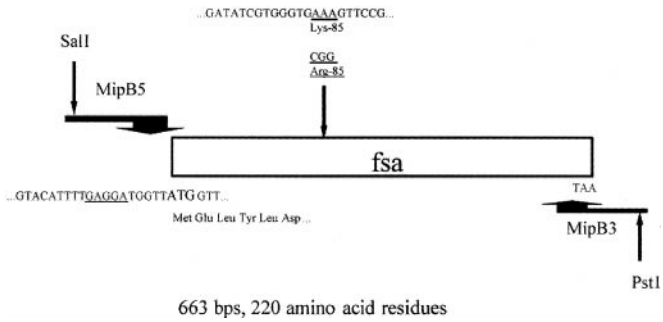


FIG. 1. **Features of the cloned *fsa* gene from *E. coli*.** PCR primer sites MipB5 and MipB3 (including the engineered *Sal*I and *Pst*I restriction sites) are denoted as well as the site of site-directed mutagenesis of the critical Lys-85 residue. The putative ribosome binding site is *underlined*.

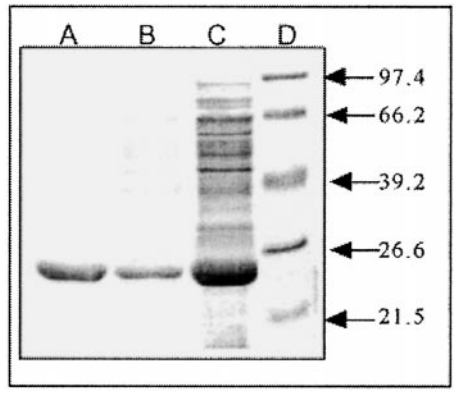


FIG. 2. **SDS-PAGE analysis of the *E. coli* aldolase purification.** The gel was run as described under "Experimental Procedures" with the following reference marker proteins in *lane D*: phosphorylase *b*, 97,400 Da; bovine serum albumin, 66,200 Da; fructose-bisphosphate aldolase, 39,200 Da; triose-phosphate isomerase, 26,600 Da; and trypsin inhibitor, 21,500 Da. In the *lanes A–C*, samples of the purification steps were applied, and 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.

enriched by subsequent steps of protein purification. In the homogeneous state, a fructose-6-phosphate aldolase activity (at 30 °C in glycylglycine buffer, pH 8.5) of 7 units per mg of protein was found (Table IV). 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 polyvinylidene difluoride membrane, and stained with Amido Black. The first 10 amino acid residues were determined by an automated Edman degradation and analyzed by reversed phase high performance liquid chromatography. The sequence was determined as H_2N-(Met)-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[2] (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 pro-

TABLE III
Purification scheme for E. coli fructose-6-phosphate aldolase

Sample	Purification factor	Yield	Total activity	Total protein content	Specific activity aldolization
			units	*mg*	*units/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 IV
Kinetics of fructose-6-P aldolase FSA

Substrate	K_m	V_{max}
	mM	*units/mg*
Fructose 6-phosphate	9	7
Dihydroxyacetone	35	45
Glyceraldehyde 3-phosphate	0.8	45

teins indicated a subunit mass for the purified protein of 24,000 ± 1,000 (Fig. 2). By 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 of 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 10 or 12 identical subunits, respectively.

The influence of different buffer substances, pH values, and temperature on the activity of the enzyme as well as the storage stability were analyzed 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, 3-(cyclohexylamino)-1-propanesulfonic acid, or phosphate were used. Of these, glycylglycine (50 mM) was the best buffer compound. Optimal activity was found around pH 8.5, with a broad range of activity in buffers from pH 6.0–12.0.

FSA displayed a broad temperature optimum and was active in the range from 20 to 75 °C. Although no significant loss of activity was detected after 600 h 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 dithiothreitol 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 10 mM). Rapid loss of activity was seen if kept in contact with glycerol (see Fig. 4*a*). 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. 4*b*). 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_{max} were determined in 50 mM 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_{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 phosphoglucose-isomerase and glucose-6-phosphate dehydrogenase (assay II). Aldol formation took place at a faster rate than the cleavage reaction (V_{max} was calculated to be at 45 units/mg). By using high pressure liquid chromatography 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). Dihydroxyacetone phosphate 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—Data bank searches with total genome sequences from various eu- and archaebacterial microorganisms revealed sequences with apparent homology to FSA. Data bank searches were done using the NCBI Blast server (25). Preliminary sequence data were obtained from The Institute for Genomic Research. 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*, and *Bacillus stearothermophilus*, in the extreme thermophilic eubacteria *Aquifex aeolicus* and *Thermotoga maritima*, and in the archaebacterium *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 23–24 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 1 lysine residue appeared (at position 85 of FSA).

To test whether this conserved lysyl residue indeed fulfills a

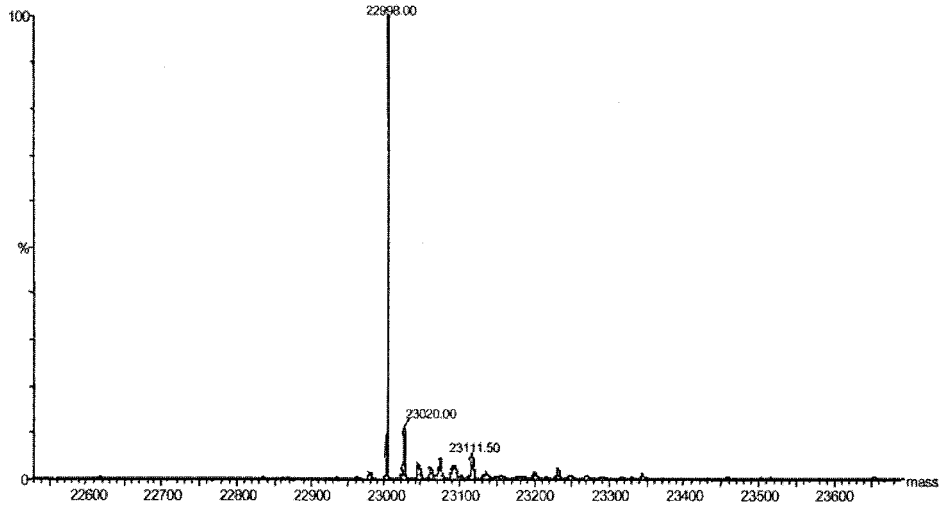


FIG. 3. **Electrospray tandem mass spectroscopy of FSA.** 5 μl of purified FSA solution at a concentration of 10 mg/ml was used according to "Experimental Procedures."

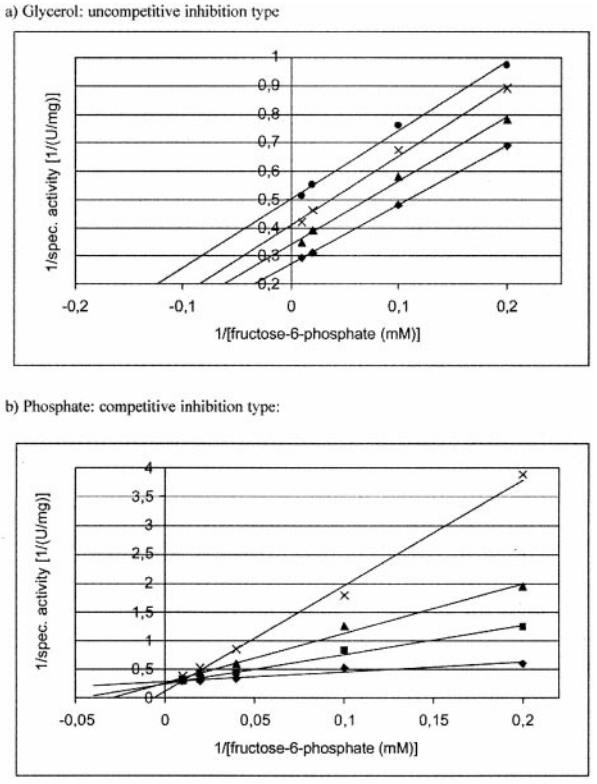


FIG. 4. **Inhibitory effects of glycerol and inorganic phosphate.** *a,* inhibitory effect of glycerol at various inhibitor concentrations, shown as a double-reciprocal Lineweaver-Burk plot. ♦, without inhibitor; ■, 20 mM glycerol; ▲, 59 mM glycerol; ×, 118 mM glycerol; ●, 230 mM glycerol. *b,* inhibition 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.

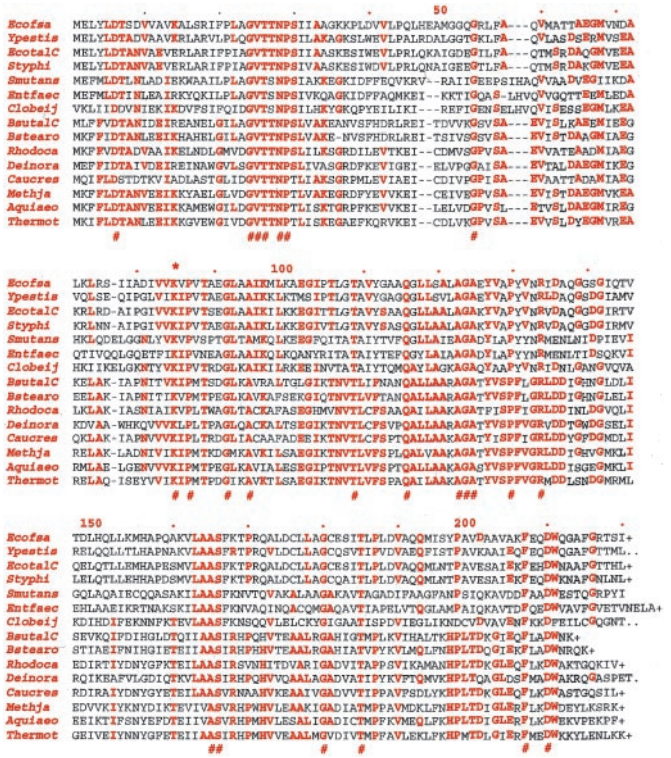


FIG. 5. **Alignment of *E. coli* FSA with related derived protein sequences.** Residues in *bold face* (*red*) are conserved in at least 50% of the sequences. # denotes residues that are conserved throughout all sequences. The * above residue 85 denotes the putative reactive lysyl residue of the novel aldolase. Abbreviations are as follows: *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; see Ref. 31); *BsutalC, B. subtilis* "transaldolase C," GenBank™ accession number AL009126; *Bstearo, B. stearothermophilus* (unfinished genome; OU-ACGT); *Rhodoca, Rhodobacter capsulatus* (ORF M3.gl379 start, 347190; end, 346540, unfinished genome, TIGR); *Deinora, Deinococcus radiodurans* R1, GenBank™ accession numbers AE000513 and AE001825; *Caucres, Caulobacter crescentus* (TIGR); *Methja, M. jannaschii,* L77117; *Aquiaeo, A. aeolicus,* GenBank™ accession number AE000657; *Thermot, T. maritima,* GenBank™ accession number AE000512.

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 units/ 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). 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 *B. subtilis* (where no transaldolase gene had been functionally assigned so far) and from the hyperthermophilic bacterium *T. maritima. TM0295* was amplified as a 680-bp *Pst*I-*Sal*I fragment and *ywjH* as a 790-bp *Pst*I-*Sal*I fragment (Table I). Both genes were amplified by PCR,[3] cloned into suitable expression vectors, and transformed in *E. coli* strain JM109. Both genes led to formation of extra protein bands visible in SDS-PAGE (subunit size ~24 kDa). Crude extracts from the recombinant strains showed elevated transaldolase activities but no fructose-6-P aldolase activity.[4] To our best knowledge, this is the first proof for a transaldolase gene and enzyme function in *B. subtilis* as well as in *T. maritima*.

DISCUSSION

We have cloned an open reading frame from the *E. coli* chromosome that had been assumed earlier to be a transaldolase or a transaldolase-related protein (19).[2] 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* instead of *mipB* (whose true function is unknown so far). 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 the following: (*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,[5] 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; see Ref. 16) or from plants and cyanobacteria (average size about 42 kDa; see Ref. 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 dihydroxyacetone phosphate to an aldehyde (32). Thus, the limits between the two enzyme activities (aldolase *versus* 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*. By 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 what circumstances *fsa* and *talC* are transcribed (if at all) and to what amounts. Experiments to elucidate the structure and function of FSA are under way.

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 $\Delta G^{0\prime}$ of + 32 kJ mol^{-1}, which is about 10 kJ mol^{-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 *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 phospho*enol*pyruvate-dependent phosphorylation through a phosphotransferase system might help the cells to circumvent this activation problem. As well, an NADH-dependent glycerol dehydrogenase could withdraw dihydroxyacetone from the reaction. A glycerol dehydrogenase is known from *E. coli,* and in this context, it is of interest that the encoding *gldA* gene (34, 35) lies immediately downstream (overlapping for 28 bp in the 3′ region) of the *talC* gene of *E. coli* that encodes this second fructose-6-P aldolase. This chromosomal location indicates that both *talC* and *gldA* are part of an operon and may serve in a metabolic pathway that handles dihydroxyacetone. No such glycerol dehydrogenase gene, however, is found adjacent to the *fsa* (formerly *mipB*) gene in the chromosome. The function of both new aldolases remains to be unveiled.

Acknowledgments—We thank William J. Griffiths at the Protein Analysis Center, Department of Medical Biochemistry and Biophysics Karolinska Institute, Stockholm, Sweden, for carrying out the mass spectroscopy. We thank Rainer Kappes from our institute for chromosomal DNA of *Bacillus subtilis*; Wolfgang Liebl (University of Göttingen, Germany) for the kind donation of *T. maritima* chromosomal DNA; Gunter Schneider for critically reading the manuscript; and Hermann Sahm for continuous support.

[3] We established that our DNA sequence of the *B. subtilis ywjH* gene is in conflict with the deposited sequence in the data banks; the corrected derived YwjH peptide sequence is therefore longer at its C terminus.

[4] M. Schürmann and G. A. Sprenger, manuscript in preparation.

[5] M. Schürmann, S. Thorell, G. Schneider, Y. Lindqvist, and G. A. Sprenger, unpublished observations.

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