The *dhnA* gene of *Escherichia coli* encodes a Class I fructose biphosphate aldolase

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The gene encoding the *Escherichia coli* Class I fructose-1,6-bisphosphate aldolase (FBP aldolase) has been cloned and the protein overproduced in high amounts. This gene sequence has previously been identified as encoding an *E. coli* dehydrin in the GenBank™ database [gene *dhnA*; entry code U73760; Close and Choi (1996) Submission to GenBank™]. However, the purified protein overproduced from the *dhnA* gene shares all its properties with those known for the *E. coli* Class I FBP aldolase. The protein is an 8–10-mer with a native molecular mass of approx. 340 kDa, each subunit consisting of 349 amino acids. The Class

I enzyme shows low sequence identity with other known FBP aldolases, both Class I and Class II (in the order of 20 %), which may be reflected by some novel properties of this FBP aldolase. The active-site peptide has been isolated and the Schiff-base-forming lysine residue (Lys²³⁶) has been identified by a combination of site-directed mutagenesis, kinetics and electrospray-ionization MS. A second lysine residue (Lys²³⁸) has been implicated in substrate binding. The cloning of this gene and the high levels of overexpression obtained will facilitate future structure–function studies.

INTRODUCTION

Fructose-1,6-bisphosphate (FBP) aldolase (EC 4.1.2.13) is a key enzyme in cellular metabolism, catalysing the reversible aldol condensation/cleavage reaction between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP) to yield FBP. Two classes of FBP aldolase exist which are distinguished by their structural and mechanistic properties [1,2]. The Class I enzymes utilize an active-site lysine residue to form a Schiff base with the substrate as part of the reaction mechanism. The Class II FBP aldolases, on the other hand, are metalloenzymes which utilize a bivalent metal ion in catalysis in an otherwise similar mechanism. Class II FBP aldolases are found in bacteria, yeast, fungi and blue–green algae and are dimeric enzymes with a subunit molecular mass of approx. 40 kDa. In contrast, Class I FBP aldolases are distributed in higher forms of life, including animals, plants, protozoa and algae. Eukaryotic Class I FBP aldolases are invariably tetrameric, with a subunit molecular mass of approx. 40 kDa. Excellent crystal structures are available for several eukaryotic Class I FBP aldolases [3–5], revealing that these proteins are members of the α/β -barrel family of proteins, of which triosephosphate isomerase is the archetype. Class I FBP aldolases in prokaryotes have been discovered in *Escherichia coli* [6,7], *Micrococcus aerogenes* [8,9], *Lactobacillus casei* [10], *Mycobacterium smegmatis* [11,12], *Staphylococcus aureus* [13], *Staphylococcus carnosus* [14], *Synechocystis* sp. strain PCC6803 [15] and *Haloarcula vallismortis* [16]. These Class I FBP aldolases appear to be more diverse than their eukaryotic counterparts. They have subunit molecular masses ranging from 27 to 40 kDa and their oligomeric arrangement can be anything from monomeric to decameric, making studies of their sequences and structures particularly interesting. To date, however, only two complete

sequences of prokaryotic Class I FBP aldolases are known, those of *Staph. carnosus* [14] and *Synechocystis* sp. PCC6803 [15], compared with many known sequences for both Class II and eukaryotic Class I FBP aldolases.

Most organisms contain only one class of FBP aldolase, although a select few possess both classes [2,7,8,17]. *E. coli* is one of the few organisms to express both types of FBP aldolase activity, probably representing a catalytic redundancy that has largely been eliminated by most organisms through evolution [18]. The Class II *E. coli* enzyme has been cloned, sequenced and overproduced and has been the subject of much recent study [19–22]. The structure of the *E. coli* Class II FBP aldolase has recently been solved, revealing that, like its eukaryotic Class I counterparts, it also adopts an α/β -barrel fold [23,24]. Much less is known about the Class I enzyme in *E. coli*, and we have therefore attempted to clone the relevant gene and overproduce the protein. This will not only help our understanding of the evolutionary relationships between the two classes of aldolase, without the added complications of inter-species comparisons, but also be useful for future structure–function studies. The cloning of this gene, its overexpression and the identification of the active-site Schiff-base-forming lysine residue is described here.

EXPERIMENTAL

Materials

NaB³H₄ (100 mCi/mmol) was purchased from American Radio-labelled Chemicals Ltd. (St. Louis, MO, U.S.A.). CNBr, PMSF, Mes, trifluoroacetic acid (TFA; sequencing grade), NADH, D-FBP (trisodium salt), D-fructose 1-phosphate (F1P) and dihydroxyacetone phosphate (DHAP, lithium salt) were from

Abbreviations used: FBP, fructose 1,6-bisphosphate; F1P, fructose 1-phosphate; TFA, trifluoroacetic acid; DHAP, dihydroxyacetone phosphate; MALDI-TOF, matrix-assisted laser-desorption-ionization time-of-flight; ESI, electrospray ionization; GCG, (University of Wisconsin) Genetics Computer Group.

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Sigma Chemical Co. (Poole, Dorset, U.K.). Calf intestinal alkaline phosphatase, the restriction endonucleases *EcoRI* and *HindIII* and the mixture of glycerol-3-phosphate dehydrogenase and triosephosphate isomerase were obtained from Boehringer Mannheim G.m.b.h. (Mannheim, Germany). *T*₄ DNA ligase was from Pharmacia (Milton Keynes, U.K.) and *Pfu* polymerase was supplied by Stratagene (Cambridge, U.K.). Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Loughborough, U.K.). Fluorotrans blotting membranes were from Pall (Portsmouth, U.K.). The Nucleon Easyclene DNA purification kit was obtained from Scotlab Bioscience (Coatbridge, Lanarkshire, Scotland, U.K.) and the Wizard Miniprep DNA purification kit, λ *HindIII* DNA markers and PCR markers were from Promega (Southampton, U.K.). DEAE-cellulose (DE52) was from Whatman Biosystems Ltd. (Maidstone, Kent, U.K.). The Superdex[™] 200 (HR 16/20) column and Phenyl-Superose (HR 5/5) column were from Pharmacia and the Poros R2 (4.6 mm diameter $\times$ 50 mm length) column was supplied by PerSeptive Biosystems (Hertford, U.K.). All other reagents were of analytical grade.

Bacterial strains and plasmids

E. coli (Crookes strain) was obtained from the National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland, U.K.). *E. coli* strain KM3 [Δ (*his gnd*), Δ *lac*, *araD*, *fda*, *ptsF*, *ptsM*, *rpsL*, *pro*, NA^R/F', *pro* A⁺B⁺ *lacI*^q *lacZ* Δ M15] was as described previously [20]. The expression vector pKK223-3 was from Pharmacia.

Purification of Class I FBP aldolase

Purification from *E. coli* Crookes strain

The Class I FBP aldolase was purified from *E. coli* Crookes strain using a procedure modified from that previously published [6,7]. Cells were grown at 37 °C in 25 litres of M9 minimal medium supplemented with 100 mM sodium lactate. The lactate-grown cells (230 g) were suspended in 20 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM PMSF, and disrupted in a French pressure cell. After centrifugation (15000 *g*, 1 h at 4 °C), 0.3 vol. of 2% (w/v) protamine sulphate, pH 6.2, was added to the supernatant in a stepwise manner and the precipitate was removed by centrifugation. The supernatant was dialysed against 100 mM Tris/HCl, pH 7.5, and loaded on to a DE52 column (10 cm long $\times$ 5 cm diameter) equilibrated in the same buffer. Elution of the aldolase was carried out using a linear gradient (0–100 mM NaCl) over 5 column vol. at 3 ml/min. Fractions containing aldolase activity (eluting at approx. 80 mM NaCl) were pooled and dialysed against 50 mM Tris/HCl, pH 7.5. This fraction was loaded on to a second DE52 column (10 cm $\times$ 2.5 cm) equilibrated in 50 mM Tris/HCl, pH 7.5, and eluted with the same 0–100 mM NaCl gradient as before, except this time over 10 column vol. The aldolase activity was again pooled and adjusted to 50–65% saturation with (NH₄)₂SO₄. The resulting precipitate was collected by centrifugation and was suspended in 50 mM Tris/HCl, pH 7.5, and dialysed against the same buffer before chromatography on a Superdex[™] 200 column equilibrated in the same buffer. The flow rate was 1 ml/min. The active peak fractions collected were dialysed into 10 mM Tris/HCl, pH 7.5, containing 1 M (NH₄)₂SO₄ and loaded on to a Phenyl-Superose HR 5/5 column in the same buffer. The column was washed at 1 ml/min with 1 M (NH₄)₂SO₄ in 10 mM Tris/HCl, pH 7.5, and the aldolase was eluted at the end of a linear 1–0 M (NH₄)₂SO₄ gradient in the same buffer. The protein was then judged to be > 99% pure by SDS/PAGE (Figure 1).

Purification of the overproduced recombinant protein

Both the wild-type and mutant enzymes were expressed from the vectors pGJT1 or pGJT2 (see below). *E. coli* cells (strain KM3) containing the vector were grown in 2 $\times$ TY medium (10 g of bactotryptone, 10 g of yeast extract and 5 g of NaCl/litre) at 37 °C, harvested by centrifugation and suspended in 50 mM potassium phosphate buffer, pH 7.6, and disrupted using a French press. After centrifugation (15000 *g*, 1 h at 4 °C), the 0–40% saturated (NH₄)₂SO₄ precipitate, which contained the FBP aldolase activity, was collected and suspended in 50 mM Tris/HCl, pH 7.5, and dialysed against the same buffer. The enzyme was loaded on to a DE52 column (10 cm $\times$ 2.5 cm) run at 3 ml/min and, after washing with 80 mM NaCl, was eluted with step gradients of 120 mM and 200 mM NaCl in the same buffer. Fractions containing aldolase (eluted at both 120 and 200 mM NaCl) were pooled and concentrated by saturation to 80% with (NH₄)₂SO₄. After dialysis in 100 mM Tris/HCl, pH 8.0, the enzyme was chromatographed on a Superdex[™] 200 column at 1 ml/min in the same buffer. The peak fractions were pooled and were judged to be > 97% pure by SDS/PAGE.

N-terminal amino acid sequencing

Purified *E. coli* Class I FBP aldolase was electrophoresed on an SDS/12%-polyacrylamide gel and electroblotted on to a Fluorotrans PVDF membrane as described by Matsudaira [25]. Protein was revealed by staining with Coomassie Blue R-250 and was subjected to sequencing using an Applied Biosystems model 477A protein sequencer using the manufacturer's reagents and recommended cycles. Sequencing of the labelled active-site peptide (see below) was also carried out on an Applied Biosystems 477A sequencer.

BLAST database search

Sequences with high similarity to the N-terminal amino acid sequence of the wild-type Class I FBP aldolase were identified using the network BLAST server at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>)

Aldolase assays

FBP-cleavage activity was measured using a coupled enzyme assay as described elsewhere [20]. In some assays citrate, pH 8.0, in 50 mM Tris/HCl, was added to the assay to a final concentration of 10 mM. The cleavage activity with F1P was measured in a similar manner, except that the coupled assay contained NADH and glycerol phosphate dehydrogenase as secondary enzyme and the activity was monitored by the decrease in *A*₃₄₀. Kinetic parameters were measured by non-linear regression analysis [26], and protein concentration was measured by the method of Bradford [27].

Determination of native molecular mass

The native molecular mass of the Class I FBP aldolase was determined by gel filtration using a Pharmacia Superdex[™] 200 (HR 16/20) column equilibrated in 50 mM Tris/HCl, pH 8.0, containing 100 mM NaCl with a flow rate of 1 ml/min. Myoglobin (16.9 kDa), carbonic anhydrase (29 kDa), Class II FBP

Figure 1 SDS/PAGE of the Class I FBP aldolase

Composite photograph of SDS/12%-PAGE gels of the Class I FBP aldolase at various stages of the purification. Track 1, molecular-mass markers; tracks 2–7, purification from *E. coli* Crookes' strain (track 2, cell extract; track 3, protamine sulphate fraction; track 4, DE52 eluate; track 5, $(\text{NH}_4)_2\text{SO}_4$ fraction; track 6, eluate from SuperdexTM 200 column; track 7, Phenyl-Superose eluate); track 8, overproduced enzyme purified from *E. coli* strain KM3 transformed with plasmid pGJT1.

aldolase (*E. coli*) (78 kDa), alcohol dehydrogenase (150 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa) were used to calibrate the column.

The subunit molecular mass was estimated by SDS/PAGE on 12%-polyacrylamide gels [28].

Analytical ultracentrifugation

Sedimentation experiments were conducted using a Beckman XL-A analytical ultracentrifuge equipped with absorption optics, an An60-Ti rotor and filled epon centerpieces containing quartz windows. Sedimentation coefficients (*s*) were obtained by direct fitting of sedimentation-velocity boundary data using the program SVEDBERG [29]. These values were corrected for the density and viscosity of the buffer to obtain $s_{20,w}$ values [30]. Sedimentation-equilibrium experiments were conducted at 20 °C and 6000 rev./min for approx. 15 h. At equilibrium, scans of the solution column were acquired at 280 nm. Centrifugation at 40000 rev./min for 2 h was used to obtain the contribution of non-sedimenting species to the baseline absorbance. This baseline absorbance was less than 1 % of the initial absorbance. The data were analysed using the program SEDEQ1B (kindly provided by Dr. A. Minton, National Institutes of Health, Bethesda, MD, U.S.A.). Values for the partial specific volume (0.733 ml/g), hydration (0.39 g/g) and the axial ratio were calculated as described by Laue et al. [30].

PCR amplification and cloning

Oligonucleotides were designed to amplify both the full-length *E. coli dhna* gene [31] (encoding amino acids 1–374 of the Dhna protein) and a truncated form of the gene (encoding amino acids 25–374). The N-terminal primer used was either primer N1 [5'-ACG ACC GAA TTC ATG ATT GCC CGC AAA AGG (full-length)], or primer N2 [5'-GGG AGC GAA TTC ATG ACA GAT ATT GCG CAG (truncated)] (with *Eco*RI sites underlined) and the C-terminal oligo (primer C) was 5'-GTA TAA AGC CTT TCA GGC GAT AGT AAT TTT (containing a *Hind*III site, underlined). A total genomic DNA preparation from *E. coli* Crookes strain was prepared [32] for use as the template in the PCR reactions. The PCR was carried out in a 50 μ l reaction

mixture consisting of 20 mM Tris/HCl, pH 8.8, 2 mM MgSO_4 , 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1 % Triton X-100, 100 μ g/ml nuclease-free BSA, 0.2 mM each dNTP, 100 pmol of each N- and C-terminal primer, 0.5 μ g of template DNA and 2.5 units of *Pfu* DNA polymerase. The reaction mixture was overlaid with 50 μ l of mineral oil. Amplification involved an initial denaturation step at 95 °C for 5 min followed by cycling at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 2.5 min for 40 cycles, then a final extension for 2 min at 72 °C. PCR products were purified from agarose gels using a Nucleon Easiclean kit and then digested with *Eco*RI and *Hind*III. The gene was ligated into *Eco*RI/*Hind*III-treated pKK223-3. The resulting plasmids were termed pGJT1 (containing the truncated gene) and pGJT2 (containing the full-length gene) and were transformed into *E. coli* strain KM3 [20].

Active-site labelling

A 1.5 ml solution of purified Class I FBP aldolase (3.6 mg/ml) in 100 mM Tris/HCl buffer, pH 7.5, containing 0.01 mM 2-mercaptoethanol and 0.8 mM DHAP was incubated at 4 °C for 10 min. The solution was treated with 103 μ l of 0.5 M Mes buffer (adjusted to pH 5.9 with solid Tris) before the addition of NaB^3H_4 (100 mCi/mmol, 25 μ Ci/ μ l in 10 mM NaOH) to a final concentration of 7.4 mM. The reaction mixture was incubated in the dark for 15 min by which time the residual activity was 1 % of the initial value. Control experiments in the absence of DHAP retained full activity. The reaction was quenched by the addition of Tris/HCl, pH 9.0, to a final concentration of 40 mM. Excess reagents were removed by exhaustive dialysis against 0.2 M NH_4HCO_3 , pH 8.0, and the labelled protein solution dried using a Speed-Vac instrument.

CNBr digestion

The freeze-dried labelled protein (prepared as above) was resuspended in 350 μ l of 70 % (v/v) formic acid and a 100-fold molar excess of CNBr over methionine residues was added. The sample tube was flushed with N_2 and the reaction allowed to continue in the dark for 24 h at room temperature. The reaction was terminated by diluting 10-fold with water and drying under vacuum.

Purification of the active-site peptide

HPLC separation run 1

The CNBr cleavage products from the previous step were dissolved in 400 μ l of 50 mM Tris/HCl, pH 8.0, containing 50 mM dithiothreitol and 4 M guanidinium chloride, since they were not particularly soluble in buffer alone. Separation was performed on a Poros R2 (4.6 mm $\times$ 50 mm) reverse-phase column at 5 ml/min using a PerSeptive Biosystems BioCad Sprint Perfusion Chromatography System. The column was equilibrated in 10 % (v/v) acetonitrile containing 0.1 % (v/v) TFA and the peptides (200 μ l injected) were separated using a linear gradient [10–50 % (v/v)] of acetonitrile in 0.1 % (v/v) TFA over 240 column vol. Eluted peptides were detected by A_{220} and radioactivity determined by liquid-scintillation counting using a Canberra Packard 1900 TR liquid-scintillation analyser. The major radioactive 220 nm-absorbing peak was collected and freeze-dried. The collected peak was not a single peptide product as judged by amino acid sequencing and matrix-assisted laser-desorption-ionization time-of-flight (MALDI-TOF) MS, so a further separation was therefore performed.

HPLC Separation Run 2

The radiolabelled peptide sample from the first HPLC run was dissolved in 1.5 ml of 7% (v/v) acetonitrile and separated on the same system as described above, this time using an alternative buffer system. The column was equilibrated in 20 mM NH_4HCO_3 , pH 6.5, and a linear gradient (0–40%, v/v) of acetonitrile in 20 mM NH_4HCO_3 , pH 6.5, developed over 240 column vol. was used to elute the peptides. Detection was performed as described above, and the single radiolabelled peak was collected and freeze-dried.

MS

MALDI-TOF MS was performed on a Micromass (Micromass UK Ltd., Wythenshaw, Manchester, U.K.) ToFSpec instrument using a nitrogen laser at 337 nm. Peptide samples (2 μl , 100 pmol/ μl) were applied to the sample slide, mixed with 2 μl of matrix [α -cyano-4-hydroxycinnamic acid; 10 mg/ml in 50% ethanol/50% acetonitrile (v/v)] and air-dried. A source voltage of approx. 22.5 kV was used, with the microchannel plate detector voltage set to 1800–1900 V. Course laser energy was set to 20%, with fine adjustment used for each sample. A total of 20–50 laser shots were accumulated for each spectrum, and samples were externally calibrated with appropriate standards analysed under similar conditions.

Electrospray ionization (ESI) MS was carried out using a Platform II single quadrupole mass spectrometer (Micromass UK Ltd., Altrincham Cheshire, U.K.). Positive ESI was effected with a probe tip voltage of 3.5 kV and a counter-electrode voltage of 0.5 kV. The sampling cone voltage was set at 45 V. Nitrogen was employed as both the nebulizing and drying gas. The samples were stored in 100 mM NH_4HCO_3 and were diluted by a factor of two with a 1:1 (v/v) mixture of aq. 0.1% formic acid/acetonitrile to give a final concentration of about 20 pmol/ μl . Multiply charged data were accumulated over the range m/z 800–1600, and the spectra transformed on to a molecular-mass scale using Maximum Entropy techniques. An external calibration using horse heart myoglobin (molecular mass 16951.5 Da from Sigma Chemical Co.) was employed.

Site-directed mutagenesis

Site-directed mutagenesis of the expression plasmid was carried out using Megaprimer PCR [33]. Mutagenesis was performed to individually change the Lys^{236} codon (AAA) or the Lys^{238} codon (AAA) to an Ala codon (GCG). The mutagenic oligonucleotides 5'-GAT ATC GTC GCG CAA AAA ATG GCG (K236A) and 5'-GTC AAA CAA GCG ATG GCG GAA AAT (K238A) were used in conjunction with primers N2 and C (listed above) in two sequential PCR reactions using the PCR conditions already described. Subcloning of the mutant genes into pKK223-3 used the same method as described previously for the Class II FBP aldolase gene [20]. DNA sequencing confirmed the presence of each relevant mutation and that no spurious mutations had been introduced during the manipulations.

CD

Far-UV (190–260 nm) CD spectra of the wild-type and mutant aldolases were determined at a protein concentration of 0.5 mg/ml in 20 mM potassium phosphate buffer, pH 7.6, using a Jasco J720 spectropolarimeter with a pathlength of 1 mm. Two spectra were accumulated per sample.

RESULTS AND DISCUSSION

Identification of the Class I FBP aldolase gene

In *E. coli* the Class II FBP aldolase is constitutively expressed, whereas the Class I enzyme is induced by gluconeogenic substrates. Hence, only 0–5% of the total FBP aldolase activity is contributed by the Class I aldolase when *E. coli* is grown on glucose, but increased expression (up to 60% of the total aldolase activity) can be achieved on a C_3 source such as pyruvate or lactate [6,34]. In order to generate sequence information for the design of degenerate oligonucleotide probes for the Class I FBP aldolase gene, the enzyme was therefore purified, using a modification of the protocols described elsewhere [6,7], from a wild-type *E. coli* (Crookes) strain grown on lactate (Figure 1). A yield of approx. 40 mg of pure Class I FBP aldolase was achieved from 230 g wet weight of cells. The properties of the purified protein were consistent with those known for *E. coli* Class I FBP aldolase [6,7], with the subunit molecular mass estimated to be 36000 ± 1000 Da, by SDS/PAGE. Amino acid sequencing of the protein identified the following N-terminal sequence: TDIAQLLGK. Unfortunately, attempts at proteolysis of the protein to provide internal amino acid sequence met with failure, since the enzyme proved to be resistant to both tryptic and chymotryptic treatment. Initial attempts to amplify the Class I FBP aldolase gene by PCR were therefore made using degenerate oligonucleotides based upon the N-terminal sequence and upon a consensus sequence of the active-site region of known Class I FBP aldolases. However, this approach was unsuccessful.

A search of the GenBank[®] database using the N-terminal amino acid sequence of the purified *E. coli* Class I FBP aldolase revealed one sequence (gene *dhnA*; accession number U73760) [31] which perfectly matched all nine amino acid positions. The encoded protein, consisting of 374 amino acids, had been identified as *E. coli* dehydrin. The N-terminal residues of the FBP aldolase corresponded to residues 26–34 in the dehydrin sequence (Figure 2). Dehydrin proteins are stress proteins which are induced in plants in response to dehydration in order to enhance cell survival under conditions of decreased water availability [35,36]. Current thinking suggests that they may have a chaperone-like role in photosynthetic organisms in order to prevent stress-induced damage to the cell [37]. No evidence has, however, been presented to suggest that dehydrins have any catalytic activities. Dehydrins range in size from 9 to 200 kDa

MIARKRRART IHSRYPIGIY GSIV	<u>MTDIAQ</u>	LLGKDADNLL OHRGMTIPSD	26
QLYLPGHDIYV DRVMIDNNRP PAVLRNMOTL YNTGRLAGTG YLSILPVDQ			76
VEHSAGASFA ANPLYFDPKN IVELAIEAGC NCVASTYGVV ASVSRRYHR			126
TPPLVKLNHN ETLSPNTYD OTLYASVEQA FNMGAUAVGA TIYFGSEESR			176
ROIEEISAFA ERAHELGMVT VLWAYLRNSA FKKGVDYHYV SADLTGOANH			226
LAATIGADIV KOKMAENNGG YKAINGYTD DRVYSKLTSE NPIDLVRVOL			276
ANCYMGAGL INSGGAAGGE TDLSDAVRTA VINKRAGGMG LILGRKAFKK			326
SMADGVKLN AVODVYLDK ITIA			350

Figure 2 The primary amino acid sequence of the *E. coli* *dhnA* gene product

The primary sequence of the *dhnA* gene product [31] is shown. The sequence of the expressed Class I FBP aldolase is shown underlined. The first 24 amino acids (not underlined) and the initial methionine are not found in the mature aldolase protein. The corrected DNA sequence of the cloned *dhnA* gene results in Leu and Asn at positions 191 and 308 (shown double-underlined) respectively.

Table 1 Amino acid composition of the *E. coli* Class I FBP aldolase

The previously reported amino acid compositions of the Class I FBP-aldolase of *E. coli* [6,7] are compared with that deduced from the *dhnA* gene [31] (residues 26–374 as corrected in the present study and allowing for the removal of the initial methionine residue). Abbreviation: ND, not determined.

Amino acid (single-letter code)	Composition (residues/subunit)			<i>dhnA</i> (residues 26–374)
	Stribling and Perham [7]	Baldwin and Perham [6]	Average	
K	14	13	13.5	15
H	6	8	7	8
R	13	18	15.5	18
C	2	5	3.5	4
D (+N)	40	39	39.5	44
T	17	18	17.5	18
S	21	19	20	20
E (+Q)	28	21	24.5	26
P	11	5	8	10
G	28	29	29.5	30
A	38	41	39.5	41
V	21	22	21.5	25
M	6	8	7	9
I	17	18	17.5	20
L	31	30	30.5	32
Y	16	18	17	20
F	10	7	8.5	8
W	ND	ND		1

and are characterized by a highly conserved lysine-rich consensus sequence of 15 amino acids (EKKGIMDKIKEKLP_G), and also in many cases by a tract of serine residues and another consensus region, DEYGNP [37].

Several lines of evidence led us to believe that the protein identified as dehydrin might be the Class I FBP aldolase of *E. coli*. First, the estimated size of the protein (residues 26–374) is approx. 38 kDa, similar to the value estimated by SDS/PAGE for Class I FBP aldolase. Secondly, the previously reported amino acid composition of the aldolase [6,7] is very similar to that of residues 26–374 of the dehydrin (Table 1). Thirdly, the postulated dehydrin does not contain any of the characteristic motifs and the overall identity with known dehydrins is in the order of 13–20%. Finally, there are no reports, as yet, of any proteins with primary sequence similarity to dehydrins in any organism other than plants, although immunologically related osmotic-stress proteins have been identified in cyanobacteria [38].

Amplification and over-expression of the Class I FBP aldolase

The *E. coli dhnA* gene was cloned using PCR with primers based on the known sequence. Since the FBP aldolase might have been degraded during purification, resulting in the loss of the first 24 residues, oligonucleotides were designed to amplify the gene for both the full 374-amino-acid protein and the shortened 350 amino acid polypeptide (residues 25–374). PCR products of approximately the expected size, namely 1122 and 1050 bp, were generated (results not shown) and these were ligated into the expression vector pKK223-3, to generate the expression vectors pGJT2 and pGJT1 respectively.

A high degree of overproduction, in the order of 200 mg of pure Class I FBP aldolase/10 g wet weight of cells, was achieved from both pGJT1 and pGJT2, without the need for isopropyl β -D-thiogalactoside induction, and the protein was isolated using a

Table 2 Molecular masses of wild-type and mutant aldolases

The molecular masses as determined by ESI MS of the wild-type and mutant enzymes after treatment with either NaBH₄ and DHAP, or NaBH₄ alone are compared to the masses expected for the proteins deduced from their DNA sequences allowing for processing of the initial methionine residue and the two amino acid changes V191L and I308N described in the text.

Enzyme	Molecular mass			Mass difference
	Calculated from sequence	Measured after treatment with NaBH ₄	After treatment with NaBH ₄ and DHAP	
Wild-type	37 978.2	37 979.3	38 134.3	155.0
K236A	37 921.1	37 923.8	37 921.9	–1.9
K238A	37 921.1	37 923.5	38 076.3	152.8

modification of the protocol described previously [20]. The purified overproduced protein had the same properties on SDS/PAGE as the Class I FBP aldolase purified from *E. coli* Crookes strain (Figure 1).

The structural and kinetic properties of the proteins derived from both the full-length and shortened genes were identical. DNA sequencing confirmed that the construct pGJT1 carried the shortened gene and pGJT2 the full-length gene. However, N-terminal sequencing revealed that the protein produced from both constructs had the same N-terminal sequence: TDIA. Both proteins therefore have a processed N-terminus with the initial methionine removed and the 24-amino-acid extension encoded in pGJT2 was not found in the mature protein. This is perhaps not surprising, since an *E. coli* ribosome-binding site is found immediately upstream of the second methionine residue [39]. Further studies were restricted to the protein produced from pGJT1, and the residue numbers are based on this sequence after allowing for the removal of the initial methionine residue.

Repeated DNA sequencing of the cloned *dhnA* gene in a number of pGJT1 constructs revealed two changes to the sequence previously reported. These changes (GTG to CTG in codon 191 and ATC to AAC in codon 308) result in two changes to the deduced primary sequence of the Class I FBP aldolase: Val¹⁹¹ replaced by Leu, and Ile³⁰⁸ replaced by Asn (Figure 2). The presence of these amino acid changes in the encoded protein was confirmed by the determination of the accurate subunit mass by ESI MS (Table 2).

Characterization of the FBP aldolase

Enzyme kinetics

The purified protein was shown to have FBP aldolase activity using a coupled enzyme assay [20], and detailed steady-state kinetic studies were carried out using FBP as substrate. Previous studies had found difficulties in determining 'true' kinetic constants owing to a combination of enzyme proteolysis and the presence of contaminants in commercial preparations of substrate [6,7]. The experimental data measured here were, however, completely consistent with Michaelis–Menten kinetics. The K_m and k_{cat} values for FBP (Table 3) were 20 μ M and 13 min^{–1} respectively, similar to those for rabbit muscle Class I FBP aldolase [40] and distinct from the Class II FBP aldolase of *E. coli*. Like other Class I FBP aldolases, the *E. coli* enzyme could also utilize FIP, albeit at a much lower rate than with FBP (Table 3).

Baldwin and Perham [6] demonstrated that the FBP-cleavage activity of the *E. coli* Class I FBP aldolase was greatly enhanced

Table 3 FBP-cleavage kinetics of the wild-type and mutant *E. coli* FBP aldolases

Assays were carried out as described in the Experimental section both in the presence and in the absence of 10 mM citrate. Kinetic parameters were determined by non-linear regression analysis [26] and protein concentration measured by the method of Bradford [27]. Abbreviations: ND, not determined; NM, not measurable.

	FBP				F1P	
	Minus citrate		Plus citrate		Minus citrate	
	K_m (mM)	k_{cat} (min ⁻¹)	K_m (mM)	k_{cat} (min ⁻¹)	K_m (mM)	k_{cat} (min ⁻¹)
Class I FBP aldolase	0.02 ± 0.002	13 ± 0.4	0.07 ± 0.012	190 ± 9.5	33 ± 6	7 ± 0.8
Class II FBP aldolase*	0.19 ± 0.03	490 ± 26	ND	ND	ND	ND
K237A mutant Class I FBP aldolase	NM	NM	0.06 ± 0.02	0.52 ± 0.004	NM	NM
K239A mutant Class I FBP aldolase	0.27 ± 0.05	13 ± 0.62	6.3 ± 0.5	230 ± 10	24 ± 6	6 ± 1

* Values taken from [22].

in the presence of citrate. Using saturating concentrations of 10 mM citrate [6], the k_{cat} of the purified protein was increased approx. 15-fold (to 190 min⁻¹), whereas the K_m remained largely unchanged (Table 3). This enhancement is consistent with that described for the Class I FBP aldolase [6]. The presence of citrate did not affect the F1P-cleavage activity of the enzyme (Table 3).

NaBH₄ treatment

Class I FBP aldolases are characterized by formation of a Schiff-base intermediate between FBP or DHAP and the enzyme as part of the reaction mechanism. The overproduced purified protein was also shown to behave in this manner, since the enzyme activity fell to 0.7% of its initial value following treatment with NaBH₄ in the presence of DHAP, whereas it was unaffected by treatment with NaBH₄ alone, losing only 7% of its initial activity. In contrast, activity was retained even in the presence of concentrations of EDTA of up to 0.5 M, showing that the enzyme did not depend on metal ions for activity and thus distinguishing it from Class II FBP aldolases.

Native molecular mass

Previous estimations have indicated that the *E. coli* Class I FBP aldolase is either a tetramer with a molecular mass of between 140 and 160 kDa [7,34] or a decamer with a molecular mass of approx. 340 kDa [6]. Gel filtration of the purified protein was in agreement with the latter, with an estimated molecular mass of 330 ± 10 kDa. Sedimentation-equilibrium measurements carried out on the overproduced protein indicated a single sedimenting species with a native molecular mass of 340 kDa (Figure 3) and a sedimentation coefficient ($s_{20,w}$) of 14.7 × 10⁻¹³ s⁻¹ (S). The molecular-mass value obtained from sedimentation-equilibrium experiments was independent of protein concentration over a starting concentration range of 0.24–4.0 mg/ml. Given the subunit molecular mass of 37978 Da calculated from the corrected gene sequence after removal of the N-terminal methionine, the molecular-mass value from sedimentation equilibrium indicates a stoichiometry of approx. 8.9 subunits in the native complex. This ratio is lower than the value expected for a decamer, as previously reported for the Class I FBP aldolase from the halophilic archaeobacterium *Haloarcula vallismortis* [16], and, excluding an unlikely arrangement of nine subunits, suggests the enzyme may be an octamer of identical subunits (perhaps related to the tetrameric eukaryotic aldolases but associated as a dimer

of tetramers). The high molecular-mass value determined for the enzyme compared with the value predicted for an octamer (303824 Da) may be due to the uncertainty in the value used for the partial specific volume. The molecular mass and sedimentation coefficient determined for the enzyme yields an axial ratio of 1.7 assuming a prolate ellipsoid [30].

Taken together, these data indicate that the protein overproduced in these studies has all of the characteristics expected of a Class I FBP aldolase and clearly distinguish it from the previously cloned and overproduced Class II FBP aldolase of *E. coli* [19]. The gene cloned, previously identified as the *dhnA* gene encoding a dehydrin, therefore encodes the *E. coli* Class I FBP aldolase. However, there remains a possibility that the Class I FBP aldolase is up-regulated in *E. coli* in response to water drought stress, thus accounting for its previous identification as a dehydrin [31].

Sequence comparisons

The *E. coli* Class I FBP aldolase appears to be a unique enzyme with respect to both its primary amino acid sequence and properties. No significant identity exists between the Class I and Class II FBP aldolases, leading to the suggestion that the two classes have evolved independently [18]. However, comparisons between the classes have been complicated because FBP aldolase sequences from different species were being compared. The identification of the *E. coli* Class I gene along with the previously known *E. coli* Class II sequence [19] means that the complete sequence of both classes of FBP aldolase from the same organism are known. No significant identity (16%) is found between the two *E. coli* enzymes, adding weight to the theory of independent evolution. A more interesting comparison is to examine the homology between the eukaryotic and prokaryotic Class I FBP aldolases. Although these enzymes share a similar mechanism, there is little to suggest that the enzymes are closely related. While the identity between eukaryotic FBP aldolases is at least 50% [41], the identity between the bacterial and eukaryotic Class I FBP aldolases is much lower, namely between 17 and 28%. The size and multimeric nature of the prokaryotic Class I FBP aldolases also vary dramatically, ranging from a high-order multimer of approx. 340 kDa in the case of the *E. coli* Class I enzyme to a monomer of 33 kDa for the *Staph. aureus* enzyme [13]. None of the prokaryotic Class I enzymes, including the cloned *E. coli* FBP aldolase (Figure 2), contains the conserved C-

Figure 3 Sedimentation-equilibrium distribution of FBP aldolase

The absorbance at 280 nm as a function of radial distance was obtained after centrifugation at 6000 rev./min for 16 h at 20 °C. The initial A_{280} was 0.6 and the solution conditions were 50 mM potassium phosphate buffer, pH 7.0.

*		
<i>E. coli</i>	221	-GQANHLAAT IGADIVKQKM AENN
<i>S. carnosus</i>	197	KAELDNL-KK DQYVMLKLTl PTKV
<i>S. aureus</i>		KGLASEQ-DD VQ-VMLKPIN LTDE
<i>Synechocystis</i>	203	LKHLNQL-PK GQWVMLKLTl PEQD
Human A	214	KALSDHH-IY LEGTLLKPNM VTPG

Figure 4 Sequence alignment around the Schiff-base lysine of the Class I FBP aldolases

The region of the primary sequence alignment [carried out using the (University of Wisconsin) Genetics Computer Group (GCG) program GAP [48] using a gap weight of 3.0 and a gap length of 0.1] around the active-site lysine (Lys²³⁶) of the *E. coli* Class I FBP aldolase. For references to sequences, see the text.

terminal tyrosine residue found in vertebrate aldolases, and their kinetic properties are all unaffected by carboxypeptidase A treatment [9,14,16,42]. Despite such differences one might expect that conservation around the essential Schiff-base lysine residue in both eukaryotic and prokaryotic Class I FBP aldolases would be high. However, sequence alignment of the active-site region (see below; Figure 4), reveals that the sequences in this area are

Figure 5 Phylogenetic analysis of the Class I and Class II FBP aldolases

Phylogenetic tree of known Class I and Class II FBP aldolases was created using the GCG program GROWTREE [48].

generally varied. The arrangement of hydrophobic and hydrophilic residues around the Schiff-base lysine are, however, perhaps suggestive of a common fold which has been conserved throughout active-site evolution.

The relationship of the *E. coli* Class I FBP aldolase with other FBP aldolases is shown in a phylogenetic analysis of all the known sequences (Figure 5). One may speculate that, whereas at least two subclasses of the Class II FBP aldolases exist, there are at least three subclasses of Class I FBP aldolases comprising: (i) the eukaryotes; (ii) the prokaryotes typified by *Staph. carnosus* and *Synechocystis* and (iii) the prokaryotes typified by *E. coli*. It will be of interest to determine whether any homologues of the *E. coli* Class I FBP aldolase exist to add to this putative subclassification. It also remains to be determined whether this protein adopts an α/β -barrel fold found in all other known FBP aldolases. Attempts to model the Class I *E. coli* FBP aldolase on the known crystal structure of the *Drosophila* enzyme [4] using the threading program Swiss-Model [43] proved unsuccessful, although the *Staph. carnosus* Class I FBP aldolase could be modelled using this program. This may be due to the fact that the *E. coli* enzyme has an unusual multimeric structure.

The general overall lack of identity of the *E. coli* Class I FBP aldolase with the other FBP aldolases may also be, in part, reflected in its unusual properties. Although the deoxyribose 5-phosphate aldolases from both rat liver and *Lactobacillus plantarum* [44,45] are activated by citrate, only the *E. coli* Class I FBP aldolase is activated by citrate [6]. Rosen et al. [46] have

Figure 6 HPLC separation of the labelled active-site peptide

A sample of the Class I FBP aldolase, labelled at the active site with NaB^3H_4 in the presence of DHAP, was cleaved with CNBr and the resulting peptides were separated on a Poros R2 reverse-phase column on a BioCAD Sprint Chromatography system using (A) an acetonitrile/water/TFA buffer system, and the labelled peptide (*) was further purified using (B) an acetonitrile/ NH_4HCO_3 system (see the Experimental section).

proposed that carboxylic acids are important for the formation of the enzyme-bound substrate carbanion in the deoxyribose 5-phosphate aldolases, and it is possible that citrate performs a similar role in the *E. coli* Class I FBP aldolase. However, the precise effect of citrate on the FBP aldolase and its metabolic significance remain unclear.

Identification of the Schiff-base-forming lysine

Isolation of the active-site peptide

Modification of the Class I FBP aldolase with NaB^3H_4 in the presence of DHAP led to a loss of 99% of the enzyme activity. The labelled enzyme was subjected to an exhaustive CNBr digestion, and the resulting mixture of peptides was fractionated on a Poros R2 reverse-phase column. The elution profile is shown in Figure 6(A). The major peak of radioactivity, eluted after 17–18 min, was collected. Several peptide species were present in this fraction (as determined by MALDI-TOF MS), and therefore an additional chromatography run was performed to isolate the labelled peptide from the contaminants. Using a solvent system more basic than that used previously, further separation was achieved and a single peak of radioactivity was eluted after 30–31 min (Figure 6B).

ESI MS identified two peptides within this fraction with molecular masses of 3752.0 and 5139.4 Da, with the latter being

the major component. These masses correspond to the expected masses of the CNBr fragments of the Class I FBP aldolase sequence from residues 159–193 (3753.2 Da) and residues 194–239 covalently modified with DHAP (5141.7 Da). The first of these peptides (159–193) contains no lysine residues and has the expected mass, and thus can be ruled out as the active-site-containing peptide. The second, modified peptide contains four lysine residues (Lys¹⁹⁷, Lys¹⁹⁸, Lys²³⁶ and Lys²³⁸), any of which might be the active-site Schiff-base-forming residue. Amino acid sequencing from the N-terminus of this peptide identified the first two lysine residues (Lys¹⁹⁷ and Lys¹⁹⁸) as unmodified, thus ruling out both as the site of modification by DHAP. Unfortunately the successive yields during the N-terminal sequencing of the peptide were such that we could not distinguish the modified residue by this means. We therefore used a combination of molecular biology, enzyme kinetics and ESI MS to identify the active-site lysine residue.

Site-directed mutagenesis of Lys²³⁶ and Lys²³⁸

Two mutants, K236A and K238A, were created by PCR using mutagenic primers, and the proteins were purified to homogeneity. The purified mutant proteins were identical with the wild-type enzyme with respect to oligomeric arrangement, as judged by gel filtration, and secondary structure, as judged by CD (results not shown). The kinetic parameters of the two mutants were measured and are shown in Table 3.

The FBP-cleavage activity of the K236A mutant was not measurable in the absence of citrate, even when up to 1.7 mg of protein was used per assay. In the presence of citrate a low enzyme rate was measurable. Although the K_m was unaffected, the k_{cat} of the mutant was vastly decreased compared with the wild-type when assayed under the same conditions, decreasing from 194 min^{-1} to 0.5 min^{-1} (Table 3). These data indicate that Lys²³⁶ is critically involved in enzyme catalysis. In contrast the k_{cat} of the K238A mutant was virtually identical with that of the wild-type enzyme, whether in the presence or absence of citrate. Treatment with NaBH_4 in the presence of DHAP showed a complete inactivation of this enzyme (K238A), indicating that the Schiff base could still be formed in this mutant. ESI MS analysis of the K236A and K238A mutant proteins after treatment with NaBH_4 in the presence of DHAP indicated that a covalent modification of the enzyme was only formed in the K238A mutant (Table 2). This modification resulted in the mass difference expected for a protein containing a reduced Schiff base of DHAP (154 Da). Under the same conditions, the mass of the K236A mutant after NaBH_4 trapping was unchanged (Table 2). Taken together, these data indicate that Lys²³⁶, and not Lys²³⁸, is the Schiff-base-forming lysine in *E. coli* Class I FBP aldolase.

It is noteworthy that, whereas there was no change in the k_{cat} of the K238A mutant, the K_m value for FBP was drastically altered compared with the wild-type enzyme. A 13-fold increase in K_m in the absence of citrate and a 90-fold increase in its presence suggests that this residue is located in the substrate-binding region of the enzyme. Morris et al. [47] have proposed that the rabbit muscle Class I FBP aldolase requires a pair of spatially adjacent lysine residues (Lys¹⁴⁶ and Lys²²⁹) for efficient catalysis. The Schiff-base-forming lysine residue (Lys²²⁹) is located on a β -strand pointing towards the centre of the β -barrel [3–5] and Lys¹⁴⁶ is charged and effectively lowers the $\text{p}K_a$ of Lys²²⁹, so increasing the concentration of the nucleophile for Schiff-base formation. If Lys²³⁶ and Lys²³⁸ in the *E. coli* Class I enzyme lie in a β -sheet, it is possible that Lys²³⁸ plays a role analogous to Lys¹⁴⁶. However, the finding that the K238A mutation did not affect the rate of catalysis, but instead increased

the K_m for FBP, especially in the presence of citrate, suggests rather that Lys²³⁸ is playing a part in recognizing and binding the substrate. Interestingly, the K238A mutant had virtually identical kinetic constants for F1P cleavage as the wild-type enzyme (Table 3). This suggests that Lys²³⁸ plays a role in the binding of the C-6 phosphate of the natural substrate, FBP. The precise role for Lys²³⁸ in the mechanism of the Class I FBP aldolase will have to await X-ray-crystallographic analysis of the enzyme.

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