Volume 318, number 1, 11–16 FEBS 12115 February 1993

Identification of zinc-binding ligands in the Class II fructose-1,6-bisphosphate aldolase of *Escherichia coli*

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Received 7 December 1992; revised version received 8 January 1993

An expression and mutagenesis system for the *E. coli* Class II fructose-1,6-bisphosphate aldolase has been created by modification of the vector pKfda (Biochem. J. 257 (1989) 529–534). Large amounts of Class II aldolase (about 1 g/l in crude extracts), with properties consistent with those previously reported for the naturally occurring enzyme (Biochem. J. 169 (1978) 633–641) are obtained. The enzyme contains 2 zinc ions per enzyme dimer. We have investigated the nature of the zinc-binding site of the enzyme by site-directed mutagenesis. His-108, His-111, Cys-112 and His-142 were identified as possible zinc-binding ligands by sequence alignments and comparisons with other known zinc-containing enzymes. Mutation of these residues identified His-108 and His-111 as two of the ligands directly responsible for the tight binding of zinc. Mutation of the other two residues results in only a small effect on the amount of zinc bound per monomer and a corresponding change in specific activity. These residues are, therefore, unlikely to be directly involved in zinc binding, but may be indirectly involved in some manner in the zinc-binding environment.

Aldolase; Zinc binding; Protein engineering

1. INTRODUCTION

Fructose-1,6-bisphosphate aldolase (FBP-aldolase) catalyses the reversible aldol cleavage of fructose 1,6-bisphosphate into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Two classes of this enzyme, designated Class I and Class II, are recognised based on their catalytic and structural properties [1,2]. The catalytic mechanism of the Class I aldolases has been extensively studied [3,4]. The reaction proceeds via a carbanion intermediate which is stabilised by a protonated Schiff base formed between the substrate C-2 carbonyl and the N6-amino group of an essential lysine residue in the active site. On the other hand, the Class II aldolases utilise a divalent metal ion in an apparently similar catalytic mechanism [5].

X-ray structures of the Class I fructose-1,6-bisphosphate aldolases from several sources [6–10], and the phosphogluconate aldolase from *Pseudomonas putida* [11,12] have been determined. The Class I aldolases are, in general, tetramers of molecular weight around 160,000 Da. The molecular architecture of these enzymes is that of an eight-stranded α/β barrel [13].

In contrast to the wealth of structural information available for the Class I aldolases, comparatively little is known about the Class II enzymes. They are dimers of molecular weight around 80,000 Da and contain 2 zinc atoms per dimer, but no detailed structural information is available for any Class II aldolase. Unlike the Class I enzymes, the Class II aldolases do not utilise Schiff base formation in catalysis; instead, the zinc atoms perform the essential catalytic role [14]. In this paper we describe the overexpression, purification and characterisation of the wild-type Class II aldolase from *E. coli*. In addition, by site-directed mutagenesis of histidine and cysteine residues, the identity of two of the zinc-binding ligands of the protein are determined and the role of a further histidine and cysteine residue conserved in various Class II aldolases has been investigated.

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2. EXPERIMENTAL

2.1. *Materials*

[^{35}S]dATP-αS triethylammonium salt (400 Ci/mmol) for DNA sequencing was supplied by Amersham International. The mixture of glycerol-3-phosphate dehydrogenase and triose phosphate isomerase for the assay of aldolase and calf intestinal alkaline phosphatase were obtained from the Boehringer Mannheim GmbH, Germany. Restriction enzymes, T4 DNA ligase and T4 polynucleotide kinase were from Pharmacia. Buffers were treated by passage through a column of Chelating resin (Sigma Chemical Co.) whenever metal-free buffers were required. The plasmid pKfda was as described by Alefounder et al. [15], and was kindly obtained from Prof. R.N. Perham (University of Cambridge).

E. coli strain TG1 [K12, *Δ(lac-pro)*, *sup* E, *thi*, *hsd* D5, /F′ *tra* D36, *pro* A$^+$B$^+$, *lac* I^q, *lac* Z *Δ*M15] was from Amersham International. *E. coli* strain HB2151 [K12, *ara*, *Δ(lac-pro)*, *sup* E, *thi*, /F *pro* A$^+$B$^+$, *lac* I^q, *lac* Z *Δ*M15] was a gift from Dr G. Winter, MRC Laboratory of Molecular Biology, Cambridge. *E coli* strain JM2087 [*Δ(his gnd)*, *Δlac*,

ara D, *fda*, *pts* F, *pts* M, *rps*L] was as previously described [16] and was a gift from Dr M.C. Jones-Mortimer (University of Cambridge).

2.2. *Site-directed mutagenesis, DNA sequencing and plasmid construction*

Site-directed mutagenesis was carried out on M13fda or M13fda2 (see below). Mutants were constructed using the phosphorothioate method [17]. Putative mutants were screened directly by dideoxy sequencing. The mutated gene was resequenced to ensure that no spurious mutations had been introduced. Plasmid and bacteriophage RF DNA was prepared as described by Maniatis et al. [18]. The mutated gene was isolated by restricting bacteriophage RF DNA with *Eco*RI and *Hin*dIII, and the *fda* gene fragment was subcloned into the expression vector pKK223-3 restricted with the same enzymes.

2.3 *Construction of the expression/mutagenesis system*

The construction of the expression vector pKfda2 is summarised in Fig. 1. The 1139 bp *Eco*RI/*Bam*HI fragment from pKfda [15] containing the *fda* gene was subcloned into bacteriophage M13mp19. Site-directed mutagenesis of the resulting bacteriophage (M13fda) was used to alter the codon for Glu-308 from GAA to GAG, thus destroying a *Hin*dIII site found within the gene. The resultant recombinant bacteriophage was designated M13fda2. Subsequent mutations were introduced into M13fda2 as described above. The *fda* gene was excised from M13fda2 on a 1169 bp *Eco*RI/*Hin*dIII fragment and ligated directionally into the expression vector pKK223-3 previously cut with *Eco*RI and *Hin*dIII to yield pKfda2 (Fig. 1).

E. coli strain KM3, a *lac* I^q and *fda*$^-$ strain was constructed by first converting strain JM2087 (*fda*$^-$) into a proline auxotroph (strain KM1) by ampicillin enrichment [19] and then making the strain resistant to nalidixic acid (strain KM2) by selecting for growth on nalidixic acid containing (20 μg/ml) agar plates. Finally, an F′ episome encoding the *lac* I^q gene and the *pro* AB locus from *E. coli* strain HB2151 was introduced by conjugation, yielding strain KM3. Strain KM3 was shown directly to lack fructose-1,6-bisphosphate aldolase by appropriate enzyme assay.

2.4. *Purification of Class II FBP-aldolase*

pKfda2 transformed *E. coli* strain KM3 cells, grown in the presence of 0.3 mM $ZnCl_2$ and induced with 2 mM IPTG, were suspended in 50 mM potassium phosphate buffer pH 7.0 and disrupted in a French Press at 4°C and cell pressure of 140 MPa. After centrifugation (14,000 × *g*, 1 h), the 40–80%-saturation $(NH_4)_2SO_4$ precipitate, which contained the enzyme activity, was collected and dissolved in 50 mM Tris-HCl buffer pH 8.0 and was dialysed against the same buffer. The enzyme was then applied to a DE52 column and, after washing with 80 mM NaCl, the enzyme was eluted with 120 mM NaCl in the same buffer. Fractions containing the aldolase were pooled and concentrated by precipitation with 80% saturation $(NH_4)_2SO_4$. After dialysis against 100 mM Tris-HCl buffer pH 8.0 the enzyme was applied to a Superdex 200 column equilibrated with 100 mM Tris-HCl buffer pH 8.0. The active peak fractions were pooled and the protein was judged to be homogeneous by SDS-PAGE.

2.5. *Other methods*

The native M_r of the overexpressed *E. coli* Class II FBP-aldolase was determined by gel-filtration FPLC on a Pharmacia Superose 12 column in 50 mM potassium phosphate buffer pH 7.5. The subunit M_r was estimated by SDS-PAGE on 10% polyacrylamide slab gels [20].

A coupled enzymic assay was used for FBP-aldolase [21]. The decrease in A_{340} in an assay containing fructose 1,6-bisphosphate (3 mM), NADH (20 mM), coupling enzymes (2 μl of a 10 mg/ml mixture of glycerol phosphate dehydrogenase/triose phosphate isomerase) and a suitable aliquot of the aldolase was measured in 50 mM Tris-HCl buffer pH 8.0 at 30°C. One unit of enzyme activity is defined as that causing the oxidation of 2 μmol of NADH/min in this assay. Kinetic parameters were estimated by non-linear regression analysis [22]. Protein concentration was determined by the method of Bradford [23].

Zinc was determined by atomic absorption spectrometry using a Perkin-Elmer model 380 atomic absorption spectrometer. Absorbance peak areas were measured at 213.9 nm. All values are the averages of at least 10 readings. Dilutions were made with metal-free 100 mM Tris-HCl buffer, pH 8.0. The detection limit was about 0.5×10^{-5} M zinc.

3. RESULTS AND DISCUSSION

3.1. *Overexpression, purification and characterisation of the Class II FBP-aldolase from* E. coli

The original overexpression system for the *E. coli fda* gene (pKfda) [15] has been adapted to allow repeated directional subcloning between an M13 based mutagenesis vector and the pKK223-3 based expression vector (Fig. 1). The new expression vector, pKfda2, can be efficiently transformed into the desirable *fda*$^-$ strain of *E. coli*, KM3 and the aldolase is expressed at extremely high levels. The expression of the wild-type and mutant Class II aldolases was routinely carried out in cells grown in the presence of 0.3 mM $ZnCl_2$ to avoid the formation of inactive apoenzyme.

The purified wild-type enzyme had the expected specific activity of 6.1 U/mg and analysis by SDS-PAGE showed the preparation to be homogeneous. The amino acid composition of the purified enzyme was, within error, identical to that of the naturally expressed *E. coli* enzyme [24] and with that expected from the DNA sequence of the *fda* gene [15]. The N-terminal sequence, Ser-Lys-Ile-Phe-Asp, matched exactly the predicted sequence except that the N-terminal methionine was absent. Thus, despite the enormous overexpression, the enzyme is still efficiently processed in a manner identical to that of the naturally expressed protein [24]. The native and subunit molecular weights of the overexpressed aldolase were determined as 78,000 Da and 39,000 Da, respectively, in excellent agreement with the predicted value of 39,016 Da and confirming that the overexpressed protein correctly assembles into a dimer of identical subunits [14,24,25]. The K_m of the purified enzyme for fructose 1,6-bisphosphate was estimated to be 240 μM and agrees closely with that previously reported for the *E. coli* and other Class II enzymes [24,25]. Stimulation (2- to 35-fold) of the activity of Class II aldolases in Tris buffers by ca. 100 mM potassium ions has been reported [25]. As expected, at pH 8.0 in 50 mM Tris-HCl, the addition of 100 mM KCl stimulated the overexpressed enzyme about threefold. Under these conditions, there is no significant change in the K_m for FBP (Table I).

3.2. *Measurement of zinc and identification of the zinc-binding ligands*

The zinc content of the overexpressed enzyme, measured after gel filtration of the enzyme in metal-free buffer, was 2.3 ± 0.2 zinc atoms per enzyme dimer (assuming a subunit molecular weight of 39,000 Da) (Table

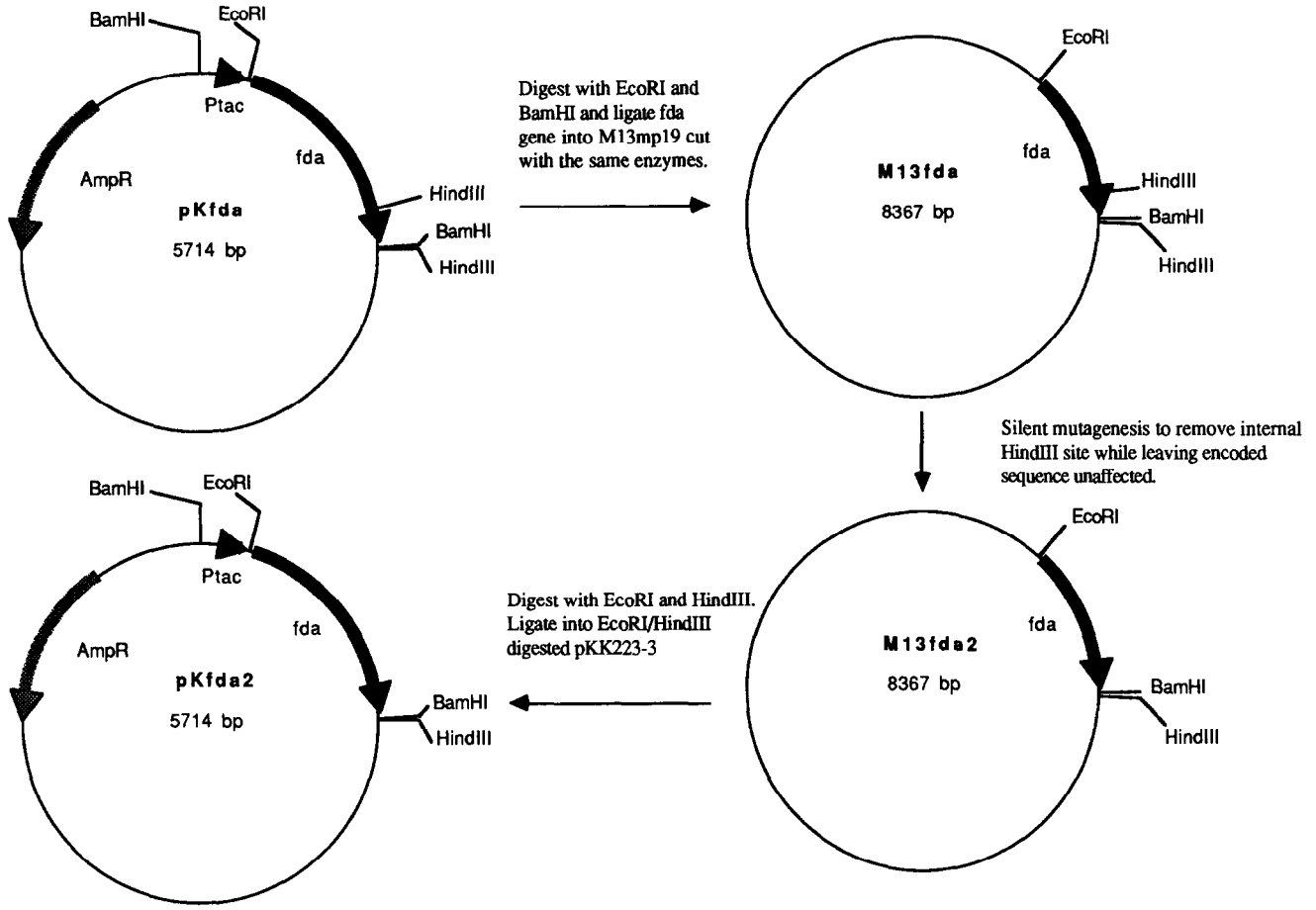

Fig. 1. The construction of the expression vector pKfda2. The *fda*-containing fragment from pKfda [15] was subcloned into M13mp19 RF DNA. Site directed mutagenesis was used to remove the internal *Hin*dIII site, and the *fda* gene was cloned into pKK223-3 to yield the expression vector pKfda2.

I), confirming that the holoenzyme contains a full complement of zinc ions.

Comparison of the structures of a number of zinc-containing enzymes has revealed a zinc-binding motif [26]. In enzymes where zinc fulfills a catalytic role, the metal is usually tetrahedrally coordinated to 3 amino acid ligands and a water molecule. The binding motif consists of two amino acid ligands, L_1 and L_2, separated

Table I

Kinetic parameters and zinc content of wild-type and mutant *E. coli* Class II FBP-aldolases

Enzyme	Buffer/Additions	Specific activity (U/mg)	Fold stimulation with K^+	K_m FBP (μM)	k_{cat} (min^{-1})	Zn ions per enzyme dimer
Wild-type	50 mM Tris-HCl pH 8.0	6.1	–	240 ± 30	620 ± 18	2.3 ± 0.2
Wild-type	Ditto + 100 mM potassium acetate	20.6	3.4	290 ± 40	1,820 ± 50	–
H108A	50 mM Tris-HCl pH 8.0	N.D.	–	–	–	0.13 ± 0.01
H111A	Ditto	N.D.	–	–	–	0.15 ± 0.01
C112A	Ditto	1.9	–	260 ± 30	240 ± 12	0.73 ± 0.06
H142A	Ditto	4.7	–	350 ± 17	330 ± 6	1.78 ± 0.1

The initial rates of reaction at various concentration of fructose 1,6-bisphosphate were measured by following the decrease in A_{340} in a coupled assay system [21]. The kinetic parameters were estimated by non-linear regression analysis [22]. Zinc, protein concentration and enzyme activity were all determined as described in section 2. The zinc content per dimer was calculated based on a molecular weight of the enzyme subunit of 39,016 Da. N.D., not detectable.

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                    1                                                  50
E.coli               MSKIFDFVK PGVITGDDVQ KVFQVAKENN FALPAVNCVG TDSINAVLET
Yeast               MGVEQILKRK TGVIVGEDVH NLFTYAKEHK FAIPAINVTS SSTAVAALEA
C.glutamicum                   PIATPEVYN EMLDRAKEGG FAFPAINCTS SETINAALKG

                    51                                                 100
E.coli              AAKVKAPVIV QFSNGGASFI AGKGVKSDVP QGAAILGAIS GAHHVHQMAE
Yeast               ARDSKSPIIL QTSNGGAAYF AGKGI.SNEG QNASIKGAIA AAHYIRSIAP
C.glutamicum        FAEAESDGII QFSTGGAEFG SGLAVKNKVK ......GAVA LAAFAHEAAK

                    101                                                150
E.coli              HYGVPVILHT DHCAKKLL.P WIDGLLDAGE KHFAATGKPL FSSHMIDLSE
Yeast               AYGIPVVLHS DHCAKKLL.P WFDGMLEADE AYFKEHGEPL FSSHMLDLSE
C.glutamicum        SYGINVALHT DHCQKEVLDE YVRPLLAISQ ERVDRGELPL FQSHMWDGSA
                          =====                                    ====

                    151                                                200
E.coli              ESLQENIEIC SKYLERMSKI GMTLEIELGC TGGEEDGVDN SHMDASALYT
Yeast               ETDEENISTC VKYFKRMAAM DQWLEMEIGI TGGEEDGVNN ENADKEDLYT
C.glutamicum        VPIDENLEIA QELLAKAKAA NIILEVEIGV VGGEEDGVEA ..KAGANLYT
                                          ====     ===

                    201                                                250
E.coli              QPEDVDYAYT ELSK.ISPRF TIAASFGNVH GVYKPGNVVL TPTILRDSQE
Yeast               KPEQVYNVYK AL.HPISPNF SIAAAFGNCH GLY.AGDIAL RPEILAEHQK
C.glutamicum        SPEDFEKTID AIGTGEKGRY LLAATFGNVH GVYKPGNVKL RPEVLLEGQQ

                    251                                                300
E.coli              YVSKKHN.LP HN.SLNFVFH GGSGSTAQEI KDSVSYGVVK MNIDTDTQWA
Yeast               YTREQVGC.K EEKPLFLVFH GGSGSTVQEF HTGIDNGVVK VNLDTDCQYA
C.glutamicum        VARKKLGLAD DALPFDFVFH GGSGSEKEKI EEALTYGVIK MNVDTDTHYA
                                                                   ===

                    301                                                350
E.coli               TWEGVLNYY KANEAYLQGQ LGNPKGEDQP NKKYYDPRVW LRAGQTSMIA
Yeast               Y.LTGIRDYV LNKKDYIMSP VGNPEGPEKP NKKFFDPRVW VREGEKTMGA
C.glutamicum        FTRPIVSHMF ENYNGVLK.. ....IDGEVG NKKAYDPRSY MKKAEQSMSE

                    351            368
E.coli              RLEKAFQELN AIDVL...
Yeast               KITKSLETFR TTNTL...
C.glutamicum        RIIESCQDLK SVGKTTSK
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Fig. 2. Alignment of the primary sequences of the Class II FBP-aldolases from *E. coli*, *C. glutamicum* and yeast. The primary sequences for the fructose-1,6-bisphosphate aldolases from *E. coli* [15], *S. cerevisiae* [32] and *C glutamicum* [33] were aligned using the computer program GAP [34] with a gap weight of 3.0 and a gap length weight of 0.1. The five regions of the proteins where conserved histidine, cysteine, glutamic acid or aspartic acid residues are found in positions which align with the observed L_1, L_2, L_3 ligand pattern [26] and hence might act as the zinc-binding ligands are shown underlined.

by 1–3 amino acids and a third amino acid ligand, L_3, separated from the other two by either about 20 or about 120 amino acids. The predominant amino acid ligand in these enzymes is histidine, with much lower frequencies of glutamic acid, aspartic acid and cysteine. We used the L_1, L_2, L_3 pattern described above to search for a potential zinc-binding site in the *E. coli* Class II aldolase. This approach has been successfully used even when, as in this case, the degree of identity between the primary sequences of the unknown structure and any of the known ones is low (<20%) [27]. Residues important in zinc binding were presumed to be conserved in the Class II aldolases and the search therefore focussed on conserved histidine, cysteine, glutamic and aspartic acid residues. An alignment of the primary sequences of the Class II aldolases from *E. coli*, *C. glutamicum* and *S. cerevisiae* is shown in Fig. 2. There are five regions where conserved residues are suitably positioned to act as the L_1 and L_2 ligands (Fig. 2). Only two of these regions contain histidine, the most likely of which would be that encompassing residues 108 and 111 since it uniquely contains two conserved histidine residues. This section of polypeptide (His-Thr-Asp-His-Cys) also includes the only conserved cysteine residue (Fig. 2). In addition, 32 residues nearer the C-terminus there is a third conserved histidine residue (His-142) which could act as L_3. Residues His-108, His-111, Cys-112 and His-142 were, therefore, each changed to alanine.

All the mutant proteins were expressed in *E. coli* strain KM3 and purified as described for the wild-type enzyme. All were dimeric proteins with the correct subunit molecular weights. The close similarity of the far UV CD spectra of the mutant and wild-type proteins (Fig. 3) indicates that no major structural changes result from the mutations. The mutants H108A and H111A contained very low levels of zinc and were virtually inactive (Table I). Addition of an excess of zinc (up to 100-fold) did not result in an increase in activity. Clearly His-108 and His-111 are two of the zinc-binding ligands. The concomitant loss of enzyme activity and zinc content in these mutants confirms the essential role of zinc in catalysis. By contrast, the contribution of zinc

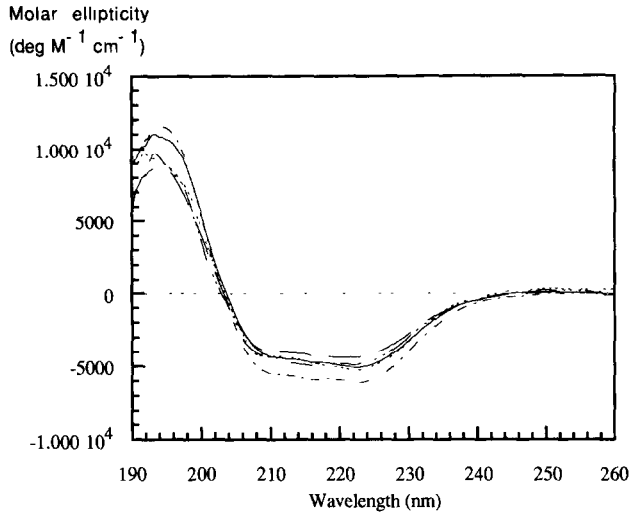

Fig. 3. Far UV Circular dichroism spectra of wild-type and mutant Class II aldolases. The far UV circular dichroism spectra of wild-type and mutant Class II FBP-aldolases (ca. 1 mg/ml) in 20 mM potassium phosphate buffer pH 7.6 were measured on a Jobin Yvon CD6 instrument in a cell with pathlength 0.1 mm. Spectra were smoothed using the Sabitzky Golay algorithm using a sliding average over 7.5 nm. The exact concentration of each enzyme was determined by amino acid analysis. Wild-type (—); H108A mutant (– – –); H111A mutant (–·–·–); C112A mutant (–···–); H142A mutant (·····).

to the maintenance of tertiary structure must be very limited (Fig. 3). Such a His-X-X-His motif is also found in the related Class II enzyme fuculose-1-phosphate aldolase [28] and in the *cfx*A gene tentatively identified as a Class II FBP-aldolase in *Rhodobacter sphaeroides* [29] and should be a strong candidate for zinc binding in these enzymes.

In contrast to mutations H108A and H111A, the mutant H142A was found to have a specific activity of 4.7 units/mg protein and a zinc content of 1.78 zinc ions per dimer (Table I). Substituting His-142 by alanine had little effect on the kinetic parameters of the enzyme (Table I). On transfer of this enzyme into metal-free buffer, the enzyme activity and the zinc content fell to almost zero. Addition of excess zinc to this inactive enzyme resulted in restoration of the original specific activity and a maximum zinc content of 1.8 ions per dimer. His-142, therefore is unlikely to be the third zinc-binding ligand. It may be, however, that His-142 is a weak, but direct ligand to the zinc ion, alternatively, the effect of its mutation might be via a local change in the zinc-binding site.

It has previously been suggested [30,31] that cysteine groups might be important for zinc-binding in the yeast Class II aldolase. Only one cysteine residue, Cys-112, is conserved in the three Class II enzymes (Fig. 2) and thus this residue was mutated to an alanine residue. The resulting protein (C112A) had a specific activity of only 1.9 U/mg, about 30% of the wild-type activity (Table I). Similarly, a zinc content of 0.7 Zn per dimer (Table I) was observed. The addition of excess zinc (up to 100-fold) did not result in any increase in activity. Mutation of Cys-112 does not, therefore, totally abolish zinc binding as would be expected for a direct metal-binding ligand [27]. The kinetic parameters for this enzyme were also similar to those of the wild-type enzyme (Table I). The lower zinc content in this mutant enzyme may result from the close proximity of Cys-112 to His-111, a residue already identified as a ligand to the zinc ion (see above). Mutation of such a close neighbour could cause localised changes resulting in a more unfavourable environment for zinc binding. That any such changes cannot be a consequence of major changes in structure can be seen from the similarity of the far UV CD spectrum of the C112A mutant to the wild-type enzyme (Fig. 3).

The present study has shown that the pKfda2 expression system can be used efficiently to produce large amounts of the *E. coli* FBP-aldolase. By sequence alignments, putative zinc ligands in the enzyme were pinpointed (Fig. 2) and have been individually mutated. Two ligands, His-108 and His-111, have been identified unequivocally as zinc-binding ligands. Residue His-142, which is at an appropriate distance in the primary sequence to act as the L_3 ligand [26] does not appear to be a direct zinc-binding ligand. Similarly, the only conserved cysteine residue, Cys-112, does not appear to be directly involved in zinc binding. Mutation of these residues may disrupt the environment of the zinc-binding site without their being direct ligands. The role of the other 2 conserved histidine residues (Fig. 2) and the identity of the L_3 ligand in the *E. coli* Class II aldolase will have to await further site-directed mutagenesis experiments.

Acknowledgements: We thank Mrs Z.J. Jacoby, Mr M. Wheldon and Dr J. McCormick of the Wellcome Trust/SERC-funded protein and nucleic acid facility (University of Cambridge) for amino acid analysis, oligonucleotide synthesis and N-terminal sequence analysis and Dr. S.E. Radford for critical reading of this manuscript. This work was supported by the Royal Society. We thank the Science and Engineering Research Council for a Research Studentship (to K.E.M.). A.B. is a Royal Society 1983 University Research Fellow.

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