

## Initiating a Crystallographic Study of a Class II Fructose-1,6-bisphosphate Aldolase

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We have reproducibly crystallized the metal-dependent Class II fructose-1,6-bisphosphate aldolase from *Escherichia coli*. Crystals in the shape of truncated hexagonal bipyramids have unit cell dimensions of  $a = b = 78.4 \text{ \AA}$ ,  $c = 290.6 \text{ \AA}$  and are suitable for a detailed structural analysis. The space group has been identified as  $P6_122$  or enantiomorph. Data sets to approximately  $2.9 \text{ \AA}$  resolution have been recorded using both the Rigaku R-AXIS IIc image plate area detector coupled to a copper target rotating anode X-ray source and using the MAR image plate systems with synchrotron radiation at the EMBL outstation DESY in Hamburg, and at S.R.S. Daresbury. Diffraction beyond  $2.5 \text{ \AA}$  has been observed when large freshly grown crystals are used with the synchrotron beam. A data set to this resolution has been collected. Several putative heavy-atom derivative data sets have also been measured using synchrotron radiation facilities and analysis of these data sets is in progress.

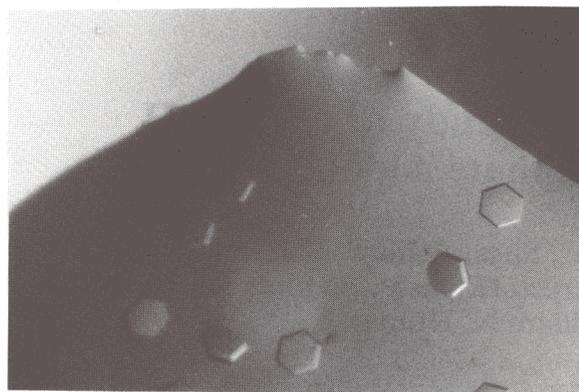
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Fructose-1,6-bisphosphate aldolases (FBP-aldolases, EC.4.1.2.13) catalyze the cleavage of this biphosphate to a ketose, dihydroxyacetone phosphate and an aldose, glyceraldehyde 3-phosphate (Horecker *et al.*, 1972). This reaction represents a distinctive stage in glycolysis involving the change-over from six to three-carbon units for subsequent steps in this metabolic pathway. Aldolases are widely dispersed in nature and can be separated into two groups termed Class I and II. The Class I enzymes generally occur in eukaryotes although they have also been identified in some bacteria (Baldwin & Perham, 1978, and references therein). They are homotetramers with a total relative molecular weight of approximately 160,000. They exist as isoforms, depending on the type of tissue in which they are located and show high sequence

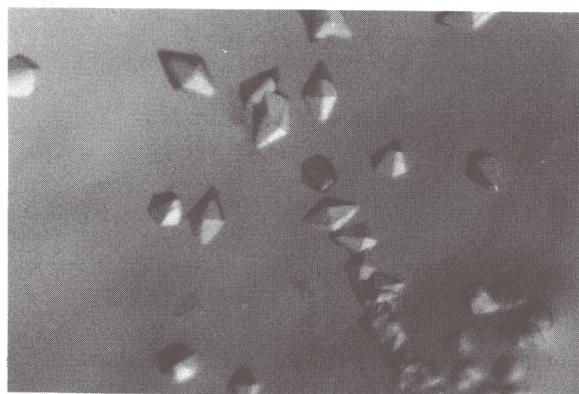
homology with each other. A number of crystal structures of the Class I enzyme have been determined. These include the enzymes isolated from rabbit muscle (Sygush *et al.*, 1987) and rabbit liver (White *et al.*, 1991), human muscle (Gamblin *et al.*, 1990, 1991), *Drosophila melanogaster* (Hester *et al.*, 1991) and the bacterial phosphogluconate aldolase (Lebioda *et al.*, 1982). The architecture of the Class I enzyme is that of an eight-stranded  $\alpha/\beta$ -barrel similar to that first observed in triose-phosphate isomerase (Banner *et al.*, 1975) and subsequently in more than a dozen other enzymes (Farber & Petsko, 1990). Unlike other  $\alpha/\beta$ -barrel structures, the aldolases have the active site located in the center of the barrel. The mechanism of the Class I enzyme has been studied extensively and involves an active site lysine residue which is able to stabilize the reaction intermediate *via* Schiff-base formation with the substrate (Horecker *et al.*, 1972).

In contrast to the structural detail now available for the Class I enzymes, little is known about the

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(a)



(b)



(c)

**Figure 1.** Three morphologies of the Class II aldolase from *E. coli*. (a) Hexagonal plates; (b) hexagonal bipyramids; and (c) truncated hexagonal bipyramids. Typical sizes are presented in the text.

Class II system. The latter proteins have proven difficult to purify in the quantities necessary for structural and mechanistic studies. It is known that they are functionally active as homodimers ( $M_r$  per subunit for the *E. coli* enzyme is approximately 39,000) and are metal dependent. Zinc is thought to act as the electron sink, during catalysis, in place of the Class I lysine residue (Mildvan *et al.*, 1971). The recent cloning and over-expression of the gene for the *E. coli* Class II FBP-aldolase (Alefounder *et al.*, 1989) has allowed access to large quantities of enzyme for our structural and protein engineering

studies. Knowledge of the three-dimensional structure combined with site-directed mutagenesis should allow us to derive the structure-activity relationships of this enzyme.

The enzyme was isolated from *E. coli* containing the expression vector pkfda for the Class II enzyme (Alefounder *et al.*, 1989). Purification was carried out by a modification of the method described by Baldwin *et al.* (1978) using ammonium sulfate fractionation, ion-exchange chromatography and gel filtration (K.M. & A.J.B., unpublished results). This resulted in sufficient quantities of enzyme for crystallization experiments. Different techniques for achieving supersaturation of the enzyme were tried (McPherson, 1982). These included the use of dialysis buttons and bags, vapor diffusion with hanging and sitting drops and microbatch methods. A variety of precipitants at 4 and 20°C were also tested. Initial experiments resulted only in amorphous precipitate. The exceptionally high expression of the enzyme, approximately 70% soluble protein, produces a mixture of holo and apo-enzyme. The apo-form can be easily reconstituted to holo-enzyme by incubation with excess zinc ions. It was quickly apparent that, in the presence of excess zinc, the enzyme crystallizes readily under a wide range of conditions using vapor diffusion, dialysis or batch methods. The last technique has proved most convenient for growth of crystals. Three related crystal morphologies have so far been observed. These are hexagonal plates, hexagonal bipyramids and truncated hexagonal bipyramids (Fig. 1). The plates are typically 0.3 mm across the face and less than 0.1 mm thick. The full and truncated bipyramids are grown from drops containing 18 mg protein/ml, 30 mM-Tris·HCl (pH 7.5), 7% (w/v) polyethylene glycol (mol. wt. 4000), 2.5 mM-ZnCl<sub>2</sub>. The former were typically less than 0.2 mm in the largest dimension, the latter were typically 0.6 mm in length and 0.4 mm thick. On occasion, truncated bipyramid crystals with all dimensions in excess of 1 mm have been obtained. These crystals grow to full size in one week at 4°C.

In order to characterize the diffraction of the truncated bipyramids, crystals were mounted in thin-walled glass capillaries and then sealed in the presence of mother liquor to maintain high humidity conditions. Preliminary X-ray photography, using oscillation and precession geometry, indicated the presence of one long cell edge (approximately 300 Å: 1 Å = 0.1 nm), two edges of length about 80 Å and hexagonal symmetry. To characterize the crystals further, they were exposed to X-rays (Rigaku rotating anode with a copper target operating at 50 kV 180 mA, utilizing a graphite monochromator,  $\lambda = 1.54$  Å and a 0.3 mm collimator) and data collected using the Rigaku R-AXIS IIc image plate detector. The crystal-to-detector distance was set at 203 mm and an exposure time of 10 minutes with an oscillation range of 0.75° was employed. Cooling the crystals to 6°C significantly enhanced their lifetime, allowing a data set to be collected to 2.9 Å resolution. The crystals were mounted such

**Table 1**  
Data collection statistics

	Synchrotron data		Rotating anode data	
Measurements	55,206		30,849	
Independent reflections†	11,843 (20,466)		10,945 (17,467)	
$R_{\text{merge}}$ (%)‡	8.5 (7.7)		9.9 (8.4)	
Resolution (Å)	8–3.5	3.5–2.9	20–3.5	3.5–2.9
Completeness (%)†	97 (95)	98 (97)	98 (96)	68 (53)

† Data with  $I \geq 1\sigma I$  where  $I$  is the intensity and  $\sigma$  represents the standard deviation. Values for data processed in Laue group  $6/m$  are given in parentheses.

‡ Defined as  $R = \Sigma |I(k) - \langle I \rangle| / \Sigma I(k)$ , where  $I(k)$  and  $\langle I \rangle$  represent the diffraction intensity values of individual measurements and the corresponding mean values. The summation is over all measurements with  $I \geq 1\sigma I$ .

that the  $\phi$  rotation was about the unique axis. The unit cell was determined, using the autoindexing method of Higashi (1990a), as  $a = b = 78.40(5)$  Å,  $c = 290.61(1)$  Å,  $\alpha = \beta = 90^\circ$ ,  $\gamma = 120^\circ$ . Data processing was carried out using PROCESS (Higashi, 1990b). Statistics relevant to the data collection are given in Table 1.

The best  $R_{\text{merge}}$  value for these data was obtained using Laue group  $6/m$  with an overall value of 8.4%. However, in Laue group  $6/mmm$   $R_{\text{merge}} = 9.9\%$ . This is a modest increase, given that the number of reflections being merged has increased significantly. In  $6/mmm$  this data set was 98% complete between 20 and 3.5 Å resolution and 68% complete between 3.5 and 2.9 Å.

In order to enhance the completeness, further data were collected using a synchrotron radiation source at the EMBL outstation, Hamburg and the MAR prototype image plate detector on the X31 beamline. The experimental design of this beamline has been described by Wilson (1989). The wavelength used was 1.009 Å, 0.35 mm slits were employed and the crystal-to-detector distance was 290 mm. Exposure time was linked to flux and with a current of 36 mA (4 GeV) this gave a typical exposure time of 3 minutes for a  $1^\circ$  oscillation. The crystals were cooled as before during data collection. Data processing was carried out using the MOSFLM package (Leslie *et al.*, 1986) as modified for image plate data by Leslie (personal communication) and Dauter *et al.* (personal communication). Relevant statistics are given in Table 1. This data set now comprises 98% of the theoretical diffraction data with  $I \geq 1\sigma I$  between 3.5 and 2.9 Å resolution.

From an examination of the intensities along the  $c$ -axis ( $00L$ ) we observe strong reflections that suggest the condition  $L = 6n$  applies. Thus the space group could be  $P6_122$  or the enantiomorph  $P6_522$ . With 12 equivalent positions the volume per asymmetric unit is approximately 128,900 Å<sup>3</sup> which gives values of 3.3 Å<sup>3</sup>/Da, 63% solvent content (Matthews, 1968) for a monomer of Class II FBP-aldolase in the asymmetric unit. The range of values typically observed for a selection of protein crystals is 1.68 to 3.53 Å<sup>3</sup>/Da and 27 to 65% solvent volume (Matthews, 1968). With a dimer in the asymmetric unit, values of 1.65 Å<sup>3</sup>/Da and solvent

volume of 26% are calculated. It would be unusual for a protein of this size to crystallize in such a tightly packed unit cell so that only one-quarter of the volume was solvent. It is more likely that a monomer would constitute the asymmetric unit. This is supported by preliminary self-rotation function studies, which have been unable to locate the presence of any non-crystallographic 2-fold axis.

In the lower symmetry  $P6_1$  or  $P6_5$  space groups there are six equivalent positions with an asymmetric volume of 257,800 Å<sup>3</sup>. Values of 6.6 Å<sup>3</sup>/Da, 81% solvent content are obtained for a monomer in the asymmetric unit. It would be more likely in this case that a functional dimer would constitute the asymmetric unit with 3.3 Å<sup>3</sup>/Da (63% solvent content). Self-rotation function calculations in  $6/m$  have so far failed to identify any non-crystallographic 2-fold symmetry, rather supporting  $6/mmm$  as the correct Laue group.

We note two recent instances where strong pseudo-symmetry in the diffraction pattern have complicated a protein structure analysis. These involve recombinant human interleukin 1- $\beta$  (Finzel *et al.*, 1989) and chicken citrate synthase (Liao *et al.*, 1991). In both cases crystals display apparent  $4/mmm$  symmetry but are really in Laue group  $4/m$ . So, whilst we observe consistency to suggest  $P6_122$  or  $P6_522$  as the correct space group choice, we realize that the molecular 2-fold of this dimeric FBP-aldolase may be contributing elements of pseudo-symmetry to our crystals. Unambiguous proof will result only from the structure solution and successful refinement of this protein. Until such time we will keep an open mind about other choices.

Some of the large crystals, mentioned above, of the protein have been grown immediately prior to exposure to X-rays. They have been examined using synchrotron radiation sources. A resolution test on Station PX7.2 of the Daresbury synchrotron radiation facility using a wavelength of 1.488 Å and film as the detector showed that good diffraction to 2.5 Å was observed. The experimental configuration of this station is described by Helliwell *et al.* (1982). We have subsequently measured a data set to this resolution on Station PX9.5 at Daresbury and these data are being processed. The facilities at the EMBL outstation and at Daresbury have also been used to

measure data from several putative heavy-atom derivatives. These data sets are being processed and evaluated for their usefulness.

As both classes of FBP-aldolase catalyze the same reaction the solution of the structure by molecular replacement is an inviting possibility. However, the sequence homology between the two classes of enzyme is low (Alefounder *et al.*, 1989; von der Osten, 1989). We have determined, using the GCG program GAP with a gap weight of 3.0 and length weight of 0.1 (Devereux *et al.*, 1984), that the Class I and Class II enzymes share about 15% sequence identity. Molecular replacement could therefore be problematic. This, coupled with space group considerations discussed above, suggests that multiple isomorphous replacement may prove the most suitable approach for the structure determination hence a search for isomorphous heavy-atom derivatives has been initiated.

In summary, we have successfully crystallized a Class II aldolase. The crystals are well ordered and diffract to an effective resolution of 2.5 Å resolution despite having a long unit cell edge of almost 300 Å. The crystals offer the opportunity for a detailed structural analysis to complement the biochemical studies being carried out on this system.

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