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Crystallization and preliminary crystallographic analysis of the snake muscle fructose 1,6-bisphosphatase

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The snake muscle fructose 1,6-bisphosphatase, a typical allosteric enzyme which plays important roles in gluconeogenesis, was crystallized in the presence of polyethylene glycol 3350 and magnesium chloride at pH 8.5. The crystals diffract to 2.3 Å on a rotating-anode X-ray source. The space group was determined to be either $P3_121$ or its enantiomorph $P3_221$, with unit-cell parameters $a = b = 83.7$, $c = 202.41$ Å, $\alpha = \beta = 90$ and $\gamma = 120^\circ$. There are two subunits in the asymmetric unit. Preliminary molecular-replacement studies indicate that the first enantiomorph is the correct one.

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1. Introduction

Fructose-1,6-bisphosphatase (Fru-1,6-Pase; E.C. 3.1.3.11) is a typical allosteric enzyme which catalyzes the hydrolysis of fructose 1,6-bisphosphate (Fru-1,6-P_2) to form fructose 6-phosphate (F6p) and ortho-phosphate. It works together with phosphofructo-1-kinase to constitute the substrate cycle at the expense of ATP. The liver and kidney enzymes have been extensively studied (Benkovic & de Maine, 1982; Pontremoli & Horecker, 1971; Tejwani, 1983; Van Schaftingen, 1987). However, the physiological function of the muscle enzyme remains unclear (Black *et al.*, 1972; Krebs & Woodford, 1965). Apart from some earlier reports showing that in the bumblebee this enzyme is responsible for heat production (Storey, 1978), most studies on the mechanism of the muscle enzymes to date have been carried out on Fru-1,6-Pase from the snake muscle.

An understanding of the snake muscle Fru-1,6-Pase function, along with a comparison with the isoforms from other sources involved in heat production, may provide important clues to the comprehension of the physiological role of the muscle enzyme.

The sequence of this enzyme has recently been determined (Xu *et al.*, unpublished work). A comparison of the primary structure between snake muscle and pig kidney Fru-1,6-Pases has shown a high sequence homology. In fact, about 75% of the sequence is identical to that of the pig kidney enzyme. Most of the differences occur at the N-terminus and at the C-terminus (MacGregor *et al.*, 1982). The snake muscle enzyme displays a cooperativity which has been studied in detail (Xu *et al.*, 1982, 1985; Yu & Xu, 1992; Zhao *et al.*, 1984, 1995). It has been found that the snake enzyme catalyzes the hydrolysis of Fru-1,6-P_2 by a ping-pong mechanism instead of a random Bi Bi as found in bovine liver enzyme. The snake muscle enzyme displays a unique feature: AMP performs a dual regulatory function, being both an allosteric inhibitor at pH 7.5 and an activator of the same enzyme at pH 9.2 (Zhao *et al.*, 1998). The physiological concentration of AMP is about 100 μM. At such a concentration the enzyme is almost completely inhibited. A slight shift in pH would thus alter the activity of Fru-1,6-Pase and consequently the substrate cycle.

The structural studies of Fru-1,6-Pase complexes, especially the three-dimensional structures of the enzyme complexed with different inhibitors or activators, will contribute to the understanding of the enzyme cooperativity. These studies will certainly contribute to the elucidation of the enzyme's structure–function relationship and hence its physiological role.

Interesting comparisons can be made with respect to crystallographic studies of chicken heart, pig kidney and human liver Fru-1,6-Pase (Anderson & Matthews, 1977; Soloway & McPherson, 1978; Iversen *et al.*, 1997). The pig kidney enzyme has been crystallized and its three-dimensional structure determined. Two interesting points are that the structures of the unligated enzyme and its fructose bisphosphate complex showed important quaternary and tertiary conformational changes (Ke *et al.*, 1989, 1990), and that an allosteric transition has been demonstrated in the modification of the tertiary structure (Liang *et al.*, 1993). Using X-ray crystallography, the structure–function studies have greatly progressed in recent years, *e.g.* for the pig kidney enzyme (Stec *et al.*, 1996; Lu *et al.*, 1995; Kurbanov *et al.*, 1998; Choe *et al.*, 1998).

Since insulin-resistant obese and/or diabetic patients have an elevated level of blood

glucose, it is believed that Fru-1,6-Pase plays an important role in type II diabetes. This high blood glucose may be produced by the gluconeogenesis pathway even without taking carbohydrates in the diet (Consoli & Nurjhan, 1990; Fujiwara *et al.*, 1995). The decrease of Fru-1,6-Pase activity will certainly suppress the pathway of hepatic gluconeogenesis, thus making Fru-1,6-Pase a pharmaceutical target for type II diabetes.

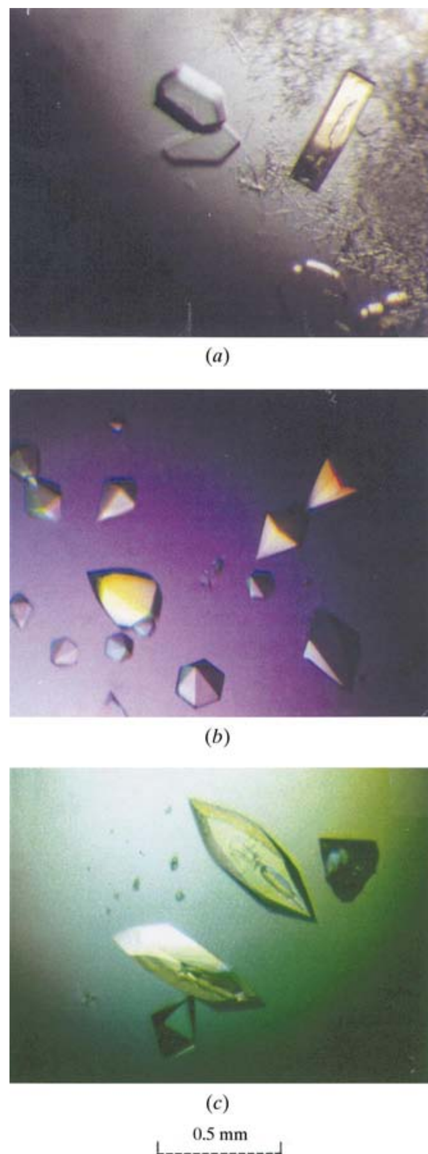

Figure 1
Different Fru-1,6-Pase crystals obtained in the screening of crystallization conditions. The crystallization was initiated by mixing equal volumes of the protein sample and of one of the reservoir solutions. The protein sample includes 20 mg ml^{-1} enzyme protein in 20% glycerol, 0.2 m*M* EDTA, 0.4 m*M* DTT and 10 m*M* Tris–HCl. Crystals were grown with the following reservoir solutions: (*a*) 30%(*w*/*v*) PEG 4K, 0.2 *M* sodium acetate and 0.1 *M* Tris–HCl pH 8.5, (*b*) 0.5 *M* Li_2SO_4 and 15%(*w*/*v*) PEG 8K, (*c*) 30%(*w*/*v*) PEG 400, 0.2 *M* $MgCl_2$ and 0.1 *M* HEPES at pH 7.5.

Since the muscle enzyme has an affinity which is tenfold higher than that of the same enzyme from other origins, comparative studies of the allosteric site may provide new insight leading to better inhibitor design. Here, we report the crystallization and preliminary crystallographic study of the snake (*Zaoeys dhumnades*) muscle Fru-1,6-Pase. The structure determination is under way (see §3).

2. Experimental

2.1. Preparation of the sample

The native form of the enzyme was prepared according to the method described previously (Xu *et al.*, 1982). The enzyme molecule consists of four subunits with a total molecular mass of about 144 kDa (4 × 36 kDa). The optimum pH for maximum activity is around 7. The activity of the enzyme was determined either by using a coupled enzyme assay measuring the formation of NADPH spectrophotometrically (Traniello *et al.*, 1972) or by measuring the release of inorganic phosphate as previously described (Xu *et al.*, 1982). The protein concentration was determined using the optical method [protein concentration (mg ml^{-1}) = A_{280}/0.72]. The absorbance was measured with a Beckman DU-70 spectrophotometer in which a micro-cell of 50 µl can be used.

2.2. Crystallization

The conventional sitting-drop vapor-diffusion method was employed. A protein sample of concentration 20 mg ml^{-1} was prepared in a buffer containing 20% glycerol, 0.2 m*M* EDTA, 0.4 m*M* DTT, 10 m*M* Tris–HCl at pH 7.5. Just prior to setting up the drop, ATP was added to the enzyme sample to a final concentration of 0.2 m*M*. The reservoir solution contained 100 m*M* Tris–HCl at pH 8.5, 0.39 *M* magnesium chloride and 27%(*w*/*v*) polyethylene glycol 3350 as the precipitant. Equal volumes of protein and reservoir solutions (10 µl) were mixed and allowed to equilibrate against the reservoir solution in the sitting drop. The final volume of the drop after equilibrium is about 14 µl, resulting in modified concentrations for the components as indicated in the legend of Fig. 2.

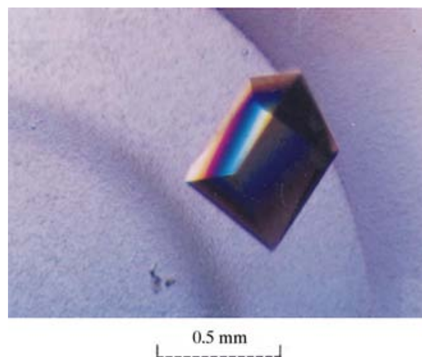

Figure 2
Fru-1,6-Pase crystals grown in the presence of 19%(*w*/*v*) PEG 4K, 0.27 *M* $MgCl_2$, 0.14 m*M* ATP, 70 m*M* Tris–HCl at pH 8.5, 14% glycerol and 14 mg ml^{-1} protein in the drop after equilibrium. The crystal has a typical size of 0.3 × 0.45 × 0.62 mm and diffracts to 2.3 Å.

2.3. X-ray diffraction analysis

The preliminary X-ray diffraction analysis of Fru-1,6-Pase crystals was performed at room temperature with an R-AXIS IIc image plate and a Rigaku rotating-anode generator. The crystal was mounted in a glass capillary which was sealed with a small amount of mother liquor at one end. The detector was placed at a distance of 160 mm from the crystal and the beam was collimated to 0.3 mm. The unit-cell parameters and the crystal orientation angles were determined from three fixed positions of the crystal, 45° apart in spindle rotation, using *R-AXIS* software. Data was collected over 90° using 1.5° oscillations and the resulting imaging plates were processed with the *R-AXIS* software.

3. Results and discussion

3.1. Crystal growth

The sparse-matrix screening method (Jancarik & Kim, 1991) was used for preliminary screening. The enzyme preparation yielded a highly active Fru-1,6-Pase preparation, which can catalyze the formation of 25 µmol of fructose 6-phosphate in one minute per milligram of enzyme protein under the assay conditions described in §2.1. Through preliminary screening, some multiple crystals were obtained in the sodium acetate/Tris–HCl/PEG (polyethylene glycol), magnesium chloride/HEPES/PEG and lithium sulfate/PEG systems (Fig. 1). Based on the above preliminary results, further refinement was carried out. A series of crystals were obtained with different magnesium chloride and PEG concentrations, but the best crystals were obtained with a reservoir solution of 0.39 *M* $MgCl_2$, 27%(*w*/*v*) PEG 4K and 0.1 *M* Tris–HCl pH 8.5. The crystals appeared overnight at room temperature and grew to a typical size of 0.30 × 0.45 × 0.62 mm in four weeks (Fig. 2).

3.2. Data collection and analysis

The crystal is trigonal with unit-cell parameters $a = b = 83.7$, $c = 202.41$ Å, $\alpha = \beta = 90$ and $\gamma = 120°$. Based on the systematic absences from the collected data, the space group was first determined to be either $P3_121$ or its enantiomorph $P3_221$. There are two subunits in the asymmetric unit. The initial data set was collected at 2.8 Å, although many diffraction spots were observed up to 2.3 Å. The data was processed with *R-AXIS* software and yielded 16670 unique reflections from 48343 independent measurements, with an $R_{\rm merge}$ based on structure factors of 5.76%. The data were 86.3% complete between 12 and 2.87 Å and 78.2% complete in the 3.0–2.87 Å shell. With the subunit mass of 36 kDa (determined by mass spectroscopy) and assuming two subunits per asymmetric unit, the Matthews coefficient, V_m, was calculated to be 2.84 $Å^3$ Da^{-1}. This is within the normal range (Matthews, 1968).

We have performed an initial molecular-replacement analysis using fructose-1,6-bisphosphatase from the pig adrenal cortex as a model (Ke *et al.*, 1990) in the molecular-replacement package *AMoRe* (Navaza, 1994). The 20 best rotation-function solutions had correlation functions ranging from 15.9 to 12.3. However, only the third (15.8) and fourth peaks (15.7) yielded translation-function solutions significantly higher than the remainder of the peaks when solved in $P3_121$. None of the rotation solutions yielded significant peaks in the translation search when $P3_221$ symmetry was used. The two solutions were refined as individual rigid bodies within *AMoRe* and were then found to be related to each other through a non-crystallographic twofold symmetry. This confirmed the results obtained previously from a self-rotation function and results in a close contact dimer. Using the graphics program *O* (Jones *et al.*, 1991), the symmetry mate of the second solution, which was also related directly by non-crystallographic twofold symmetry to the first solution, was chosen as the second subunit. The first subunit corresponded to the first solution. The large number of contacts between the two resultant subunits lie mainly along the twofold symmetry axis, which suggests a physiological importance for the subunit interactions and could be closely related to the cooperative behavior of fructose 1,6-phosphatase as demonstrated by kinetic studies (Xu *et al.*, 1982). The tetrameric molecule is generated by crystallographic transformation of the dimer produced by the non-crystallographic symmetry.

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