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Crystallization and preliminary crystallographic data of fructose-1,6-bisphosphatase from human muscle

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The enzyme human muscle fructose-1,6-bisphosphatase, which plays a critical role in gluconeogenesis, has been crystallized in the presence of 2-propanol, polyethylene glycol and magnesium chloride at pH 7.5. The space group was determined to be $P4_22_12$, with unit-cell parameters $a = b = 73.57$, $c = 146.50$ Å, $\alpha = \beta = \lambda = 90^\circ$ and one subunit in the asymmetric unit. A 99.6% complete data set to 2.04 Å has been collected at the National Synchrotron Light Source.

1. Introduction

Fructose-1,6-bisphosphatase (Fru-1,6-P2ase; E.C. 3.1.3.11) is a primary control point in gluconeogenesis and a regulator of blood glucose. The liver and kidney enzymes have been extensively studied (Pontremoli & Horecker, 1971; Benkovic & de Maine, 1982; Van Schaftingen, 1987), including the determination of crystallographic structures from several sources (Anderson & Matthews, 1977; Soloway & McPherson, 1978; Iversen *et al.*, 1997). The active site of the enzyme accommodates the substrate fructose-1,6-bisphosphate and divalent metal-ion subsites. The mutually exclusive binding of AMP and Mg^{2+} provide the basis of enzyme regulation (Kurbanov *et al.*, 1998). Since insulin-resistant obese and/or diabetic patients have elevated blood-glucose levels, it is believed that Fru-1,6-P2ase may play an important role in type II diabetes. High blood glucose may be produced by the gluconeogenic pathway even without taking carbohydrates and under starvation conditions (Consoli & Nurjhan, 1990; Fujiwara *et al.*, 1995). The decrease of Fru-1,6-P2ase activity will certainly suppress the pathway of hepatic gluconeogenesis, thus making Fru-1,6-P2ase one of the pharmaceutical targets for type II diabetes.

Muscle Fru-1,6-P2ase can be distinguished from the same enzyme from other sources by several kinetic properties. It has a tenfold higher affinity for AMP than either the liver or kidney enzymes (Feng-wen *et al.*, 2000), with AMP also acting as a pH-dependent allosteric inhibitor. At more alkaline pH values (*e.g.* pH 9.2) AMP becomes an activator of Fru-1,6-P2ase (Zhao *et al.*, 1998). Though there is no direct evidence that pH 9.2 is a physiological condition in muscle, the required pH for enzyme activation has only to be higher than neutral pH. At the latter pH, the concentration of AMP in muscle under physiological conditions (~1 m*M*) will completely inhibit Fru-1,6-P2ase. The AMP activation curve for the enzyme is bell-shaped, so that even a slight increase of pH will decrease the inhibition and increase the ability of AMP to activate the muscle enzyme. Tillmann & Eschrich (1998) cloned the human muscle enzyme and reported its sequence and some of its properties. The kinetic parameters of both snake and human muscle enzymes, which share 88.5% sequence homology, are quite similar. When compared with human liver Fru-1,6-P2ase, the muscle enzyme's sequence has an identity of 77%. This suggests these enzymes have a similar three-dimensional structure but that they are coded by distinct genes with some differences in their structure–function relationship. The muscle enzyme is a tetrameter with a molecular mass of 4×36 kDa.

The inhibition of Fru-1,6-P2ase would decrease gluconeogenesis and thereby reduce the blood glucose of diabetic patients. It could be used as an adjuvant drug for those patients, with the inhibition target mainly being the liver and kidney enzymes. AMP is a specific inhibitor for Fru-1,6-P2ase, but the inhibition constants for the liver or kidney enzymes are relatively high (at the micromolar level) and hardly useful for drug design. The tenfold higher binding ability for the muscle enzyme over that of the liver or kidney enzymes will lead to valuable structural information for inhibitor design. In order to further understand the enzyme–substrate interactions, including those between the enzyme and AMP, the crystallographic structure of muscle Fru-1,6-P2ase needs to be determined. An understanding of the binding of AMP at the allosteric site for the liver and muscle enzyme will provide new insights and the basis for the design of better inhibitors. The snake muscle enzyme has also recently been crystallized (Zhu *et al.*, 1999) and the structure is being determined. Here, we report the crystallization

and preliminary crystallographic study of the human muscle Fru-1,6-P2ase. The corresponding structure and the enzyme–AMP interactions are being determined.

2. Materials and methods

Ultrapure polyethylene glycol (PEG) 8K and 4K were purchased from Fluka; AMP, magnesium chloride and 2-propanol were purchased from Sigma. The sparse-matrix screening kits were purchased from Hampton Research (Los Angeles, USA).

The human muscle Fru-1,6-P2ase was overproduced in *Escherichia coli* and prepared according to the method described previously (Tillmann & Eschrich, 1998). The enzyme was stored at 277 K in 20% glycerol, 0.2 m*M* EDTA, 0.4 m*M* dithiothreitol (DTT), 10 m*M* Tris–HCl at pH 7.5 (sample buffer *A*, in the presence of glycerol) or 100 m*M* KCl, 0.4 m*M* EDTA, 0.4 m*M* dithiothreitol (DTT), 50 m*M* HEPES adjusted to pH 7.0 (sample buffer *B*, in the absence of glycerol). The activity of the enzyme was determined either by using a coupled enzyme assay that measures the formation of NADH spectrophotometrically or by measuring the release of inorganic phosphate as previously described (Xu *et al.*, 1982). The protein concentration was measured by optical density [protein concentration (mg ml^{-1}) = A_{280}/0.72].

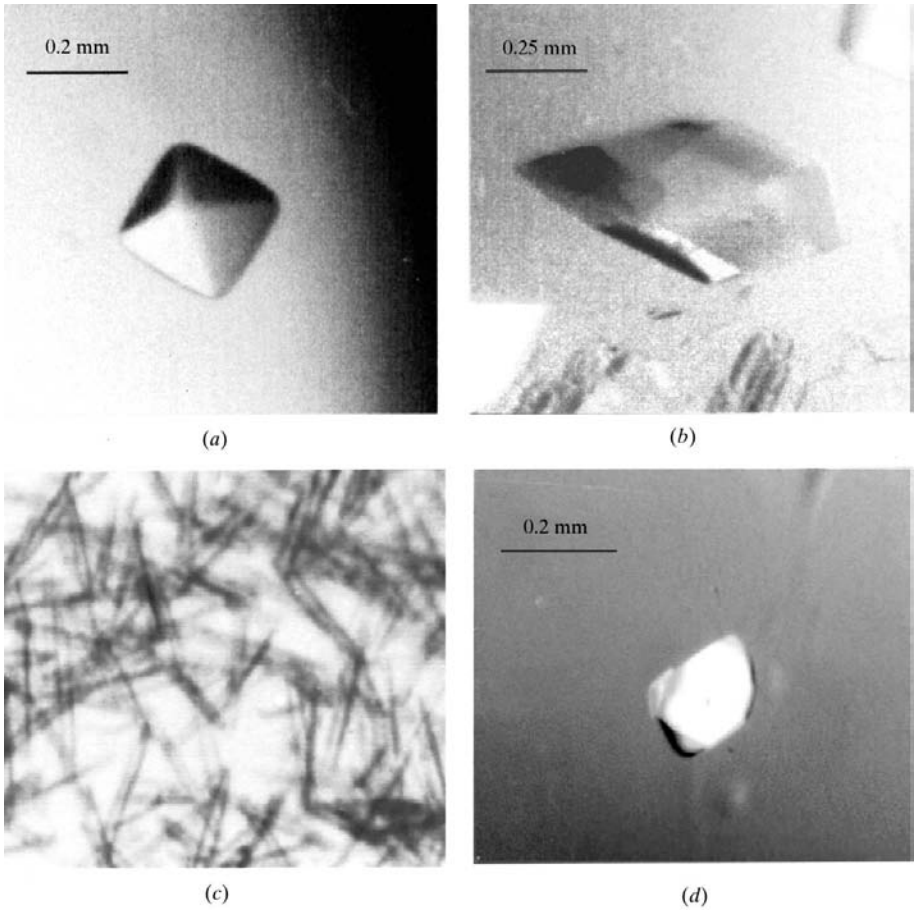

Figure 1
Fru-1,6-P2ase–AMP crystal obtained from the screening of crystallization conditions. (*a*) A protein sample was prepared in sample buffer *A* at 12 mg ml^{-1}. AMP was added to the enzyme sample to a final concentration of 0.2 m*M*. The reservoir solution contained 0.14 *M* magnesium acetate, 20%(*w*/*v*) PEG 8K and 0.1 *M* cacodylate pH 6.5. A typical crystal had dimensions 0.22 × 0.22 × 0.1 mm and diffracted to only 3.8 Å using our home facility (an R-AXIS IIc image-plate area detector on a Rigaku RU-200 rotating anode at room temperature). (*b*) A protein sample was prepared in sample buffer *B* to a concentration of 15 mg ml^{-1}. Crystals of the AMP complex were obtained in the presence of 6% 2-propanol, 14%(*w*/*v*) PEG 4K, 0.1 *M* HEPES pH 7.5, 0.2 m*M* AMP in the drop after equilibrium. The crystal dimensions are 0.380 × 0.360 × 0.28 mm; it diffracted to 3.0 Å. The same home facility was used. (*c*) Preparation of sample is similar to (*b*). The reservoir solution contained 0.5 *M* lithium sulfate and 15%(*w*/*v*) PEG 8K. Needle crystals appeared 1 d after setting the drop. (*d*) A protein sample of concentration 15 mg ml^{-1} was prepared in sample buffer *B* containing 0.1 *M* KCl, 0.4 m*M* EDTA and 0.4 m*M* DTT, 50 m*M* HEPES pH 7.0. The Fru-1,6-P2ase crystals were grown in the presence of 2%(*w*/*v*) 2-propanol, 0.1 *M* magnesium chloride, 15%(*w*/*v*) PEG 4K, 0.1 *M* HEPES pH 7.5 and 0.2 m*M* AMP. The crystals of the Fru-1,6-P2ase–AMP complex appeared in 1 d and grew to typical dimensions of 0.22 × 0.22 × 0.14 mm in two weeks. The crystals yielded data from a synchrotron-radiation source to 2.04 Å.

The crystallization was carried out by vapour diffusion in hanging drops at room temperature. The reservoir contained 1 ml of solution including the precipitant, salt and buffer. AMP was added to the enzyme sample just prior to crystallization to a final concentration of 0.2 m*M*. Equal volumes of protein and reservoir solution (3 µl) were mixed to initiate the crystallization. Initial conditions that yielded crystals were found using the sparse-matrix kits I and II.

The preliminary X-ray diffraction analysis of human Fru-1,6-P2ase–AMP complex 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 180 mm from the crystal and the beam was collimated to 0.3 mm. The unit-cell parameters and the crystal orientation angles were determined using the *HKL* software package (Otwinowski & Minor, 1997).

The best crystals of the Fru-1,6-P2ase–AMP complex had to be flash-frozen to 100 K for data collection. Data were collected using a MAR 30 cm image plate at beamline X12C at the National Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY, USA. Data collection was with the detector 160 mm from the crystal and a wavelength of 0.9 Å. All data sets were processed with the *HKL* software package (Otwinowski & Minor, 1997).

3. Results and discussion

Initial crystallization conditions were found using the sparse-matrix screening of Jancarik & Kim (1991) and were then optimized. When the protein sample was prepared in sample buffer *A* at 12 mg ml^{-1}, AMP was added to the enzyme sample to a final concentration of 0.2 m*M* (the protein:AMP molar ratio is about 1:2). A preliminary screen found that multiple crystals were obtained in sodium cacodylate/magnesium acetate/PEG 8K, 2-propanol/Na HEPES/PEG 4K and lithium sulfate/PEG 8K as precipitant solutions. For example, crystals of the AMP complex were obtained with a reservoir solution of 0.14 *M* magnesium acetate, 20%(*w*/*v*) PEG 8K, 0.1 *M* cacodylate pH 6.5 and 0.2 *M* AMP, as shown in Fig. 1(*a*). The crystals appeared after 4 d at room temperature. A typical crystal has dimensions of 0.22 × 0.22 × 0.1 mm and diffracts to only 3.8 Å using our home facility. The crystal is primitive tetragonal, with unit-cell parameters $a = b = 75.06$,

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell (2.11–2.04 Å).

No. of images	183
Oscillation range (°)	1.0
Space group	$P4_22_12$
Unit-cell parameters (Å)	$a = b = 73.57$, $c = 146.50$
Mosaicity (°)	0.66
Resolution range (Å)	40–2.04
No. of observations	235 402
No. of unique reflections	26 161
Redundancy	9.0
R_{sym} (%)	8.2 (50.5)
Completeness (%)	99.6 (99.3)
$\langle I/\sigma(I)\rangle$	21.4 (3.1)

† $R_{sym} = \sum|I - \sigma(I)|/\sum\sigma(I)$.

c = 148.67 Å, $\alpha = \beta = \gamma = 90°$. When the protein sample was prepared in sample buffer *B* to a concentration of 15 mg ml^{-1} with a protein:AMP molar ratio of 1:2, multiple crystals were also found in a preliminary screen in the above-mentioned three conditions. Initial refinement of the 2-propanol conditions yielded the Fru-1,6-P2ase–AMP complex crystals shown in Fig. 1(*b*). The reservoir solution contained 6% 2-propanol, 14%(*w*/*v*) PEG 4K, 0.1 *M* HEPES pH 7.5 and 0.2 m*M* AMP, with the crystals growing to maximum dimensions of 0.38 × 0.36 × 0.28 mm. The crystals are again primitive tetragonal, with unit-cell parameters $a = b = 74.88$, $c = 148.03$ Å, $\alpha = \beta = \gamma = 90°$, and diffract to 3.07 Å using our home facility. In Fig. 1(*c*), crystals were obtained using the same protein sample as in Fig. 1(*b*), but in the presence of 0.5 *M* lithium sulfate, 15%(*w*/*v*) PEG 8K and 0.2 m*M* AMP. Needle crystals appeared within 1 d after setting up the drop. In order to improve crystal quality, the pH, PEG 4K, salts and protein concentrations were further varied in this study. Crystals with the best morphology were obtained by mixing a protein sample in buffer *B* at 15 mg ml^{-1} and a precipitant solution consisting of 2%(*w*/*v*) 2-propanol, 0.1 *M* magnesium chloride, 15%(*w*/*v*) PEG 4K, 0.1 *M* HEPES pH 7.5 and 0.2 *M* AMP. Crystals of the AMP complex appeared within 1 d and grew to typical dimensions of 0.22 × 0.22 × 0.14 mm in two weeks (Fig. 1*d*). The crystals were flash-cooled in a cryoprotectant solution containing 80% crystallization reservoir solution and 20% ethylene glycol. The crystals were determined to be primitive tetragonal, with unit-cell parameters $a = b = 73.57$, $c = 146.50$ Å. A total of 183 images were collected using 1° oscillations and were processed using the *HKL* package (Otwinowski & Minor, 1997). Using an $I/\sigma(I)$ ratio of at least 2 for the last resolution shell as a cutoff, the resolution was determined to be 1.9 Å, with nearly 100% completeness. However, the R_{sym} of this last shell was too high and the resolution was therefore lowered to 2.04 Å. The data collection is summarized in Table 1. Based on systematic absences, the space group was determined to be $P4_22_12$. V_M was calculated to be 2.70 Å^3 Da^{-1} (Matthews, 1968) assuming one subunit in the asymmetric unit. The solvent content of the crystal was therefore calculated to be 54%. Human muscle Fru-1,6-P2ase is a tetramer in solution. The crystal unit cell can accommodate the tetramer possibly through the fourfold crystallographic axis.

The structure will be solved through molecular replacement using the previously solved structures of Fru-1,6-P2ase from either liver or kidney.

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