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Purification, crystallization and preliminary X-ray diffraction analysis of the fructose-1,6-/sedoheptulose-1,7-bisphosphatase of *Synechococcus* PCC 7942

Yoshihiro Nakamura,[a]* Toshiji Tada,[a] Kei Wada,[a] Takayoshi Kinoshita,[b] Masahiro Tamoi,[c] Shigeru Shigeoka[c] and Keiichiro Nishimura[a]

[a]Research Institute for Advanced Science and Technology, Osaka Prefecture University, Sakai, Osaka 599-8570, Japan, [b]Exploratory Research Laboratories, Fujisawa Pharmaceutical Co. Ltd, Tsukuba, Ibaraki 300-2698, Japan, and [c]Department of Food and Nutrition, Faculty of Agriculture, Kinki University, Nakamachi, Nara 631-8505, Japan

Correspondence e-mail: ynakam@biochem.osakafu-u.ac.jp

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Fructose-1,6-/sedoheptulose-1,7-bisphosphatase of *Synechococcus* PCC 7942, overexpressed from *Escherichia coli*, has been purified and crystallized by the hanging-drop vapour-diffusion method using ammonium sulfate as a precipitant. The crystals were monoclinic, with unit-cell parameters a = 80.1, b = 84.2, c = 104.3 Å, β = 101.7°. They belonged to space group $P2_1$ and diffracted to at least 2.2 Å resolution. The calculated V_M value, based on a tetramer in the asymmetric unit, was 2.2 Å^3 Da^{-1}.

1. Introduction

Fructose-1,6-bisphosphatase (FBPase; E.C. 3.1.3.11), which is distributed in the chloroplasts and cytosol of higher plants, catalyzes the hydrolysis of fructose 1,6-bisphosphate to form fructose 6-phosphate. The enzyme participates in the photosynthetic carbon-reduction (PCR) cycle, gluconeogenesis and oxidative pentose phosphate pathways of carbohydrate metabolism (Zimmermann *et al.*, 1976; Kelly *et al.*, 1982; Preiss, 1982; Marcus *et al.*, 1987). Sedoheptulose-1,7-bisphosphatase (SBPase; E.C. 3.1.3.37) catalyzes the hydrolysis of sedoheptulose 1,7-bisphosphate to sedoheptulose 7-phosphate in the PCR cycle (Buchanan *et al.*, 1976). These enzymes also work in the PCR cycle of cyanobacteria and chemoautotrophic bacteria. Crystallographic studies of FBPases have been widely conducted: *e.g.* pig kidney (Ke *et al.*, 1989), human liver (Gidh-Jain *et al.*, 1994), spinach chloroplast (Villeret *et al.*, 1995), rabbit liver (Weeks *et al.*, 1999) and pea chloroplast (Chiadmi *et al.*, 1999). Although crystal structures of SBPases have not been reported, their tertiary structures have been discussed based on theoretical models (Anderson *et al.*, 1996).

Synechococcus PCC 7942, a cyanobacterium, contained two isozymes of FBPase designated FBPase-I and FBPase-II (Tamoi *et al.*, 1996). FBPase-I possessed both FBPase and SBPase activities *in vitro*, so we named this enzyme fructose-1,6-/sedoheptulose-1,7-bisphosphatase (FBP/SBPase). FBP/SBPase is generally distributed in most of the cyanobacteria and is suggested to work in the PCR cycle as an FBPase as well as working as an SBPase *in vivo*, as no isozyme of SBPase has been found in cyanobacteria. The enzyme has unique characteristics differing from those of higher plant chloroplastic and cytosolic enzymes (Tamoi *et al.*, 1996). The enzyme is resistant to inactivation by hydrogen peroxide, unlike the light-activated chloroplastic enzymes (Tamoi *et al.*, 1996, 1998). The enzyme is inhibited by AMP but not by fructose 2,6-bisphosphate, both of which are inhibitors of cytosolic FBPase. Furthermore, when the enzyme was treated with a mixture of fructose 1,6-bisphosphate and sedoheptulose 1,7-bisphosphate, the hydrolytic activity was higher than that with each of the substrates (Nakamura *et al.*, unpublished data). The primary structure of the FBP/SBPase is completely different from those of typical FBPases and SBPases from diverse animals and higher plants (Tamoi *et al.*, 1996).

The aim of the present study was to clarify the molecular mechanisms of these novel properties of FBP/SBPase using the crystallographic structure of the enzyme. The enzyme has a molecular mass of 160 kDa and consists of four identical subunits, similar to typical FBPases (Tamoi *et al.*, 1996). The three-dimensional structure of FBP/SBPase is expected to be similar to those of FBPases. A detailed study of the three-dimensional structure of the enzyme, comparing it with those reported for FBPases, should provide some information about the recognition mechanism differentiating between the two substrates and the resistance mechanism to some inhibitors reported for other FBPases. In the present study, we report the purification, crystallization and preliminary X-ray analysis of the novel protein FBP/SBPase originating from *Synechococcus* PCC 7942.

2. Experimental

2.1. Purification

FBP/SBPase of *Synechococcus* PCC 7942 was overexpressed by *E. coli* BL21(DE3)-pLysS, which was cultured with shaking in four 1 l flasks containing 500 ml of Luria broth (Tamoi *et al.*, 1996). *E. coli* cells were

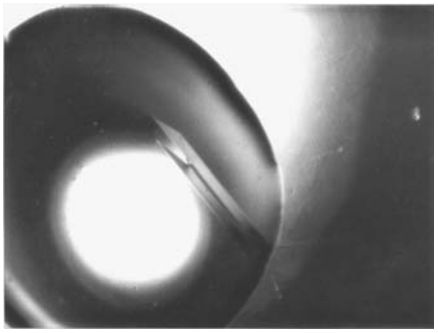

Figure 1
A typical crystal of the FBP/SBPase of *Synechococcus* PCC 7942. Its dimensions were approximately 0.7 × 0.15 × 0.15 mm.

harvested, resuspended in 30 ml buffer *A* (50 m*M* potassium phosphate buffer pH 8.0 containing 2.5 m*M* dithiothreitol, 1 m*M* glutathione reduced form and 10% sucrose) and sonicated. The lysate was centrifuged to produce a crude extract, which was then loaded onto a HiLoad 26/10 Q Sepharose column (FPLC system, Pharmacia) and eluted with a linear gradient of NaCl (0–0.6 *M*). The active fractions were combined and adjusted to 30% saturation of ammonium sulfate. After centrifugation, the supernatant was chromatographed on a HiLoad 16/10 phenyl Sepharose column (FPLC system, Pharmacia) equilibrated with buffer *A* containing 30% saturated ammonium sulfate. Protein was eluted with a descending linear gradient of 30–0% ammonium sulfate. The ammonium sulfate in the active fractions was removed by excessive dialysis against buffer *A*. The resulting enzyme solution was loaded onto a Mono-Q HR 5/5 column (FPLC system, Pharmacia) equilibrated with buffer *A* and eluted with a linear gradient of NaCl (0–0.6 *M*). The purity of the expressed FBP/SBPase was confirmed by SDS–PAGE.

2.2. Crystallization

The protein used for crystallization was at 5 mg ml^{-1} in 50 m*M* potassium phosphate buffer pH 8.0 containing 0.15 *M* NaCl. All crystallization trials were carried out using the hanging-drop vapour-diffusion method by mixing 1 µl of protein solution with 1 µl of various reservoir solutions and equilibrating the drops over the respective reservoirs at 293 K. The initial trials were performed using the commercially available sparse-matrix screening kits Crystal Screen I and II from Hampton Research (Jancarik & Kim, 1991). The initial conditions found to be the best were optimized by varying the concentrations of the protein, precipitants and buffer systems.

2.3. Data collection and analysis

Preliminary X-ray diffraction studies were carried out using an R-AXIS IIc image-plate area detector mounted on a Rigaku RU-300 rotating-anode source operating at 40 kV, 100 mA with Cu $K\alpha$ radiation. A complete data set was collected on an R-AXIS IV image-plate detector using synchrotron radiation of wavelength 0.7 Å at the BL41XU station of SPring-8, Japan. Measurements were collected at 100 K. Cryoconditions were established for one of the crystal forms in order to collect a native data set. The crystal-to-detector distance was 500 mm and 60 images were recorded at 3° intervals with an exposure time of 6 s per image. The intensity measurements were processed with the program *DENZO* and merged with the program *SCALEPACK* (Otwinowski & Minor, 1997).

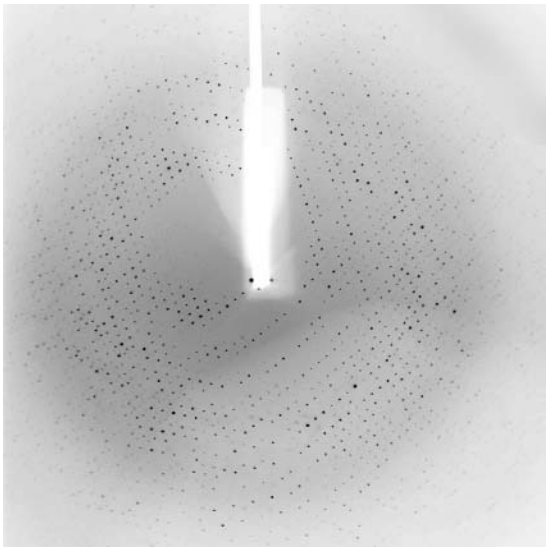

Figure 2
An X-ray diffraction pattern of the FBP/SBPase crystal. The diffraction was obtained at 100 K on beamline BL41XU at the SPring-8 synchrotron with an R-AXIS IV image plate using a crystal-to-detector distance of 500 mm, a wavelength of 0.7 Å, an exposure time of 6 s and an oscillation angle of 3°.

Table 1
Crystal data and data-collection statistics.

Values for the highest resolution shell are shown in parentheses.

X-ray source	BL41XU, SPring-8
Wavelength (Å)	0.7
Crystal dimensions (mm)	0.70 × 0.15 × 0.15
Space group	$P2_1$
Unit-cell parameters (Å,°)	a = 80.1, b = 84.2, c = 104.3, β = 101.7
Resolution (Å)	20–2.2
No. of observations	290947
No. of unique reflections	59500
$I/\sigma(I)$	4.5
Completeness (%)	88.7 (65.6)
$R_{\rm merge}$ (%)	11.6 (26.2)

3. Results and discussion

The purification produced 13 mg of protein which was greater than 95% pure as judged by SDS–PAGE inspection. In the initial crystallization trials, small crystals were obtained from several drops containing ammonium sulfate. The shape of these crystals was improved by adding a small amount of detergent or organic solvent. However, the crystals diffracted weakly to a maximum resolution of 3.7 Å and decayed quickly after a few images because of radiation damage. High-quality crystals grew in the presence of AMP, which is an inhibitor of FBP/SBPase (Tamoi *et al.*, 1996). The crystallization conditions finally established were a protein solution of 10 mg ml^{-1} in 50 m*M* potassium phosphate buffer pH 8.0 containing 0.15 *M* NaCl and 10 m*M* AMP equilibrated against a reservoir of 0.1 *M* citrate buffer pH 6.0 containing 45% saturated ammonium sulfate and 10%(*v*/*v*) glycerol. The crystals grew to maximum dimensions of 0.7 × 0.15 × 0.15 mm in a month (Fig. 1) and diffracted to 2.8 Å resolution using an R-AXIS IIc image plate.

The crystals diffracted to at least 2.2 Å resolution at SPring-8. The crystal system was determined to be monoclinic, space group $P2_1$, with unit-cell parameters a = 80.1, b = 84.2, c = 104.3 Å, β = 101.7°. The statistics for the intensity data are shown in Table 1. Assuming a tetramer in the asymmetric unit, the $V_{\rm M}$ value as defined by Matthews (1968) was 2.2 Å^3 Da^{-1}, corresponding to a solvent content of 45%. A typical X-ray diffraction pattern of the FBP/SBPase crystal is shown in Fig. 2. We have started overexpression and crystallization of the selenomethionine derivative of FBP/SBPase. The phase problem will hopefully be solved by multiple anomalous diffraction (MAD) experiments.

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