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A thermostable xylose isomerase from *Thermus caldophilus*: biochemical characterization, crystallization and preliminary X-ray analysis

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A highly thermostable xylose isomerase from *Thermus caldophilus* has been expressed in *Escherichia coli*. The purified enzyme has an optimum temperature of 363 K. It has been crystallized at room temperature using ammonium sulfate as a precipitant. The crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 84.35, b = 123.60, c = 140.24 Å. The presence of one molecule of tetrameric xylose isomerase in the asymmetric unit gives a crystal volume per protein mass (V_m) of 2.1 Å^3 Da^{-1} and a solvent content of 41% by volume. The crystals initially showed diffraction to 1.7 Å Bragg spacing with synchrotron X-rays, and a set of native data extending to 2.3 Å resolution has been collected.

1. Introduction

Xylose isomerase (XI; E.C. 5.3.1.5) catalyzes the reversible isomerization between an aldo sugar D-xylose and a keto sugar D-xylulose in the first step of xylose metabolism. It also catalyzes the isomerization between D-glucose and D-fructose (Rangarajan & Hartley, 1992; Meng *et al.*, 1991). The isomerization reaction catalyzed by xylose isomerases is an equilibrium reaction and the ratio between D-glucose and D-fructose in the product depends on the temperature. The current isomerization process operated at 331 K yields a 40–42% fructose syrup, and an additional chromatographic step is necessary to produce 55% syrup concentration. Performing the isomerization reaction at 368 K would achieve a high-fructose corn syrup without the chromatographic step (Zeikus, 1996). Therefore, there is much commercial interest in developing highly thermostable xylose isomerases for the industrial production of high-fructose corn syrup. Crystal structures of type I XIs from several mesophilic bacteria have been reported (Allen *et al.*, 1995; Rasmussen *et al.*, 1994; Lavie *et al.*, 1994; Cha *et al.*, 1994; Collyer *et al.*, 1992; Blow *et al.*, 1992; Whitlow *et al.*, 1991; Dauter *et al.*, 1990; Farber *et al.*, 1989; Henrick *et al.*, 1989; Carrell *et al.*, 1989; Rey *et al.*, 1988). They share a common $(\beta/\alpha)_8$ barrel fold with an extended domain for each subunit and a similar tetrameric arrangement of subunits. However, there have been no reports of the structure of any type I XIs with an optimum temperature near 363 K. Compared with type I XIs, type II XIs have extended N-terminal amino-acid residues. Highly thermostable type II XIs from *Thermoanaerobacterium thermosulfurigenes* and *Thermotoga neapolitana* have been crystallized (Lloyd *et al.*, 1994; Chayen *et al.*, 1997), but their crystal structures have not yet been reported.

T. caldophilus GK24, a thermophilic bacterium, produces a highly thermostable type I XI, which is a homotetrameric enzyme of 387-residue subunits (subunit M_r = 43912). In this study, the optimum reaction temperature of TcaXI is determined to be 363 K. Therefore, XI from *T. caldophilus* (TcaXI) is potentially useful for the production of high-fructose corn syrup. It would be of interest to compare the structure of this thermostable XI with those of less thermostable XIs to delineate the structural basis of the thermostability. As a first step, we report here the crystallization and preliminary X-ray analysis of TcaXI crystals.

2. Experimental

2.1. Expression, purification and biochemical characterization

The gene encoding XI (*xylA*) was obtained by the polymerase chain reaction (PCR) using the chromosome of *T. caldophilus* GK24 as the template, essentially according to Park (1996). The PCR product was initially inserted into the *Sma*I site of the cloning vector pUC19. Since the insert was found to have the incorrect orientation in all the selected plasmids, the plasmid was digested by *Eco*RI–*Hin*dIII and the insert was cloned into the same site of pUC18. This resulted in ten extra residues at the N-terminus of the expressed protein. The protein was expressed under the control of the *lac* promoter.

Cells were harvested by centrifugation of a 10 l culture (Sorvall GS3, 4200*g*, 10 min). The pellet was resuspended in 100 ml of the lysis buffer (50 m*M* potassium phosphate at pH 7.2, 1 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride, 1 m*M* β-mercaptoethanol and 0.1% pepstatin A) and then homogenized with an ultrasonic processor (Branson sonifier model

350). The solution was centrifuged (Sorvall GS3, 4200*g*, 1 h) and the cell debris was discarded. The cell extract was heated and kept between 348 and 353 K for 10 min, taking 5 min to reach 348 K from room temperature, in order to denature contaminating heat-labile proteins. After being chilled on an ice bath, the cell extract was centrifuged (Sorvall GS3, 4200*g*, 1 h). The supernatant was subject to column chromatography using DEAE–Sephacel employing a linear gradient of 0–500 m*M* NaCl in buffer *P* (50 m*M* potassium phosphate pH 7.0) and subsequently on a hydrophobic interaction column (phenyl-Toyopearl TSK) with a linear gradient of 0.8–0.4 *M* ammonium sulfate in buffer *P*.

The optimum reaction temperature was determined by incubating a mixture of the substrate (100 m*M* D-fructose), buffer (100 m*M* HEPES, pH 7.5), metal ion (2.5 m*M* $MnCl_2$) and xylose isomerase at different temperatures for 5 min. The reaction mixture was then chilled on ice to stop the reaction, and the D-glucose in solution was quantified using the peroxidase–glucose oxidase (PGO) assay kit from Sigma. The effect of divalent metal ions (Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}) and EDTA was also examined using a metal-ion concentration of 5 m*M*. The isomerization reaction was carried out at 356 K for 15 min with 100 m*M* D-fructose as the substrate.

2.2. Crystallization

The purified enzyme was concentrated to about 10 mg ml^{-1} concentration using a YM30 ultrafiltration membrane (Amicon) and was dialyzed against 50 m*M* sodium phosphate pH 7.2 for crystallization. The protein concentration was estimated by measuring the absorbance at 280 nm, assuming a correspondence of 1.0 mg ml^{-1} concentration to the unit absorbance for 1.0 cm path length. Crystallization was performed by the hanging-drop vapour-diffusion method using 24-well tissue-culture plates (Linbro) at room temperature. Hanging drops were prepared by mixing 4 µl each of the protein and the reservoir solutions. The protein solution contained TcaXI at 10 mg ml^{-1} concentration in 50 m*M* sodium phosphate buffer at pH 7.2. Initial searches for crystallization was performed using Screen I (Jancarik & Kim, 1991) and Screen II conditions (Hampton Research).

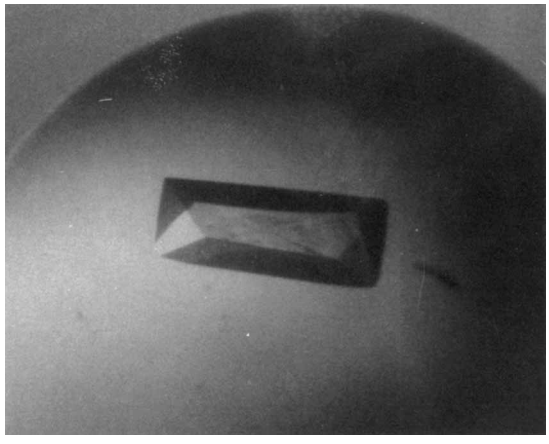

Figure 1
An orthorhombic crystal of TcaXI. Its approximate dimensions are 0.2 × 0.2 × 0.5 mm.

2.3. X-ray data collection

A crystal was mounted in a thin-walled glass capillary and the capillary was sealed with wax, after filling both ends with mother liquor. Initial X-ray experiments were carried out using graphite-monochromated Cu $K\alpha$ X-rays from a rotating-anode generator (Rigaku RU-200BH), running at 40 kV and 70 mA with a 0.3 mm focus cup. A set of native data was collected on the FAST area-detector system (Enraf-Nonius, Delft) using the *MADNES* software (Messerschmidt & Pflugrath, 1987). The unit-cell parameters were determined by the autoindexing and parameter-refinement procedure of *MADNES*. The reflection intensities were obtained by the profile-fitting procedure (Kabsch, 1988) and the data were scaled by the Fourier scaling program (Weissman, 1982).

Later, synchrotron X-rays were used to collect the data to higher resolution from two crystals mounted in different orientations. X-ray data were collected at 287 K using a Weissenberg camera for macromolecular crystallography at the BL-6A2 experimental station of Photon Factory, Tsukuba, Japan (Sakabe, 1991). The wavelength of the synchrotron X-rays was 1.00 Å and a 0.1 mm collimator was used. A Fuji image plate (type BAIII, 20 × 40 cm) was placed at a distance of 429.7 mm from the crystal. Coupling constants were either 1.2 or 1.5° mm^{-1} depending on the crystal orientation. The oscillation range per image plate was 3.5°, allowing an overlap of 0.5° between two contiguous image plates.

The diffraction patterns recorded on the image plates were digitized by a Fuji BA100 scanner. The raw data were processed using the programs *WEIS* (Higashi, 1989) and the data were scaled with *SCALA* and *AGROVATA* in the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

3. Results

3.1. Optimum temperature and the effect of metal ions

The activity of TcaXI increased as the temperature was raised and showed a maximum at 363 K (data not shown). The optimum reaction temperature of 363 K for this XI is the highest among type I XIs. When the effect of divalent metal ions on the activity of TcaXI was examined, the maximum activity was seen with either Mn^{2+} or Co^{2+}. Approximately 40% of the maximum activity was observed with Mg^{2+} and little activity with other metal ions such as Ca^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} or with EDTA (data not shown).

3.2. Crystallization and X-ray data collection

Rod-shaped crystals grew to typical dimensions of 0.2 × 0.2 × 0.5 mm within one month (Fig. 1), under the optimized reservoir-solution conditions of 1.6–1.8 *M* ammonium sulfate and 100 m*M* *N*-(2-acetamido)-2-iminodiacetic acid (ADA) (final pH 5.95).

Upon exposure to Cu $K\alpha$ X-rays, the crystals of TcaXI initially showed diffraction to 2.0 Å Bragg spacing. However, the crystals were somewhat radiation sensitive and a set of native data has been collected to approximately 3.5 Å resolution. The data consist of 37451 measurements of 17070 unique reflections with an $R_{\rm merge}$ of 4.5%. The merged data set is 89% complete in the 15–3.5 Å range. The space group is $P2_12_12_1$, as indicated by the Laue symmetry and systematic absences of the reflection data, and the unit-cell parameters are a = 84.45, b = 123.80, c = 140.81 Å. The presence of one tetrameric XI in the asymmetric unit gives a crystal volume per protein mass (V_m) of 2.1 Å^3 Da^{-1} and a solvent content of 41% by volume (Matthews, 1968).

Later, data extending to higher resolution were collected using synchrotron X-rays. Although the initial diffraction pattern showed reflections to 1.7 Å Bragg spacing, the data beyond 2.1 Å disappeared after the crystal was exposed for about 1 h. The synchrotron data, consisting of 208259 measurements of 68336 unique reflections, gave an $R_{\rm merge}$ of 10.0%. The data completeness was 76% for the 8.0–2.3 Å range and 47% for the 2.4–2.3 Å shell. Unit-cell para-

meters of a = 84.35, b = 123.60, c = 140.24 Å were obtained.

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