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Crystallization and preliminary X-ray studies of Trp138Phe/Val185Thr xylose isomerases from *Thermotoga neapolitana* and *Thermoanaerobacterium thermosulfurigenes*

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Xylose isomerases from *Thermotoga neapolitana* (TNXI) and *Thermoanaerobacterium thermosulfurigenes* (TTXI) share 70.4% sequence identity and are thermostable. The double mutants Trp138Phe/Val185Thr of TNXI and TTXI have higher catalytic efficiencies than TNXI and TTXI, respectively. The Trp138Phe/Val185Thr TNXI and TTXI mutants were overexpressed in *Escherichia coli* strain BL21(DE3) and purified. Crystals of the two proteins were grown with polyethylene glycol 8000 as the major precipitant by the hanging-drop vapour-diffusion method. Crystals of the TNXI mutant were obtained in the absence of substrate, in complex with glucose and in complex with fructose. Crystals of the TTXI mutant were obtained complexed with glucose. Diffraction data were collected at 1.9, 2.1 and 2.1 Å resolution for the fructose–TNXI mutant, glucose–TNXI mutant and substrate-unbound TNXI mutant, respectively. The diffraction data for the glucose–TTXI mutant were collected at 2.0 Å resolution. The crystals belong to the orthorhombic space groups $C222_1$ (TNXI mutant) and $P2_12_12_1$ (TTXI mutant). The TNXI and TTXI mutant crystals contain two and four monomers in the asymmetric unit, respectively.

1. Introduction

Xylose isomerase (XI) is an enzyme that catalyzes the reversible isomerization of an aldo sugar D-xylose to a keto sugar D-xylulose. This isomerization activity of XI plays an important role in the pentose phosphate pathway. XI can catalyze the isomerization of D-glucose to D-fructose *in vitro* and for this reason it is also known as 'glucose isomerase' (Takasaki *et al.*, 1969; Zhu *et al.*, 1999). Therefore, this protein is used widely in industry to produce high-fructose corn syrups (Jenkins *et al.*, 1992; Lee & Zeikus, 1991). Because of its usefulness, many biologists have tried to engineer a mutant XI that would have increased activity towards glucose. In the isomerization process, the fructose yield is dependent on the reaction temperature: the equilibrium between D-glucose and D-fructose is shifted toward D-fructose at higher temperature (Smith *et al.*, 1991). For this reason, a thermostable XI would be beneficial for fructose production.

XIs are divided into two classes: type I enzymes are shorter than type II enzymes by about 50 amino acids at their N-terminus (Vangrysperre *et al.*, 1990). Type I XIs were obtained from genera such as *Streptomyces* and *Thermus* and type II XIs from genera such as *Bacillus*, *Thermotoga* and *Thermoanaerobacterium*. Three-dimensional structures of XIs have been published only for type I enzymes (Chang *et al.*, 1999; Dauter *et al.*, 1990; Henrick *et al.*, 1989; Rey *et al.*, 1988).

Thermotoga neapolitana produces a hyperthermostable XI (TNXI) that is optimally active at 368 K and *Thermoanaerobacterium thermosulfurigenes* produces a thermostable XI (TTXI) with an optimum temperature of 358 K. The two enzyme sequences are 70.4% identical (Vieille *et al.*, 1995). TNXI and TTXI form tetramers with molecular weights of 202.8 kDa (*i.e.* 50.7 kDa monomers) and 201.2 kDa (*i.e.* 50.3 kDa monomers), respectively. Both enzymes are type II XIs. Because it is more thermostable than any other known XI (Vieille *et al.*, 1995), TNXI represents a potentially interesting catalyst for industrial fructose corn syrup production. TNXI and TTXI are significantly stabilized and activated in the presence of divalent cations, especially Mg^{2+} or Co^{2+} (Hess *et al.*, 1998).

Engineering of the active site may be very useful for the development of an effective glucose isomerase. In previous reports, the double mutation Trp138Phe/Val185Thr in TNXI and TTXI enhanced the enzymes' catalytic efficiencies on glucose by decreasing the K_M for glucose and increasing k_{cat}/K_M. This double mutation vastly improved TTXI's catalytic efficiency on glucose by 5.7 times (Meng *et al.*, 1991; Sriprapundh *et al.*, 2000). However, this double mutation in TNXI was not as catalytically efficient as that of TTXI. Understanding this difference between TNXI

and TTXI mutants is of importance because TNXI is a hyperthermostable enzyme and would have advantages in the high-fructose corn syrup production industry. We expect that crystallographic studies of the two proteins will contribute to understanding the structural basis of the different catalytic efficiencies of the TNXI and TTXI mutants. The crystallization only of wild-type TTXI and TNXI were reported several years ago (Chayen *et al.*, 1997; Lloyd *et al.*, 1994). However, until now no crystallizations have been reported of any of their mutant derivatives despite their biological and industrial significance. In this paper, we describe the crystallization and preliminary X-ray diffraction experiments of the double mutants TNXI Trp138Phe/Val185Thr and TTXI Trp138Phe/Val185Thr.

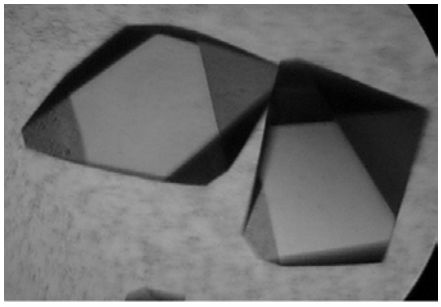
2. Materials and methods

2.1. Expression and purification

The recombinant TNXI Trp138Phe/Val185Thr and TTXI Trp138Phe/Val185Thr proteins were produced from the *Thermotoga neapolitana* and *Thermoanaerobacterium thermosulfurigenes xylA* mutant genes cloned in pET22b(+) (Sriprapundh *et al.*, 2000) and in pMMB67EH, respectively, and expressed in *E. coli* BL21(DE3). The cultures were grown at 310 K until OD_{600} = 1.4 was reached. Expression was then induced with 1 m*M* isopropyl β-D-thiogalactopyranoside at 300 K for 4 h. Harvested cells were resuspended in buffer *A* (5 m*M* $MgSO_4$ and 0.5 m*M* $CoCl_2$ in 50 m*M* Tris–HCl pH 8.0) and broken using a French press. Cell debris was removed by centrifugation at 14 000*g* for 20 min. The supernatant was heat-treated at 358 K for 15 min and cooled in ice for 10 min before being centrifuged at 14 000*g* for 20 min to remove the precipitated proteins. Ammonium sulfate at 30% saturation was then added to the supernatant to remove other impurities. Again, the solution was centrifuged at 14 000*g* for 20 min and ammonium sulfate at 80% saturation was added to the supernatant. Pellets recovered by centrifugation at 14 000*g* for 20 min were solubilized in buffer *A* and dialyzed against buffer *A*. After dialysis, the recombinant enzymes were further purified by chromatography on HighTrap Q and Superdex 200 (Pharmacia). Highly purified enzyme suitable for crystallization was obtained. Proteins were concentrated to 13 mg ml^{-1} and stored at 203 K.

2.2. Crystallization

Crystallization trials were carried out using the hanging-drop vapour-diffusion method at room temperature. An initial crystallization trial was performed using Crystal Screens I and II (Hampton Research). Microcrystals were grown in Crystal Screen II Nos. 30 and 46. Crystallization conditions were then refined by varying several conditions of precipitants, pH and additives. To obtain crystals suitable for X-ray studies, a droplet was prepared by mixing 1 µl of the protein solution (100 m*M* KCl, 5 m*M* $MgSO_4$, 0.5 m*M* $CoCl_2$, 20 m*M* Tris–HCl pH 8.0) with an equal volume of the reservoir solution [10–12%(*w*/*v*) polyethylene glycol (PEG) 8000, 10%(*v*/*v*) glycerol, 5%(*v*/*v*) 2-methyl-2,4-pentanediol (MPD), 100 m*M* HEPES–NaOH pH 7.5]. Crystals of the glucose-complex and the fructose-complex TNXI mutants were obtained by addition to the reservoir solution of glucose and fructose, respectively, to a final concentration of 100 m*M*. Single orthorhombic crystals of TNXI Trp138Phe/Val185Thr grew to typical dimensions of 0.3 × 0.3 × 0.3 mm within 10 d. Although they were grown under identical conditions, the TTXI Trp138Phe/Val185Thr crystals were smaller than those of TNXI Trp138Phe/Val185Thr (typical dimensions of 0.2 × 0.2 × 0.2 mm).



(*a*)

(*b*)

Figure 1
Photographs of orthorhombic crystals of the TNXI and TTXI mutants. (*a*) Crystals of fructose-complex TNXI mutant. The approximate dimensions are 0.3 × 0.3 × 0.3 mm. (*b*) Crystal of glucose-complex TTXI mutant. Its approximate dimensions are 0.2 × 0.2 × 0.2 mm. See the text for detailed crystallization conditions.

2.3. Data collection and processing

For the cryogenic experiments, a suitable cryoprotectant was determined to be 12% PEG 8000, 15% glycerol, 5% MPD, 100 m*M* HEPES–NaOH pH 7.5. Crystals were soaked in 5 µl of cryosolvent for 1 min. The data set was collected on an R-AXIS IV image-plate system (Rigaku Co.) attached to a Rigaku rotating-anode generator (RU-300) providing Cu $K\alpha$ radiation at 50 kV and 90 mA in a nitrogen-gas stream at 110 K (Oxford Cryosystems). The resolution of these data was 3.0 Å. To obtain high-resolution data, crystals of fructose–TNXI mutant, glucose–TNXI mutant, substrate-unbound TNXI mutant and glucose–TTXI mutant were examined at the Photon Factory in Japan using the X-ray beamline 18B from a 2.5 GeV synchrotron-radiation source and data sets were collected at 100 K. A total of 200 frames with 0.5° oscillation were measured at a wavelength of 1.00 Å using a crystal-to-detector distance of 160 mm. The images were processed with *MOSFLM* (Leslie, 1994).

3. Results and discussion

Protein purification was carried out using heat treatment, ammonium sulfate precipitation, ion exchange on a HighTrap Q column and gel filtration on a Superdex 200 column. Protein purity was judged by SDS–PAGE (data not shown).

Preliminary crystallization trials using Crystal Screens I and II from Hampton Research yielded crystals using PEG MME 550 as the precipitant. However, the size and quality of the crystals were not suitable for data collection. Several kinds of PEG were tested to improve the size and quality of the crystals. PEG 8000 was found to be an optimal precipitant for crystals suitable for data collection. Crystallization conditions were further refined using the additives introduced in §2. The enzymes were co-crystallized with fructose and glucose to visualize the interactions between the substrate and amino acids in the active site and to design enzyme mutants that are more effective in glucose isomerization than the TNXI and TTXI double mutants. They grew as single orthorhombic crystals with typical dimensions of 0.3 × 0.3 × 0.3 mm (TNXI mutant) and 0.2 × 0.2 × 0.2 mm (TTXI mutant) (Fig. 1).

The data sets were collected at 1.9, 2.1, 2.1 and 2.0 Å for the fructose–TNXI mutant,

glucose–TNXI mutant, substrate-unbound TNXI mutant and glucose–TTXI mutants, respectively, using the synchrotron-radiation source at the Photon Factory in Japan. The statistics of the data are summarized in Table 1. Crystals have an orthrhombic lattice, with unit-cell parameters a = 121.2, b = 160.8, c = 97.7 Å for the fructose–TNXI mutant, a = 121.7, b = 160.8, c = 97.5 Å for the glucose–TNXI mutant, a = 121.3, b = 161.2, c = 97.2 Å for the substrate-unbound TNXI mutant and a = 152.6, b = 155.8, c = 84.8 Å for the glucose–TTXI mutant. The space groups are $C222_1$ and $P2_12_12_1$ for the TNXI and TTXI mutants, respectively. The asymmetric unit contains two (TNXI mutant) and four (TTXI mutant) monomers, as reasonable values of 2.4 and 2.5 Å^3 Da^{-1} are obtained for the Matthews coefficient (V_M) for the TNXI and TTXI mutants, respectively. The calculated solvent contents are 47% for the TNXI mutant and 49% for the TTXI mutant: these values are well within the range of previously observed protein crystals (Matthews, 1968). To obtain the phase information, molecular replacement was performed with the program *AMoRe* (Navaza, 1994) using the models from the previously solved wild-type TNXI and TTXI structures at 2.7 and 2.5 Å resolution, respectively (PDB codes 1a0e for TNXI and 1a0c for TTXI). These structures are as yet unpublished.

Table 1
Summary of the data statistics of the TNXI and TTXI mutants.

Values in parentheses are for the highest resolution shell.

Data set	Substrate-unbound TNXI Trp138Phe/ Val185Thr	Glucose–TNXI Trp138Phe/ Val185Thr	Fructose–TNXI Trp138Phe/ Val185Thr	Glucose–TTXI Trp138Phe/ Val185Thr
X-ray source	PF (BL-18B)	PF (BL-18B)	PF (BL-18B)	PF (BL-18B)
Wavelength (Å)	1.00	1.00	1.00	1.00
Space group	$C222_1$	$C222_1$	$C222_1$	$P2_12_12_1$
Unit-cell parameters (Å)	a = 121.3	a = 121.7	a = 121.2	a = 152.6
	b = 161.2	b = 160.8	b = 160.8	b = 155.8
	c = 97.2	c = 97.5	c = 97.7	c = 84.8
Resolution (Å)	2.1	2.1	1.9	2.0
Total No. of reflections	267561	223746	274107	429438
No. of unique reflections	55823	55983	69265	133232
Completeness (%)	100 (100)	100 (100)	99.5 (99.5)	99.6 (90.3)
R_{merge}† (%)	5.7 (25.6)	5.7 (28.5)	6.2 (32.5)	10.8 (43.7)
Mean $I/\sigma(I)$	9.9 (2.9)	10.8 (2.7)	8.4 (2.0)	6.8 (1.7)

† $R_{merge}(I) = \sum_h \sum_i |I_i - I| / \sum_h \sum_i I_i$, where I is the mean intensity of i reflections h.

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