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Protein preparation, crystallization and preliminary X-ray crystallographic studies of a thermostable hypoxanthine–guanine phosphoribosyltransferase from *Thermoanaerobacter tengcongensis*

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Native and His-tagged mutant (L160I) hypoxanthine–guanine phosphoribosyltransferase (HGPRT) from *Thermoanaerobacter tengcongensis* were cloned, expressed in *Escherichia coli* and purified. Both proteins were crystallized with polyethylene glycol as the main precipitant at 293 K using the hanging-drop vapour-diffusion method. The crystal of native HGPRT belongs to space group $C222_1$, with unit-cell parameters $a = 65.77$, $b = 137.73$, $c = 95.27$ Å, and diffracted to 2.2 Å resolution on an in-house X-ray generator. The crystal of the His-tagged mutant (L160I) HGPRT belongs to the space group $I222$, with unit-cell parameters $a = 52.21$, $b = 88.36$, $c = 93.03$ Å, and diffracted to 1.7 Å resolution in-house.

1. Introduction

The *de novo* synthesis of purine nucleotides requires a minimum of ten enzymes for the production of inosine monophosphate (IMP), the major intermediate of purine metabolism. The salvage pathway of nucleosides provides an energy-saving alternative and the few enzymes involved can be divided into purine and pyrimidine phosphoribosyltransferases. Hypoxanthine–guanine phosphoribosyltransferases (HGPRT; EC 2.4.2.8) are purine-salvage enzymes that catalyze Mg^{2+}-dependent transfer of the 5-phosphoribosyl group from α-D-5-phosphoribosyl-1-pyrophosphate (PRPP) to the N9 position of 6-oxopurines (hypoxanthine, guanine) to form the corresponding ribonucleotides IMP and guanosine monophosphate (GMP), with the release of inorganic pyrophosphate (PP_i) (Hochstadt, 1978).

Purine phosphoribosyltransferases have been of significant interest both in human genetic diseases and in parasite treatments, where the enzymes are potential drug targets. The study of purine-salvage enzymes is medically important since disease states such as gouty arthritis and Lesch–Nyhan Syndrome result from amino-acid mutations in the human hypoxanthine phosphoribosyltransferases (HPRT) that cause partial or complete loss of HPRT activity (Patel & Caskey, 1985). In free-living organisms including humans, purine nucleotides can be generated *via de novo* synthesis as well as by the salvage of preformed bases. In contrast, many parasitic organisms are unable to synthesize purines *via de novo* pathways and must rely on the enzymes in salvage pathways; therefore, HPRTs have also been targeted as potential therapeutic agents for the treatment of diseases caused by parasites such as malaria (Craig & Eakin, 2000). For these reasons, considerable interest has been focused on the understanding of this class of enzymes with regard to the structural determinants for substrate binding and catalysis. Three-dimensional structures of human HPRT (Eads *et al.*, 1994; Shi, Li, Tyler, Furneaux, Grubmeyer *et al.*, 1999), *Escherichia coli* HPRT (Guddat *et al.*, 2002), *Toxoplasma gondii* HGPRT (Heroux *et al.*, 1999), *Plasmodium falciparum* HGPRT (Shi, Li, Tyler, Furneaux, Cahill *et al.*, 1999), *Trypanosoma cruzi* HPRT (Focia *et al.*, 1998) and *Tritrichomonas foetus* HGPRT (Somoza *et al.*, 1996) have so far been reported.

Thermoanaerobacter tengcongensis is a rod-shaped Gram-negative anaerobic eubacterium that grows in freshwater hot springs in the temperature range 323–353 K, optimally at 348 K, and in the pH range 5.5–9.0 (Xue *et al.*, 2001). The complete genome of *T. tengcongensis* has been sequenced (Bao *et al.*, 2002) and the gene encoding HGPRT has been identified. It encodes an open reading frame of 181 amino acids, which bears about 45% identity to HGPRT from *E. coli*. Owing to the thermal stability and alkaline resistance of *T. tengcongensis* HGPRT, it has potential industrial value for the production of nucleotide intermediates. We have thus initiated structural studies on *T. tengcongensis* HGPRT in order to further understand its catalytic mechanism and thermal stability. We report

here preliminary crystallographic results from *T. tengcongensis* HGPRT crystals in the presence of IMP or GMP.

2. Materials and methods

2.1. Protein expression and purification of native HGPRT

The native HGPRT gene was amplified by PCR from genomic *T. tengcongensis* DNA and cloned into expression vector pET-11a-DEST using the Invitrogen Gateway Cloning System (Walhout *et al.*, 2000). The protein was expressed in *E. coli* strain BL21 (DE3) pLysS. Cells carrying the HGPRT expression vector were first grown in 20 ml LB medium containing ampicillin (100 µg ml^{-1}) and chloramphenicol (34 µg ml^{-1}) at 310 K. This culture was then transferred to 1 l LB medium containing ampicillin and chloramphenicol and was grown at 310 K. When the optical density (OD) at 600 nm in the culture reached a value of 0.8, protein expression was induced by the addition of 0.5 m*M* isopropylthio-β-D-galactoside (IPTG). Cells were grown for a further 7 h at 310 K and were then harvested by centrifugation at 5000*g* for 15 min. After harvesting, the cell pellet was suspended in 20 m*M* Tris–HCl buffer pH 8.0 and lysed by sonication. After centrifugation, the supernatant was loaded at 281 K onto a diethylaminoethyl (DEAE) Sepharose column and the elution fraction between 50 and 120 m*M* NaCl was further purified using HiTrap-Q and Superdex-75 columns (Amersham Pharmacia Biotech) at 298 K. The final yield is about 10 mg purified protein per litre of culture, with at least 95% purity.

2.2. Protein preparation and purification of mutant L160I HGPRT

In order to prepare the HGPRT mutant (L160I) protein, the coding sequence for *T. tengcongensis* HGPRT was amplified using the polymerase chain reaction (PCR) technique and was then cloned into the *Nde*I and *Bam*HI sites of the pET15b vector (Novagen Inc.) using T4 DNA ligase. This resulted in an N-terminal His tag in the recombinant protein. The L160I mutation in *T. tengcongensis* HGPRT gene was created using the GeneEditor *in vitro* site-directed mutagenesis system from Promega, USA according to the technical manual. Plasmid DNA from a positive clone was sequenced to confirm the produced mutation and subsequently transformed into *E. coli* BL21 (DE3) pLysS competent cells.

The protein expression was performed in a very similar way to that of the native HGPRT described above, *i.e.* BL21 (DE3) pLysS cells carrying the pET15b-HGPRT plasmid were first grown overnight in 20 ml LB medium containing ampicillin and chloramphenicol. This culture was then transferred to 1 l LB medium in the presence of antibiotics and was grown at 310 K. When the $OD_{600\ nm}$ of the culture reached a value of 0.8, expression was induced by the addition of 0.5 m*M* IPTG. Cells were grown for a further 7 h at 310 K and were then harvested by centrifugation at 5000*g* for 15 min. The pellets were suspended in 25 ml buffer *A* (20 m*M* Tris–HCl pH 7.4, 300 m*M* NaCl) and disrupted by sonication. Cell debris was removed by centrifugation at 16 000*g* for 50 min. The crude extract was applied onto a 5 ml HiTrap affinity column (Amersham Pharmacia Biotech) charged with nickel and pre-equilibrated with buffer *A*. The column was washed with ten column volumes of buffer *A* and five column volumes of buffer *A* containing 100 m*M* imidazole. Protein was eluted with buffer *A* containing 500 m*M* imidazole. Fractions were analyzed by SDS–PAGE and those containing L160I HGPRT were pooled and concentrated to 2 ml in Centricon Plus-20 (PL-10) centrifugal filter devices (Millipore). To obtain a homogenous pool of protein for crystallization trials, the sample was loaded at 298 K onto Superdex-75 HR 16/60 (Amersham Pharmacia Biotech) equilibrated with buffer *B* (20 m*M* Tris–HCl pH 7.4, 150 m*M* NaCl) using an ÄKTA FPLC system (Amersham Pharmacia Biotech). Proteins were eluted with the same equilibration buffer with a flow rate of 1.0 ml min^{-1}. Fractions containing pure L160I HGPRT were identified by SDS–PAGE and pooled. This protocol yields approximately 28 mg of 95% pure protein from 1 l of culture.

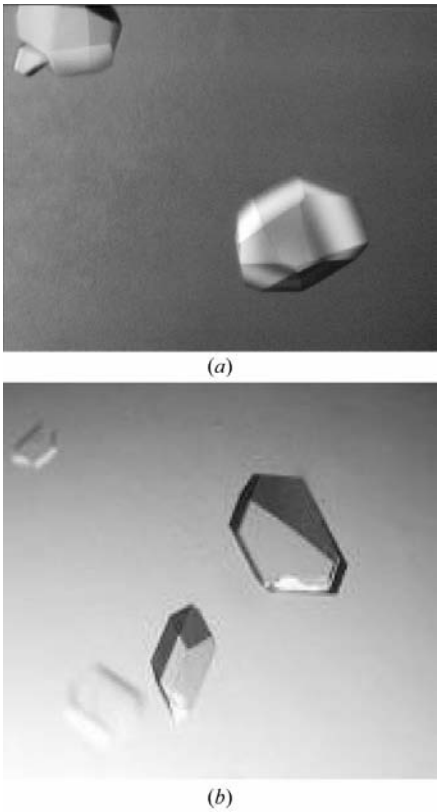

Figure 1
Microphotographs of HGPRT crystals.

2.3. Crystallization

The purified native and L160I HGPRT used for crystallization trials were concentrated to about 10 mg ml^{-1}, the buffer was exchanged to 20 m*M* Tris–HCl pH 7.4 using Centricon Plus-20 (PL-10) centrifugal filter devices (Millipore) and excess IMP and GMP were added in a 4:1 molar ratio to native and L160I HGPRT, respectively. Crystallization trials were conducted at 293 K in 16-well cell-culture plates by the hanging-drop vapour-diffusion method. Hampton Research Crystal Screen kits I and II (Hampton Research, Riverside, CA, USA) were used for initial screening. 1 µl protein solution was mixed with 1 µl crystallization solution on a siliconized glass cover slip, which was then sealed with high-vacuum grease over a well containing a 0.4 ml reservoir of the respective crystallization solutions. Single crystals of L160I HGPRT–GMP were obtained at 293 K using condition No. 14 (0.2 *M* calcium chloride dihydrate, 0.1 *M* HEPES pH 7.5, 28% polyethylene glycol 400) from Hampton Research Crystal Screen within two weeks. The crystal reached approximate dimensions of 50 × 80 × 100 µm (Fig. 1*a*). Single crystals of native HGPRT–IMP were obtained at 293 K using condition No. 18 of Hampton Research Crystal Screen within one week; the condition was then optimized to 0.2 *M* magnesium acetate tetrahydrate, 0.1 *M* sodium cacodylate pH 6.5, 12% polyethylene glycol 4000. The crystal reached approximate dimensions of 40 × 100 × 150 µm (Fig. 1*b*) within one week.

2.4. Data collection and processing

X-ray diffraction experiments were performed with a Bruker SMART-6000 CCD detector (Bruker AXS GmbH, Germany) mounted on a Bruker–Nonius FR591 X-ray generator with Cu $K\alpha$ radiation (λ = 1.5418 Å) operated at 45 kV and 110 mA. The crystal-to-detector distance

Table 1
Crystallographic parameters and data-collection statistics.

Values in parentheses are for the last resolution shell.

Crystals	L160I HGPRT–GMP	Native HGPRT–IMP
Crystal parameters		
Space group	*I*222	$C222_1$
Resolution (Å)	64.1–1.7 (1.79–1.70)	59.5–2.2 (2.30–2.20)
Unit-cell parameters		
a (Å)	52.21	65.77
b (Å)	88.36	137.73
c (Å)	93.03	95.27
Matthews coefficient ($Å^3$ Da^{-1})	2.33	2.35
Solvent content (%)	45.2	45.4
Data processing		
No. reflections observed	82823	190570
No. unique reflections	23486	28032
$I/\sigma(I)$	14.4 (3.9)	10.94 (2.14)
Completeness (%)	97.4 (87.3)	93.07 (78.9)
Multiplicity	3.53 (1.72)	9.15 (3.96)
R_{merge}† (%)	3.79 (10.76)	5.20 (19.92)

† $R_{merge} = \sum |I_{obs} - I_{avg}| / \sum I_{obs}$, where the summation is over all reflections.

was typically 5 cm, the ω oscillation angle was 0.2° and 900 frames were collected, with the exposure time typically being 60 s per frame. During the data collection, the crystal was maintained at 100 K using nitrogen gas without cryoprotection. Data reduction was performed with the Bruker *Proteum* package (Bruker Advanced X-ray Solutions, 2003).

3. Results and discussions

The L160I HGPRT crystal diffracted to better than 1.7 Å resolution. The crystal belongs to space group *I*222, with unit-cell parameters $a = 52.21$, $b = 88.36$, $c = 93.03$ Å. There is one HGPRT molecule per asymmetric unit assuming a V_M value of 2.33 $Å^3$ Da^{-1} (Matthews, 1968), corresponding to a solvent content of 45%. Overall and outer-shell completeness of the data were 97.4 and 87.3%, respectively. The R_{merge} among symmetry-related reflections was 3.79% overall (see Table 1 for data-collection statistics).

The native HGPRT crystal belongs to space group $C222_1$, with unit-cell parameters $a = 65.77$, $b = 137.7$, $c = 95.27$ Å. There are two HGPRT molecules in each asymmetric unit assuming a V_M value of 2.35 $Å^3$ Da^{-1}. The data were processed to 2.2 Å resolution. The crystallographic parameters and data-collection statistics are listed in Table 1. Structure determination by the molecular-replacement methods is now under way for both crystals.

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