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Cloning, expression, purification, crystallization and preliminary X-ray crystallographic study of the putative SAICAR synthetase (PH0239) from *Pyrococcus horikoshii* OT3

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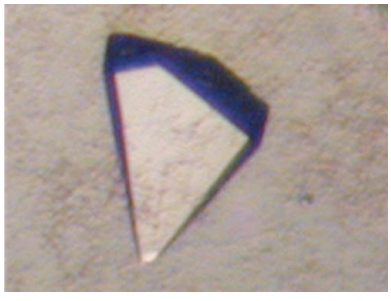

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The study of proteins involved in *de novo* biosynthesis of purine nucleotides is central in the development of antibiotics and anticancer drugs. In view of this, a protein from the hyperthermophile *Pyrococcus horikoshii* OT3 was isolated, purified and crystallized using the microbatch method. Its primary structure was found to be similar to that of SAICAR synthetase, which catalyses the seventh step of *de novo* purine biosynthesis. A diffraction-quality crystal was obtained using Hampton Research Crystal Screen II condition No. 34, consisting of 0.05 *M* cadmium sulfate hydrate, 0.1 *M* HEPES buffer pH 7.5 and 1.0 *M* sodium acetate trihydrate, with 40%(*v*/*v*) 1,4-butanediol as an additive. The crystal belonged to space group $P3_1$, with unit-cell parameters $a = b = 95.62$, $c = 149.13$ Å. Assuming the presence of a hexamer in the asymmetric unit resulted in a Matthews coefficient (V_M) of 2.3 Å^3 Da^{-1}, corresponding to a solvent content of about 46%. A detailed study of this protein will yield insights into structural stability at high temperatures and should be highly relevant to the development of antibiotics and anticancer drugs targeting the biosynthesis of purine nucleotides.

1. Introduction

Pyrococcus horikoshii OT3, which was found in a hydrothermal vent at the Okinawa Trough (González *et al.*, 1998), grows at an optimal temperature of 371 K but can survive at higher temperatures of up to 378 K. The complete genome sequence of this organism has been determined (Kawarabayasi *et al.*, 1998). The proteins found in such hyperthermophiles have comparatively high thermal stabilities. Extensive studies of hyperthermophilic proteins have revealed some important clues regarding their tertiary structure and stability. The protein from *P. horikoshii* OT3 considered in the present study is 238 amino acids in length and its primary structure is 52.8% identical to that of an enzyme involved in nucleotide biosynthesis called phosphoribosylaminoimidazole-succinocarboxamide (SAICAR) synthetase. Nucleotide biosynthesis is an essential process in all living forms, playing a major role in replication, universal energy reserves, enzyme cofactors and signal transduction molecules. Because of its role in replication and other important cellular activities, the enzymes involved are potential drug targets against disease-causing microorganisms. *De novo* biosynthesis of purine nucleotides involves the synthesis of purines from simpler compounds such as glutamine, glycine, formate, aspartate and carbon dioxide. The enzyme SAICAR synthetase (EC 6.3.2.6) catalyzes the seventh step in *de novo* purine biosynthesis (Lukens & Buchanan, 1959): the formation of *N*-succinyl-5-aminoimidazole-4-carboxamide ribonucleotide (SAICAR) from carboxyaminoimidazole ribonucleotide (CAIR) and aspartic acid in the presence of ATP (Fig. 1).

Structures of SAICAR synthetase from various sources have been obtained by X-ray crystallography, including those from *Saccharomyces cerevisiae* (Levdikov *et al.*, 1998), *Thermatoga maritima* (Zhang *et al.*, 2006), *Escherichia coli* (Ginder *et al.*, 2006), *Geobacillus kaustophilus* (PDB code 2ywv; Y. Kitamura, S. Yokoyama & S. Kuramitsu, unpublished work) and *Methanocaldococcus jannaschii* (PDB codes 2yzl and 2z02; M. Kanagawa, S. Baba, K. Fukui, S. Kuramitsu, S. Yokoyama, G. Kawai & G. Sampei, unpublished work). Two of the

above-mentioned organisms (*T. maritima* and *G. kaustophilus*) are hyperthermophilic bacteria, while *M. jannaschii* is a hyperthermophillic archaeon. Structures of the protein in several complexes, such as those with ATP, ADP, aminoimidazole 4-carboxamide ribonucleotide (AICAR) and aspartic acid, have also been investigated using X-ray crystallography (Antonyuk *et al.*, 2001; Ginder *et al.*, 2006). The enzyme SAICAR synthetase has also been found to play a role in the conversion of L-alanosine (an anticancer agent) to L-alanosine-AICOR (L-alanosyl-5-amino-4-imidazole carboxylic acid), an anabolite which inhibits the enzyme adenylosuccinate lyase, thus preventing the formation of AMP and subsequent cell division (Tyagi *et al.*, 1981). SAICAR synthetase from *P. horikoshii* (an archaeon) has a sequence identity of 52% to that from *M. jannaschii*. The survival temperature of *P. horikoshii* is much higher than those of the other hyperthermophiles mentioned. Thus, the present study should provide possible clues about the structural features that are responsible for its stability at higher temperatures. Furthermore, it is expected to enhance the research interests of structural biologists involved in the study of thermophilic organisms.

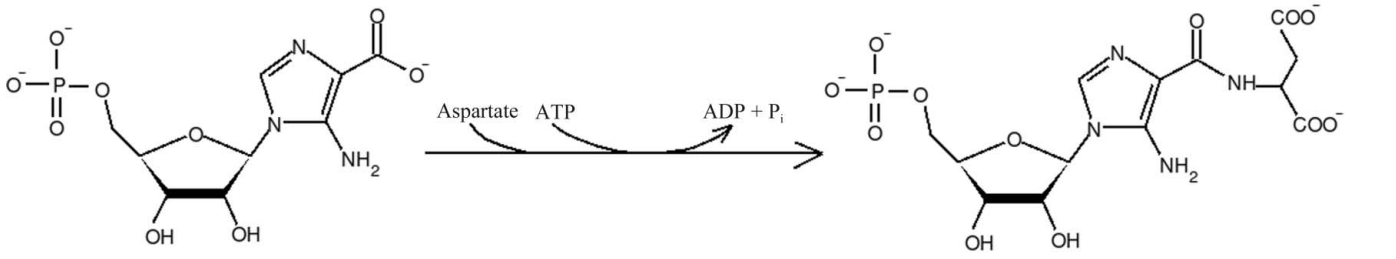
2. Materials and methods

2.1. Cloning, expression and purification

The *PH*0239 gene was amplified by the polymerase chain reaction (PCR) using *P. horikoshii* OT3 genomic DNA as a template and cloned into *E. coli* expression plasmid pET-11a. *E. coli* BL21-CodonPlus (DE3)-RIL cells were transformed with the recombinant plasmid and grown at 310 K in LB medium containing 50 µg ml^{-1} ampicillin for 20 h. The cells were harvested by centrifugation and suspended in 20 m*M* Tris–HCl pH 8.0 (buffer *A*) containing 0.5 *M* NaCl, 5 m*M* *β*-mercaptoethanol and 1 m*M* phenylmethylsulfonyl fluoride and lysed by sonication. The cell lysate was heated at 363 K for 13 min. Denatured proteins were then removed by centrifugation (15 000 rev min^{-1}, 30 min) and the supernatant solution was used as the crude extract for purification. The crude extract was desalted using a HiPrep 26/10 desalting column (GE Healthcare) and applied onto a Super Q Toyopearl 650M column (Tosoh) equilibrated with buffer *A*. The protein was eluted with a linear gradient of 0–0.3 *M* NaCl in buffer *A*. It was then desalted using a HiPrep 26/10 desalting column with buffer *A* and subjected to a Resource Q column (GE Healthcare) equilibrated with buffer *A*. The protein was eluted with a linear gradient of 0–0.3 *M* NaCl in buffer *A*. The fractions containing PH0239 protein were desalted using a HiPrep 26/10 desalting column with 10 m*M* sodium phosphate pH 7.0 and applied onto a Bio-Scale CHT-20-I column (Bio-Rad) equilibrated with the same buffer. The protein was eluted with a linear gradient of 10–300 m*M* sodium phosphate pH 7.0. The fractions containing the PH0239 protein were pooled, concentrated by ultrafiltration (Vivaspin, 5k cutoff) and loaded onto a HiLoad 16/60 Superdex 200 prep-grade column (GE Healthcare) equilibrated with buffer *A* containing 0.2 *M* NaCl. The purified protein was homogeneous on native PAGE. The protein concentration was determined by measuring the absorbance at 280 nm (Kuramitsu *et al.*, 1990). The yield of the purified protein was 14.0 mg per litre of culture medium.

2.2. Crystallization experiments

The concentration of the purified protein was 14.0 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl. Freshly purified protein was used for crystallization trials using Hampton Research Crystal Screens I and II. Small-sized crystals (Fig. 2) were obtained by the



Carboxyaminoimidazole ribonucleotide (CAIR)
[5-amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxylate]

N-Succinyl-5-aminoimidazole-4-carboxamide ribonucleotide (SAICAIR)
{(*S*)-2-[5-amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamido]succinate}

Figure 1
The reaction catalyzed by phosphoribosylaminoimidazole-succinocarboxamide synthetase (SAICAR synthetase).

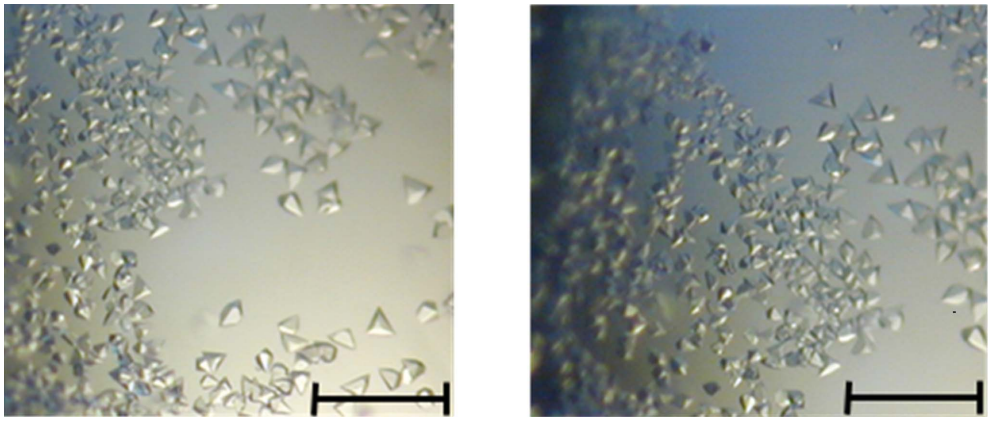


Figure 2
Small crystals obtained using the initial crystallization conditions. The scale bar is 100 µm in length.

Table 1
X-ray data-collection and processing statistics for SAICAR synthetase.

Values in parentheses are for the last resolution bin.

	Native	Se peak	Edge	Remote
X-ray source	BL12B2, SPring-8	BL12B2, SPring-8		
Wavelength (Å)	1.0000	0.9790	0.9798	0.9646
Detector	Q4R CCD, ADSC	Q4R CCD, ADSC		
Temperature (K)	100	100		
Crystal-to-detector distance (mm)	180	200		
Space group	$P3_1$	$P3_1$		
Unit-cell parameters (Å)	$a = b = 95.62$, $c = 149.13$	$a = b = 95.61$, $c = 149.24$		
Resolution range (Å)	30–2.2 (2.28–2.2)	30–2.4 (2.49–2.4)	30–2.4 (2.49–2.4)	30–2.5 (2.59–2.5)
Total reflections	299115	232338	232845	206201
Unique reflections	77374	59800	59929	53047
Completeness (%)	99.9 (99.9)	99.9 (99.9)	99.9 (99.9)	99.9 (99.9)
$R_{\rm merge}$†	9.2 (39.3)	8.9 (37.7)	9.4 (45.3)	6.6 (31.6)
$\langle I/\sigma(I)\rangle$	9.6 (2.5)	10.8 (3.1)	9.9 (2.5)	9.8 (2.3)
Redundancy	3.9	3.9	3.9	3.9
$V_{\rm M}$ (Å^3 Da^{-1})	2.3			
Z	6			
Solvent content (%)	46.1			

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl)\rangle$ is the average intensity for multiple measurements.

hanging-drop vapour-diffusion method with drops consisting of 1 µl protein solution and 1 µl reservoir solution equilibrated against 500 µl well solution at 293 K. Using a reservoir solution composed of 0.05 *M* cadmium sulfate hydrate, 0.1 *M* HEPES buffer pH 7.5 and 1.0 *M* sodium acetate trihydrate (Crystal Screen II condition No. 34), small crystals appeared in about 30 d. Optimization of promising conditions was carried out using Additive Screens (Hampton Research) with the microbatch (diffusion through paraffin oil) method in Nunc HLA plates (Nalge Nunc International) at 293 K. The best result was obtained in the presence of 40%(*w*/*v*) 1,4-butanediol; the droplet consisted of 1.0 µl protein solution, 1.0 µl additive solution and 1.0 µl reservoir solution and the drop was covered with 6 µl of paraffin oil. Diffraction-quality crystals appeared after one week (Fig. 3). A selenomethionine-derivative crystal has recently been obtained using a slight variation of the conditions used for the native crystal [0.05 *M* cadmium sulfate hydrate, 0.1 *M* HEPES buffer pH 7.3, 1.5 *M* sodium acetate trihydrate and 40%(*v*/*v*) 1,4-butanediol]. Prior to immersion in liquid nitrogen for storage and data collection, the crystal was transferred to mother liquor containing 20%(*v*/*v*) glycerol for cryoprotection.

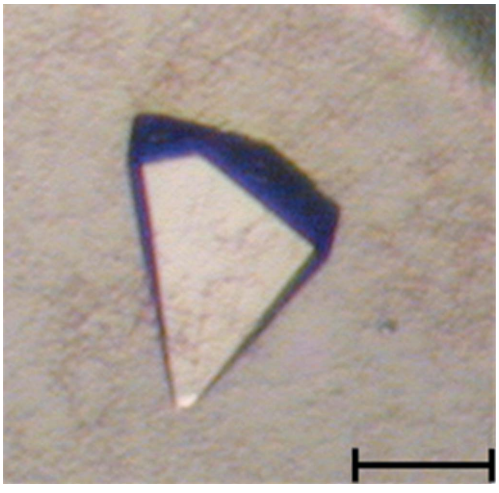


Figure 3
Crystal used for X-ray data collection. The scale bar is 200 µm in length.

2.3. Data collection and processing

A crystal of native SAICAR (PH0239) with dimensions of 0.1 × 0.08 × 0.08 mm was mounted in a loop (Hampton Research) and flash-cooled to 100 K in a nitrogen-gas stream. The X-ray wavelength was set to 1.0 Å and the crystal-to-detector distance was set to 180 mm. The X-ray absorption spectrum at the Se *K* edge was measured from the SeMet crystal. Based on the fluorescence spectrum, energy levels were chosen for data collection [the energy levels were near to the absorption edge of the Se atom: 12.6644 keV (λ = 0.9790 Å; peak), 12.6540 keV (λ = 0.9798 Å; edge) and 12.8540 keV (λ = 0.9646 Å; remote)]. The crystal-to-detector distance was maintained at 200 mm. Diffraction data sets were collected from both crystals on beamline BL12B2 equipped with a CCD detector (Quantum 4R, ADSC) at SPring-8, Japan. The diffraction data were processed and scaled with the *HKL*-2000 package (Otwinowski & Minor, 1997). The crystallographic data for the native crystals and the multiple-wavelength anomalous dispersion (MAD) data statistics for SeMet-containing crystals are summarized in Table 1.

2.4. Dynamic light-scattering studies

Dynamic light-scattering experiments were performed using a DynaPro MS/X instrument (Protein Solutions, Lakewood, New Jersey, USA). The measurements were made at 291 K using the purified protein at 0.5–1.0 mg ml^{-1} in buffer solution containing 20 m*M* Tris–HCl and 500 m*M* sodium chloride.

3. Results and discussion

The crystal belonged to space group $P3_1$, with unit-cell parameters $a = b = 95.62$, $c = 149.13$ Å. A total of 299 115 measured reflections in the resolution range 30–2.2 Å were merged to 77 374 unique reflections with an $R_{\rm merge}$ of 9.2%. The protein contained 238 amino-acid residues and had a molecular weight of 27 kDa. The Matthews coefficient (Matthews, 1968) was 2.3 Å^3 Da^{-1} and the solvent content was 46%(*v*/*v*) assuming the presence of a hexamer in the asymmetric unit. Dynamic light-scattering experiments showed that the protein has a hexameric structure in solution. Details of data collection and processing details are given in Table 1. The primary structure of the enzyme showed 52.8% sequence identity to SAICAR synthetase from *M. jannaschii* (PDB code 2yzl; M. Kanagawa, S. Baba, K. Fukui, S. Kuramitsu, S. Yokoyama, G. Kawai & G. Sampei, unpublished work). Attempts to use the molecular-replacement method using SAICAR synthetase as a search model with *AMoRe* (Navaza, 1994), *CNS* v.1.2 (Brünger *et al.*, 1998) and *Phaser* (Read, 2001; Storoni *et al.*, 2004) did not yield a clear solution. Recently, a selenomethionine derivative of the protein has been obtained and further attempts to determine the structure using SeMet data sets for MAD phasing (Hendrickson *et al.*, 1990) are in progress.

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