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Purification, crystallization and preliminary crystallographic analysis of the putative thiamine-biosynthesis protein PH1313 from *Pyrococcus horikoshii* OT3

The putative thiamine-biosynthesis protein PH1313 from *Pyrococcus horikoshii* OT3 has been overexpressed and purified. Crystallization was performed by the oil-microbatch method using 28%(*v*/*v*) 2-methyl-2,4-pentanediol as a precipitant at 291 K. A native X-ray diffraction data set at 1.9 Å resolution and a single anomalous dispersion data set from a selenomethionine-derivative crystal at 2.1 Å resolution were collected using synchrotron radiation at 100 K. The native crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 71.7, b = 71.2, c = 141.8 Å.

1. Introduction

The bifunctional enzyme ThiI is required for the biosynthesis of both thiamine and 4-thiouridine in tRNA. The thiamine-biosynthetic pathway in prokaryotes involves the separate synthesis of the thiazole [5-methyl-4-(β-hydroxyethyl)thiazole phosphate] and the pyrimidine (4-amino-5-hydroxymethylpyrimidine pyrophosphate) moieties, which are then coupled to produce thiamine phosphate (Begley *et al.*, 1999). The biochemical properties of ThiI from *Escherichia coli* (*Ec*ThiI; Taylor *et al.*, 1998; Leonardi & Roach, 2004) and *Salmonella typhimurium* (Webb *et al.*, 1997) have been reported. ThiI catalyzes the transfer of the sulfur of cysteine to the ThiS protein in thiazole synthesis. In addition, ThiI is responsible for the 4-thiouridine (s^4U) modification at position 8 in some prokaryotic tRNAs (Mueller *et al.*, 1998; Lauhon *et al.*, 2004). The importance of the invariant residues Cys344 and Cys456 for s^4U generation in *Ec*ThiI has been reported (Mueller *et al.*, 2001).

Recently, the crystal structure of ThiI from *Bacillus anthracis* (*Ba*ThiI) has been reported (Waterman *et al.*, 2006). The polypeptide chain of *Ba*ThiI folds into three domains: a ferredoxin-like domain, a THUMP (thiouridine synthases, methylases and pseudouridine synthases) domain and a catalytic PP-loop pyrophosphatase domain. The *Ba*ThiI structure suggests that the ferredoxin-like and THUMP domains jointly form the tRNA-binding surface. The putative thiamine-biosynthesis protein PH1313 from the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3 shares 29% sequence identity with *Ba*ThiI. However, there is no conventional PP-loop in PH1313 as found in the ThiI proteins from most bacteria and archaea (Waterman *et al.*, 2006). In addition, the catalytic residues Cys344 and Cys456 identified in *Ec*ThiI are not conserved in PH1313. These facts suggest that PH1313 may retain a regulatory RNA-binding role but does not have catalytic roles in thiamine biosynthesis and 4-thiouridine generation, as previously discussed (Waterman *et al.*, 2006). In order to understand its function, we intend to determine the crystal structure of PH1313. Here, we report the expression, purification and preliminary crystallographic analysis.

2. Experimental

2.1. Protein expression and purification

The putative thiamine-biosynthesis protein PH1313, which was cloned from *P. horikoshii* OT3 genomic DNA, has a molecular weight of 35.15 kDa and consists of 307 amino-acid residues. The plasmid encoding PH1313 (residues 1–307) was digested with *Nde*I and *Bgl*II

and the fragment was inserted into the expression vector pET-11a (Novagen) linearized with *Nde*I and *Bam*HI. The recombinant pET-11a encodes the full-length wild-type enzyme without any N- or C-terminal modifications. *E. coli* BL21 Codon Plus (DE3)-RIL cells were transformed with the recombinant plasmid and grown at 310 K in Luria–Bertani medium containing 50 µg ml^{-1} ampicillin for 20 h. The cells were harvested by centrifugation at 4500*g* for 5 min at 277 K, suspended in 20 m*M* Tris–HCl pH 8.0 containing 0.5 *M* NaCl, 5 m*M* 2-mercaptoethanol and 1 m*M* phenylmethylsulfonyl fluoride and finally disrupted by sonication and heated at 343 K for 13 min. The cell debris and denaturated protein were removed by centrifugation at 20 000*g* for 30 min. The supernatant solution was used as the crude extract for purification. The crude extract was desalted with a HiPrep 26/10 desalting column (Amersham Biosciences) and applied onto a Super Q Toyopearl 650M (Tosoh) column equilibrated with 20 m*M* Tris–HCl pH 8.0 (buffer *A*). Proteins were eluted with a linear gradient of 0–0.3 *M* NaCl. After buffer replacement with 20 m*M* MES–NaOH pH 6.0 (buffer *B*), the fraction containing PH1313 was subjected to a Resource-S column (Amersham Biosciences) equilibrated with buffer *B* and eluted with a linear gradient of 0–0.4 *M* NaCl in buffer *B*. The sample was then applied onto a Bio-Scale CHT-20-I column (Bio-Rad) equilibrated with 10 m*M* potassium phosphate pH 7.0 and eluted with a linear gradient of 10–400 m*M* potassium phosphate pH 7.0. The sample was concentrated by ultrafiltration (Vivaspin, 10 kDa cutoff) and loaded onto a HiLoad 16/60 Superdex 200 prep-grade column (Amersham Biosciences) equilibrated with buffer *A* containing 0.2 *M* NaCl. The homogeneity and identity of the purified sample were assessed by SDS–PAGE (Laemmli, 1970) and N-terminal sequence analysis. Finally, the purified PH1313 was concentrated by ultrafiltration to 43.0 mg ml^{-1} in buffer *A* with 0.2 *M* NaCl and stored at 203 K. Selenomethionine (SeMet) substituted protein was prepared in an analogous way and its preparations showed virtually identical results to those of the native protein.

2.2. Dynamic light-scattering study

Purified PH1313 was examined by a dynamic light-scattering experiment using a DynaPro MS/X (Protein Solutions) instrument at a protein concentration of 1.0 mg ml^{-1} in 0.2 *M* NaCl and 20 m*M* Tris–HCl pH 8.0. Several measurements were taken at 291 K and analyzed using the program *DYNAMICS* v.3.30 (Protein Solutions). A bimodal analysis revealed an estimated molecular weight of 63.7 kDa, which is consistent with a homodimeric state of PH1313 in solution.

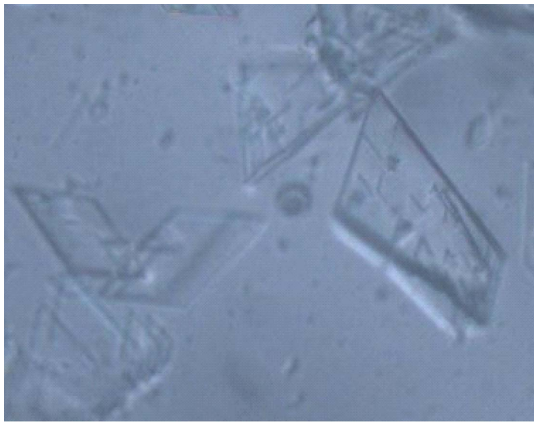

Figure 1
Crystals of PH1313 from *P. horikoshii* OT3. The crystals have approximate dimensions of 0.15 × 0.10 × 0.01 mm.

Table 1
Data-collection statistics.

Values in parentheses correspond to the highest resolution shell.

	Native	SeMet derivative
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)		
a	71.7	72.2
b	71.2	71.9
c	141.8	142.0
Wavelength (Å)	1.0000	0.9791
Resolution range (Å)	40–1.9 (1.97–1.90)	40–2.1 (2.18–2.10)
No. of unique reflections	57832 (5719)	43889 (4335)
Redundancy	5.7 (5.7)	7.0 (7.1)
Completeness (%)	99.9 (100)	100 (100)
R_{sym}† (%)	7.2 (48.9)	11.8 (44.2)
$I/\sigma(I)$	9.0 (3.8)	5.7 (4.0)

† $R_{sym} = \sum_{hkl} |I_{hkl} - \langle I_{hkl}\rangle| / \sum_{hkl} I_{hkl}$.

2.3. Crystallization and X-ray data collection

PH1313 crystals from both native and SeMet-substituted samples were obtained by the oil-microbatch method (Chayen *et al.*, 1990) using Nunc HLA plates (Nalge Nunc International). Each crystallization drop was prepared by mixing 0.5 µl precipitant solution and 0.5 µl protein solution of 43.0 mg ml^{-1} concentration. The crystallization drop was overlaid with a 1:1 mixture of silicone and paraffin oils, allowing slow evaporation of water in the drop, and stored at 291 K. Initial crystallization conditions for PH1313 were established using the TERA (automatic crystallization) system (Sugahara & Miyano, 2002) from 144 independent conditions. Diffraction-quality crystals were obtained using 28%(*v*/*v*) MPD, 0.1 *M* acetate–NaOH pH 5.4 as the precipitant solution. The crystals of PH1313 were mounted in cryoloops, covered with an emulsion mixture of Paratone-N and 10% glycerol (Kwong & Liu, 1999) and then flash-cooled in a nitrogen-gas stream at 100 K. X-ray diffraction data were collected from the flash-cooled crystals at 100 K using synchrotron radiation with a Rigaku R-AXIS V image-plate detector at BL26B1 of SPring-8, Japan. All data were processed and scaled with *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results

We have established the expression, purification and crystallization of PH1313. Crystals appeared about 4–5 d after setup and grew to approximate dimensions of 0.15 × 0.10 × 0.01 mm in two weeks (Fig. 1). An X-ray diffraction data set was collected to 2.1 Å resolution from a single SeMet-derivative crystal using a wavelength corresponding to the peak of a measured selenium f'' spectrum (Table 1). The program *SOLVE* (Terwilliger & Berendzen, 1999) was used to find 16 selenium sites out of the 18 possible. On the basis of phases obtained from the single anomalous dispersion (SAD) experiment, the structure was built, followed by a refinement against the native data to 1.9 Å resolution. The coordinates have been deposited in the PDB with the accession code 1vbk. A dynamic light-scattering experiment shows a dimeric state of PH1313 in solution (see §2), which is in agreement with the determined dimeric crystal structure in the asymmetric unit. The crystal structure of *Ba*ThiI was also reported as a dimer (Waterman *et al.*, 2006), but its subunit arrangement differs from that of PH1313. Therefore, a structural

comparison between PH1313 and *Ba*ThiI might be useful to gain insight into the biological role of the dimeric state. A detailed discussion of the refined structure will be published elsewhere.

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