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Purification, crystallization and preliminary crystallographic analysis of archaeal 6-pyruvoyl tetrahydrobiopterin synthase homologue PH0634 from *Pyrococcus horikoshii* OT3

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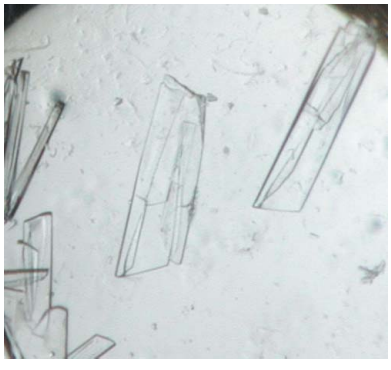
6-Pyruvoyl tetrahydrobiopterin synthase (PTPS) catalyses the conversion of dihydroneopterin triphosphate to 6-pyruvoyl tetrahydropterin, the second of the three enzymatic steps in the synthesis of tetrahydrobiopterin from GTP. PH0634, a 13.51 kDa archaeal PTPS homologue from *Pyrococcus horikoshii* OT3, was overexpressed as native and selenomethionine-substituted protein and the purified protein was crystallized by the oil-microbatch method at 295 K. X-ray diffraction data were collected to 2.1 Å resolution from the native crystal using synchrotron radiation at 100 K. The crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 35.83$, $b = 95.71$, $c = 105.65$ Å. Threefold noncrystallographic symmetry was identified from self-rotation calculations. Assuming the presence of a trimer in the asymmetric unit, the solvent content is 45% ($V_M = 2.24$ Å^3 Da^{-1}). The selenomethionine-substituted crystal is isomorphous to the native crystal and diffracts X-rays to 2.9 Å.

1. Introduction

The PH0634 protein (115 residues) from *Pyrococcus horikoshii* OT3 shares 37% (36 out of 97 amino acids) sequence identity with the 6-pyruvoyl tetrahydrobiopterin synthase (PTPS) of known structure from rat liver (Nar *et al.*, 1994). PTPS is the second enzyme in the biosynthetic pathway from GTP to tetrahydrobiopterin and catalyses the conversion of dihydroneopterin triphosphate to 6-pyruvoyl tetrahydropterin (Thöny *et al.*, 2000). The product of the three-step pathway, tetrahydrobiopterin, is an essential cofactor for several aromatic amino-acid monooxygenases and nitric oxide synthases. The crystal structure of PTPS from rat liver (144 residues) revealed that it functions as a hexamer composed of a pair of trimers arranged in a head-to-head fashion (Nar *et al.*, 1994; Ploom *et al.*, 1999). In the rat liver PTPS hexamer, the active site at the trimer–trimer interface comprises two adjacent subunits from different trimers: Cys42 from one trimer and Asp88 and His89 from the other trimer. These residues might serve as proton donors and acceptors during catalysis; the mutant Cys42Ala causes a complete loss of enzymatic activity (Bürgisser *et al.*, 1995). The PTPS reaction requires divalent cations such as Zn^{2+} and Mg^{2+}, which bind to the pocket formed by three histidine residues, His23, His48 and His50, on the same subunit. Site-directed mutagenesis of each of these three histidine residues resulted in a complete loss of metal binding and enzymatic activity (Bürgisser *et al.*, 1995). In spite of the considerable sequence homology of PH0634 to rat liver PTPS, the N-terminal regions (residues 1–26 of the former and 1–47 of the latter) show no significant similarity, indicating that the active-site residues Cys42 and His23 of the rat liver enzyme are not conserved in PH0634. Furthermore, the oligomeric state of PH0634 is a trimer, as described below. Therefore, determining the crystal structure of PH0634 may help us to understand the difference in the N-terminal region and the new oligomeric state. In this paper, we report the expression, purification and preliminary crystallographic analysis of the archaeal PTPS homologue PH0634 from *P. horikoshii* OT3.



2. Experimental

2.1. Protein expression and purification

The PH0634 protein from *P. horikoshii* OT3 used in this study has a molecular weight of 13.51 kDa and consists of 115 amino-acid residues. Protein expression and purification were performed by the Structurome Research Group at the RIKEN SPring-8 Center, Harima Institute, Japan. The plasmid encoding PH0634 (residues 1–115) 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. *Escherichia 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 363 K for 10 min. The cell debris and heat-denaturated proteins were removed by centrifugation at 20 000*g* for 30 min. The supernatant solution containing PH0634 was used as the crude extract for purification. The crude extract was desalted on 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*). After elution with a linear gradient of 0–0.3 *M* NaCl, the fraction containing PH0634 was desalted with a HiPrep 26/10 desalting column in 10 m*M* potassium phosphate pH 7.0. 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 10–300 m*M* potassium phosphate pH 7.0. The sample was concentrated by ultrafiltration (Vivaspin, 5 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 PH0634 was concentrated to 29.1 mg ml^{-1} using ultrafiltration and stored at 203 K. For the preparation of selenomethionine-substituted protein, the BL21 Codon Plus (DE3)-RIL *E. coli* cells (Stratagene) were grown in M9 medium until they reached an absorbance at 600 nm (A_{600}) of 0.4. At this point, 100 mg L-lysine, 100 mg L-phenylalanine, 100 mg L-threonine, 50 mg L-isoleucine, 50 mg L-leucine and 60 mg selenomethionine (SeMet) were added to 1 l of culture and the cells were grown at 310 K for a further 1 h before inducing expression with 1 m*M* IPTG overnight at 298 K. SeMet-substituted protein was purified as described for the native protein and its preparations showed virtually identical results to those of the native protein.

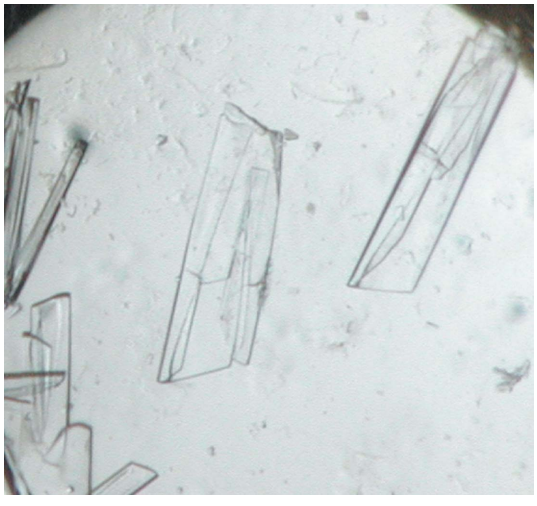


Figure 1
Crystals of the PH0634 protein. The crystal on the right has approximate dimensions of 0.1 × 0.4 × 0.02 mm.

Table 1
Data collection and phasing.

Values in parentheses correspond to the highest resolution shell.

	Native	SeMet derivative		
		Remote	Peak	Edge
Space group	$P2_12_12_1$	$P2_12_12_1$		
Unit-cell parameters (Å)				
a	35.83	37.51		
b	95.71	97.60		
c	105.65	106.82		
Wavelength (Å)	1.00000	0.96500	0.97945	0.97970
Resolution range (Å)	50–2.1 (2.18–2.10)	50–2.90 (3.00–2.90)		
Total observations	99630	42508	47617	45404
Unique reflections	21576	8706	8632	8659
Completeness (%)	98.3 (99.6)	95.6 (77.3)	95.8 (76.2)	95.3 (74.7)
Mean $I/\sigma(I)$	9.9 (3.7)	11.6 (2.0)	11.7 (2.12)	11.9 (2.0)
$R_{\rm merge}$ (%)	7.2 (27.6)	6.7 (35.5)	6.8 (35.3)	6.7 (33.6)
Phasing (20–3.0 Å)				
$R_{\rm Cullis}$† (centric/acentric)			0.50/0.61	
Mean FOM after *RESOLVE* phasing‡ (centric/acentric)			0.70/0.61	

† $R_{\rm merge} = \sum_{hkl}\sum_j |I_j(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_j \langle I(hkl)\rangle$, where $I_j(hkl)$ and $\langle I(hkl)\rangle$ are the observed intensity of measurement j and the mean intensity of the reflection with indices hkl, respectively. † $R_{\rm Cullis} = \sum_{hkl} \big||F_{\rm ph}| \pm F_{\rm p}| - F_{\rm h(calc)}\big| / \sum_{hkl} |F_{\rm ph} \pm F_{\rm p}|$, where $F_{\rm p}$ and $F_{\rm ph}$ are the structure-factor amplitudes of protein and of the heavy-atom derivative, respectively. $F_{\rm h(calc)}$ is the calculated heavy-atom structure factor. ‡ Mean figure of merit (FOM) = $\langle \sum P(\alpha)\exp(i\alpha) / \sum P(\alpha)\rangle$, where α is the phase and $P(\alpha)$ is the phase probability distribution.

2.1.1. Dynamic light-scattering study. The oligomerization state of the purified PH0634 was examined by a dynamic light-scattering experiment using a DynaPro MS/X instrument (Protein Solutions), which was performed at a protein concentration of 29.1 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0 with 200 m*M* NaCl. Several measurements were taken at 291 K and analyzed using the *DYNAMICS* software v.3.30 (Protein Solutions). The result showed a monomodal profile centred at a 2.99 nm radius, corresponding to a molecular weight of 43.7 kDa, suggesting a trimeric state of PH0634 in solution.

2.1.2. Crystallization and X-ray data collection. The initial crystallization conditions were obtained by the oil-microbatch method (Chayen *et al.*, 1990) using the TERA (automatic crystallization) system and a screening kit designed for high-throughput protein crystallization (Sugahara & Miyano, 2002). Using Nunc HLA plates (Nalge Nunc International), a 0.5 µl aliquot of the screening solution was mixed with 0.5 µl protein solution (29.1 mg ml^{-1} protein, 20 m*M* Tris–HCl pH 8.0 and 200 m*M* NaCl) and covered with 15 µl of a 1:1 mixture of silicone and paraffin oils, allowing slow evaporation of water in the drop, and stored at 291 K. Many small colourless protein crystals appeared after two weeks using a screening solution composed of 16.5% PEG 20 000, 0.1 *M* acetate–NaOH pH 6.25 (Fig. 1). The flattened rod-shaped crystals were transferred using a nylon loop (Hampton Research) from the crystallization drop into a cryoprotectant solution comprising 16.5% PEG 20 000, 0.1 *M* acetate–NaOH pH 6.25 and 10%(*v*/*v*) glycerol. After soaking for 10 s, the crystals were flash-cooled in a nitrogen-gas stream at 100 K. X-ray diffraction data were collected at 100 K using synchrotron radiation on a Jupiter 210 CCD detector at beamline BL26B1 of SPring-8, Japan. The native diffraction data consisted of 180 images in total, each exposed for 45 s with a 1° oscillation at a crystal-to-detector distance of 200 mm. For the selenomethionine-derivative crystal, multiwavelength anomalous diffraction data were collected using wavelengths determined from

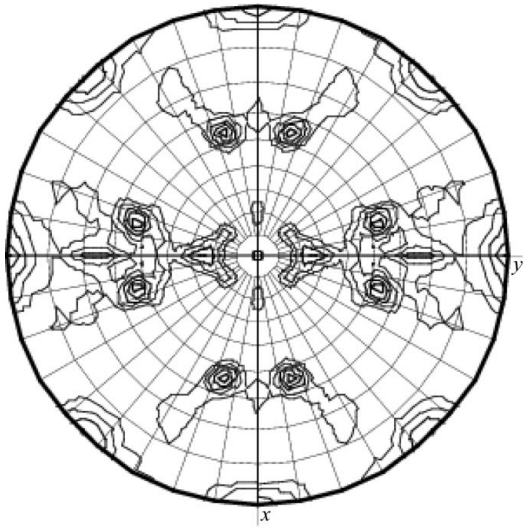


Figure 2
Projection map of the self-rotation function at the $\chi = 120^\circ$ section. The radius of integration was 19.7 Å and the higher resolution limit was 2.7 Å. On the map, contour lines are drawn at 0.5σ from a lowest level of $+0.5\sigma$. A strong signal arising from threefold noncrystallographic symmetry is observed at $\theta = 54.25^\circ$, $\varphi = 74.05^\circ$. The observed height of 2.4σ for the corresponding self-rotation peak is comparable with the height of 2.9σ for the twofold crystallographic peak in the $\chi = 180^\circ$ section (not shown). The plot was generated with the help of *MOLREP* (Collaborative Computational Project, Number 4, 1994; Vagin & Teplyakov, 1997).

the selenium-absorption spectrum: peak (0.97945 Å), edge (0.97970 Å) and remote (0.96500 Å). In these three data sets, composed of 160 images each, 30 s exposures with a 1° oscillation at a crystal-to-detector distance of 250 mm were used. Data were indexed, integrated and scaled using the *HKL*-2000 suite of programs (Otwinowski & Minor, 1997).

3. Results

We have established the expression, purification and crystallization of PH0634. Crystals appeared about two weeks after setup and grew to approximate dimensions of 0.1 × 0.4 × 0.02 mm within a month. Observation of systematic absences along the three crystallographic axes indicated the space group to be $P2_12_12_1$. Crystal parameters and data-collection statistics are summarized in Table 1. Assuming the presence of three monomeric protomers of PH0634 in the asymmetric unit of the orthorhombic crystal, a typical Matthews coefficient of 2.24 $Å^3\,Da^{-1}$ and a solvent content of 45% were calculated (Matthews, 1968). To examine the local symmetry in the asymmetric unit, self-rotation functions were calculated using *MOLREP* (Collaborative Computational Project, Number 4, 1994; Vagin & Teplyakov, 1997; Fig. 2). The search was carried out for χ angles of 180, 120, 90 and 60° to check for the presence of twofold, threefold, fourfold and sixfold axes, respectively. Local peaks with a signal-to-noise ratio of 2.4σ were found at $\chi = 120^\circ$, clearly demonstrating the existence of threefold noncrystallographic symmetry. Therefore, the asymmetric unit may contain a homotrimer of PH0634 with threefold symmetry. The selenomethionine-derivative crystal with six possible selenium sites in the asymmetric unit allowed us to apply the multiple anomalous dispersion (MAD) method (Hendrickson *et al.*, 1990). The program *SOLVE* (Terwilliger & Berendzen, 1999) identified all six selenium sites and yielded a 67% complete initial model including the N-terminal region based on the MAD phases. Crystallographic refinement is presently in progress. From the incomplete present model, we have confirmed the trimeric quaternary structure of PH0634 in the crystal, which is consistent with the dynamic light-scattering results, which show a trimeric state of PH0634 in solution (see §2). A detailed discussion of the refined structure will be published elsewhere.

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