Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Purification, crystallization and preliminary crystallographic analysis of RecA superfamily ATPase PH0284 from *Pyrococcus horikoshii* OT3

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Received 1 February 2006
Accepted 17 March 2006

Circadian (daily) protein clocks are found in cyanobacteria, where a complex of the KaiA, KaiB and KaiC proteins generates circadian rhythms. The 28.09 kDa KaiC homologue PH0284 protein from *Pyrococcus horikoshii* OT3 was cloned and expressed and the purified protein was crystallized by the oil-microbatch method at 295 K. X-ray diffraction data from the crystal were collected to 2.0 Å resolution using synchrotron radiation at 100 K. The crystal belongs to the trigonal space group $P3_221$, with unit-cell parameters $a = b = 96.06$, $c = 298.90$ Å. Assuming the presence of one hexamer in the asymmetric unit gives a V_M value of 2.36 Å^3 Da^{-1} and a solvent content of 47.9%. A cocrystal with ATP was prepared and a diffraction data set was collected at 2.3 Å resolution.

1. Introduction

Circadian clocks are self-sustained biochemical oscillators. Their properties include a time constant of approximately 24 h (daily cycle) and accurate temperature compensation. In the circadian-clock machinery found in cyanobacteria, direct protein–protein interactions between the KaiA, KaiB and KaiC proteins may be a critical process in the generation of circadian rhythms through cyclic regulation of gene expression (Ishiura *et al.*, 1998). A RecA superfamily protein, KaiC, is the core component of the clock-protein complex KaiABC (Johnson, 2004). Recently, KaiC homologous proteins have also been found in archaea as RecA superfamily ATPases (Johnson & Golden, 1999; Dvornyk *et al.*, 2003). Most of the archaeal RecA superfamily ATPases are hypothetical and more than one copy is sometimes found. The crystal structure of KaiC from *Synechococcus elongatus* has been reported (Pattanayek *et al.*, 2004; Xu *et al.*, 2004). The structure of KaiC reveals two domains, CI and CII, which adopt similar overall folds characteristic of the RecA superfamily and assemble into a pseudosymmetric double-layered hexameric ring. The CI domain shows 34% (81/236) sequence identity to the KaiC homologue PH0284 protein from the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3. Amino acids consistent with the CII domain, where the key phosphorylation sites in KaiC were detected (Xu *et al.*, 2004), were absent from the PH0284 protein. According to genome research, most of the KaiC homologues in archaea are short single-domain versions composed of CI or CII homologous domains (Dvornyk *et al.*, 2003). Furthermore, the archaea are usually found in extreme environments such as deep sea, methane vents and hot springs, for which daily cycles would not appear to be adaptive. It is likely that PH0284 has a regulatory function that is distinct from but closely related to the circadian clock. Therefore, characterizing the structures, functions and interactions between the molecular components of the putative regulation system in archaea could help us to understand much about the evolution of circadian rhythms. In this paper, we report the expression, purification and preliminary crystallographic analysis of the RecA superfamily ATPase PH0284 and its complex with ATP.

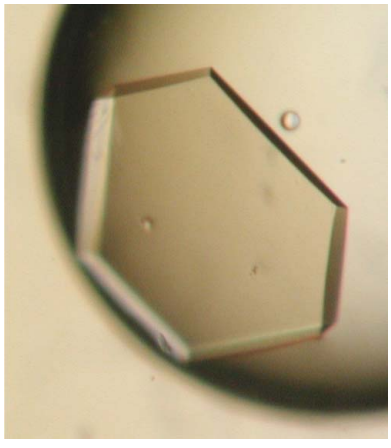

Table 1
Statistics of data collection.

Values in parentheses correspond to the highest resolution shell.

	Liganded forms	
	ADP	ATP
Space group	$P3_221$	$P3_221$
Unit-cell parameters (Å)		
a	96.06	96.26
c	298.90	300.49
Wavelength (Å)	1.00000	1.00000
Resolution range (Å)	40–2.0 (2.07–2.00)	50–2.3 (2.38–2.30)
Total observations	519711	363265
Unique reflections	101337	71885
Completeness (%)	92.9 (87.5)	98.5 (98.0)
Mean $I/\sigma(I)$	16.2 (4.3)	13.8 (3.7)
$R_{\rm merge}$† (%)	10.4 (47.5)	7.1 (52.4)

† $R_{\rm merge} = \sum_{hkl}\sum_j |I_j(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_j \langle I(hkl)\rangle$, 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.

2. Experimental

2.1. Protein expression and purification

The PH0284 protein from *P. horikoshii* OT3 used in this study has a molecular weight of 28.09 kDa and consists of 252 amino-acid residues. Protein expression and purification were performed routinely by the Structurome Research Group in RIKEN SPring-8 Center. The plasmid encoding PH0284 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* PMSF and finally disrupted by sonication and heated at 363 K for 11.5 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*). After elution with a linear gradient of 0–0.3 *M* NaCl, the fraction containing PH0284 was desalted with a HiPrep 26/10 desalting column with 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 PH0284 was concentrated to 18.9 mg ml^{-1} using ultrafiltration and stored at 203 K.

2.2. Dynamic light-scattering study

The oligomerization state of the purified PH0284 was examined by a dynamic light-scattering experiment using a DynaPro MS/X instrument (Protein Solutions), which was performed at a protein concentration of 18.9 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 sharp monomodal profile centred at 5.12 nm radius corresponding to a molecular weight of 153.9 kDa, supporting the hexameric state of PH0284 in solution.

2.3. Crystallization and X-ray data collection

A Hampton Crystal Screen kit (Jancarik & Kim, 1991) was used to determine the initial crystallization conditions for the PH0284 protein. Crystallization was carried out by the oil-microbatch technique using Nunc HLA plates (Nalge Nunc International). Each crystallization drop was prepared by mixing 1.0 µl precipitant solution and 1.0 µl protein solution (18.9 mg ml^{-1} protein, 200 m*M* NaCl and 20 m*M* Tris–HCl pH 8.0). 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 295 K. After 5–6 d equilibration, plate-like crystals occasionally grew using precipitant solution No. 4 of Crystal Screen Cryo, which was composed of 1.5 *M* ammonium sulfate, 25%(*v*/*v*) glycerol and 75 m*M* Tris–HCl pH 8.5. X-ray diffraction data were collected from flash-cooled crystals at 100 K on a Rigaku R-AXIS V image-plate detector using synchrotron radiation at beamline BL26B1 of SPring-8, Japan. As the crystal mother liquor contained cryoprotectant, the crystals were directly picked up in a nylon loop (Hampton Research) and placed in a nitrogen-gas flow at 100 K. Each frame was exposed for 45 s with a 0.5° oscillation at a crystal-to-detector distance of 370 mm. Data were processed and scaled using *HKL*2000 (Otwinowski & Minor, 1997).

3. Results

We have established the expression, purification and crystallization of the PH0284 protein. Crystals appeared about 5–6 d after setup and grew to approximate dimensions of 0.40 × 0.40 × 0.07 mm after two weeks (Fig. 1). Data-collection statistics are summarized in Table 1. Assuming six chains of PH0284 in the asymmetric unit gives a crystal volume per protein weight ($V_{\rm M}$) of 2.36 Å^3 Da^{-1} and a solvent content of 47.9% (Matthews, 1968). The self-rotation searches did not

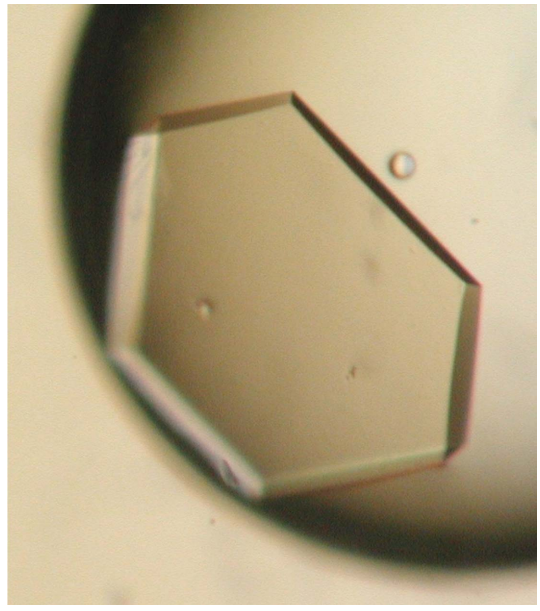

Figure 1
Crystal of the RecA superfamily ATPase PH0284. The crystal has approximate dimensions of 0.40 × 0.40 × 0.07 mm.

reveal any significant signal corresponding to the sixfold non-crystallographic symmetry characteristic of RecA superfamily proteins. It is likely that non-crystallographic and crystallographic symmetry axes nearly coincide in this case, thereby effectively masking the presence of the former. Structure determination by molecular replacement with the program *AMoRe* (Navaza, 1994) using the CI domain from the KaiC crystal structure (PDB code 1u9i; Xu *et al.*, 2004) yields an obvious solution. The homohexameric structure of PH0284 appears with six ADP molecules bound at the intersubunit interface. Crystals also grew in the presence of 5 m*M* ATP and a diffraction data set was collected to 2.3 Å resolution (Table 1). A detailed discussion of both structures will be published elsewhere.

The authors thank the staff of RIKEN Genomic Sciences Center for providing the plasmid and the technical staff of RIKEN SPring-8 Center for large-scale protein production and the dynamic light-scattering experiment. We also thank N. Ono for assistance during crystallization and M. Yamomoto and his staff for assistance during data collection at beamline BL26B1 of SPring-8. This work (PH0284/HTPF11797) was supported by the 'National Project on Protein Structural and Functional Analysis' funded by the MEXT of Japan.

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