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Expression, purification, crystallization and preliminary X-ray analysis of the KaiC-like protein PH0187 from the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3

KaiC is the central protein in the circadian rhythm in cyanobacteria. The 28 kDa KaiC-like protein PH0187 from the hyperthermophilic archaeon *Pyrococcus horikoshii* was expressed in *Escherichia coli*, purified and crystallized using the sitting-drop vapour-diffusion method at 293 K. Crystals of PH0187 were obtained using a reservoir solution consisting of 1.0 M ammonium phosphate monobasic and 0.1 M sodium citrate tribasic pH 5.3 (the final pH value of the reservoir solution was 4.8) and diffracted X-rays to 2.75 Å resolution. The crystal of PH0187 belonged to space group $P6_322$, with unit-cell parameters $a = b = 239.1$, $c = 106.5$ Å. The crystal contained four PH0187 molecules in the asymmetric unit.

1. Introduction

Circadian rhythms are self-sustained biochemical oscillators with a period of 24 h. These rhythms are found in a wide spectrum of organisms and enhance their fitness in a day/night cycle. The simplest cells that are known to exhibit circadian rhythms are prokaryotic cyanobacteria (Golden *et al.*, 1997), for which considerable progress has recently been achieved in the identification of essential clock proteins and their structures. In cyanobacteria, the circadian rhythm is controlled by a cluster of three genes: *kaiA*, *kaiB* and *kaiC* (Ishiura *et al.*, 1998). Of these genes, *kaiC* encodes a crucial protein KaiC that forms a stable homo-hexamer upon the binding of ATP. KaiC has a double-domain structure consisting of an N-terminal domain (KaiCI) and a C-terminal domain (KaiCII). KaiCI is responsible for the ATP-induced hexamerization of KaiC, while KaiCII is flexible and is responsible for the phosphorylation of KaiC (Hayashi *et al.*, 2004). Autophosphorylation of KaiC is stimulated by KaiA, whereas KaiB antagonizes the effect of KaiA on KaiC autophosphorylation (Xu *et al.*, 2003).

Archaea are usually found in extreme environments such as deep-sea areas, methane vents and hot springs, and the phenomenon of KaiC-dependent circadian rhythms has not yet been reported in archaea. However, KaiC homologues have been found in almost all species of archaea, including *Pyrococcus* and *Sulfolobus* (Dvornyk *et al.*, 2003). Most of the KaiC homologues in archaea are short (single-domain) versions that differ from those of cyanobacteria. The function of homologues of KaiC in archaea remains unknown. However, understanding this homologous protein in archaea is important to understanding the evolution of circadian rhythms.

P. horikoshii OT3 has homologous proteins to the KaiC domain, PH0186 and PH0187, which seem to form an operon on the genome similar to the double-domain architecture of cyanobacterial KaiC. We have determined the structure of PH0186 (Kang *et al.*, 2009). PH0187 shows amino-acid sequence similarity to RecA-superfamily ATPase proteins, including KaiC. The most similar protein for which the structure has been determined is the putative RecA-superfamily ATPase PH0284 (PDB code 2dr3; 30% amino-acid sequence identity; Bagautdinov & Kunishima, 2006). Here, we report the cloning, expression, purification, crystallization and preliminary crystallographic analysis of PH0187.



2. Materials and results

2.1. Overexpression and purification

The PH0187 coding sequence was obtained by PCR from genomic DNA of *P. horikoshii* OT3 and was cloned into the *NdeI/BamHI* site of the pET-28a(+) plasmid (Novagen). PH0187 was overexpressed in *Escherichia coli* Rosetta (DE3) (Novagen) harbouring the constructed plasmid. Expression of PH0187 was induced by the addition of 0.5 mM (final concentration) isopropyl β -D-1-thiogalactopyranoside (IPTG) when the optical density of the medium at 600 nm reached 0.5. The cells were harvested after overnight culturing at 298 K. The harvested cells were resuspended in 10 mM Tris-HCl pH 7.5 containing 100 mM NaCl and then disrupted by sonication. After centrifugation at 40 000g for 30 min, the supernatant was heated at 353 K for 30 min to denature heat-labile *E. coli* proteins. After centrifugation at 40 000g for 30 min, the supernatant was applied onto a Ni-Sepharose (GE Healthcare) column equilibrated with 10 mM Tris-HCl pH 7.5 containing 100 mM NaCl. His-tagged PH0187 was eluted with a buffer solution consisting of 10 mM Tris-HCl pH 7.5, 100 mM NaCl and 200 mM imidazole. The eluted fraction was treated with thrombin to remove the N-terminal His tag of PH0187. The PH0187 was further purified using a Resource Q (GE Healthcare) column pre-equilibrated with 20 mM Tris-HCl pH 8.0 and was eluted with a linear gradient of 0–1 M NaCl. The purified protein was dialyzed against 20 mM Tris-HCl pH 8.0 and concentrated to 12 mg ml⁻¹ for crystallization.

2.2. Crystallization

All crystallization experiments were performed at 293 K using the sitting-drop vapour-diffusion method. Initial crystallization screening of PH0187 was carried out with the Crystal Screen HT (Hampton Research) and Wizard I, II and III (Emerald BioSystems) screening kits. After refinement of the crystallization conditions, the best crystals of PH0187 were obtained by mixing 1.0 μ l PH0187 solution, 0.8 μ l reservoir solution consisting of 1.0 M ammonium phosphate monobasic and 0.1 M sodium citrate tribasic pH 5.3 (the final pH value of the reservoir solution is 4.8) and 0.2 μ l 0.1 M cobalt(II) chloride. Fig. 1 shows a typical crystal (0.50 \times 0.20 \times 0.10 mm) of PH0187.

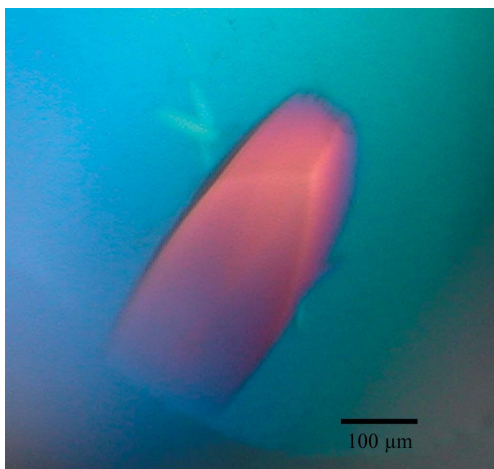


Figure 1
Crystal of PH0187. The scale bar is 100 μ m in length.

Table 1

Summary of data-collection statistics for the PH0187 crystal.

Values in parentheses are for the highest resolution shell.

Beamline	PF BL5A
Wavelength (\AA)	1.07156
Space group	<i>P</i> 6 ₃ 22
Unit-cell parameters	
<i>a</i> = <i>b</i> (\AA)	239.1
<i>c</i> (\AA)	106.5
Resolution range (\AA)	15.0–2.75 (2.82–2.75)
No. of measurements	971665 (54977)
No. of unique reflections	87775 (6469)
Multiplicity	11.1 (8.5)
Completeness (%)	99.2 (98.9)
$R_{\text{merge}}^{\dagger}$ (%)	6.0 (62.8)
$\langle I \rangle / \langle \sigma(I) \rangle$	27.4 (3.5)

$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ is the i th intensity measurement of reflection hkl , including symmetry-related reflections, and $\langle I(hkl) \rangle$ is its average.

2.3. Data collection and processing

The crystal of PH0187 was flash-cooled at 95 K in a nitrogen-gas stream for data collection. For cryoprotection, the crystal of PH0187 was soaked in reservoir solution supplemented with 30% (v/v) ethylene glycol for a few seconds. An X-ray diffraction data set for PH0187 was collected on the BL5A beamline at Photon Factory (Tsukuba, Japan) using an ADSC Quantum 315r CCD detector. The

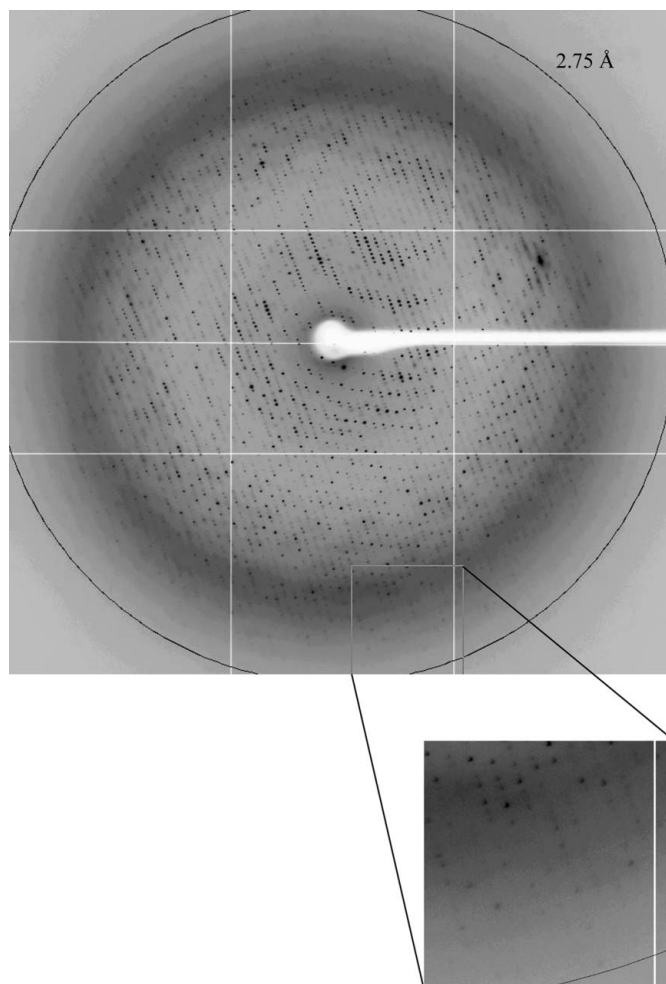


Figure 2
X-ray diffraction image of PH0187. The circle indicates a resolution of 2.75 \AA .

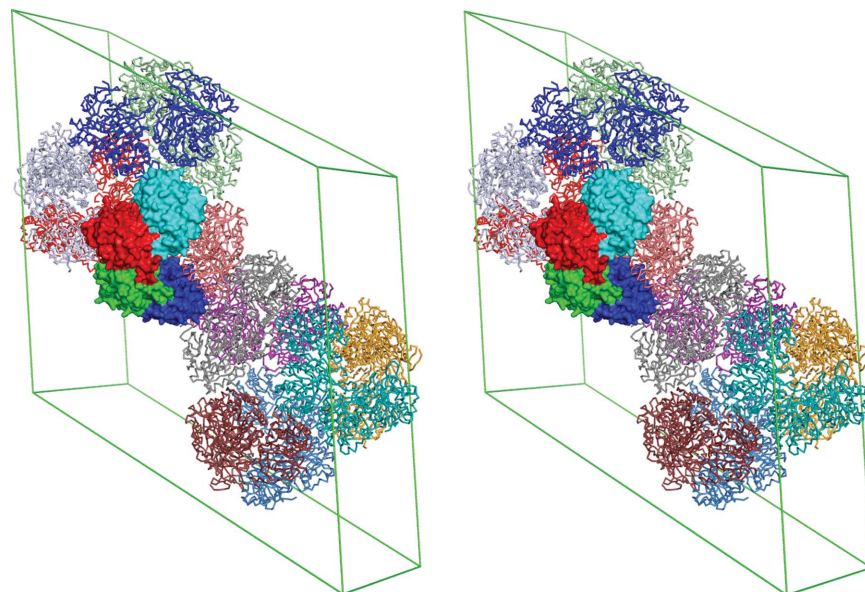


Figure 3

Stereoview of the crystal packing of PH0187 in the primitive hexagonal cell. The molecules in the asymmetric unit are shown as blue, green, red and cyan surfaces, while symmetry-related molecules within the unit cell are shown as C α -atom traces. The image was created with *PyMOL* (DeLano, 2002).

best crystal of PH0187 diffracted X-rays to 2.75 Å resolution (Fig. 2). The diffraction data were indexed, integrated and scaled with *XDS* (Kabsch, 2010). The space group of the crystal was determined to be *P6₃22*, with unit-cell parameters $a = b = 239.1$, $c = 106.5$ Å. Statistics of data collection are summarized in Table 1. Structure determination was performed by molecular replacement with *MOLREP* (Vagin & Teplyakov, 2010) using the coordinates of the RecA-superfamily ATPase PH0284 (PDB code 2dr3; Bagautdinov & Kunishima, 2006) as the template model. The results of molecular replacement suggest that the crystal contains four molecules per asymmetric unit, with a Matthews coefficient (Matthews, 1968) and a solvent content of 3.83 Å³ Da⁻¹ and 67.95%, respectively. Although both KaiC and PH0186 show a hexameric assembly, a tetrameric state of PH0187 was found in the crystal. The disruption of the hexameric state observed in this study may correlate with the flexibility of the KaiCII domain of KaiC. The *R* factor and *MOLREP* score after *MOLREP* using the structure of chains *ABEF* of PH0284 as a template were 0.582 and 0.137, respectively (Fig. 3). A total of 5% of the reflections were randomly selected to provide a test set for *R*_{free} calculations. The *R* factor, *R*_{free} and FOM values after ten cycles of restrained refinement using *REFMAC5* (Murshudov *et al.*, 1997) were 0.509, 0.525 and 0.303, respectively. Further model building and refinement are in progress.

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