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Cloning, expression, purification, crystallization and preliminary crystallographic analysis of selenomethionine-labelled KaiC-like protein PH0186 from *Pyrococcus horikoshii* OT3

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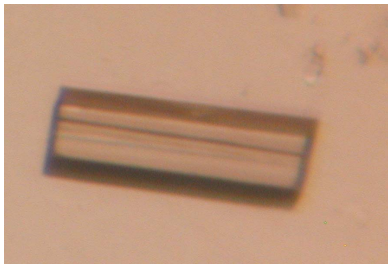
KaiC is the central protein in the circadian-clock system of cyanobacteria. A selenomethionine-labelled KaiC-homologous protein from *Pyrococcus horikoshii* OT3 (PH0186; 28 kDa) was crystallized by the sitting-drop vapour-diffusion method using ethanol as a precipitant. The crystals diffracted X-rays to beyond 2.0 Å resolution using a synchrotron-radiation source. The space group of the crystals was determined to be *C*2, with unit-cell parameters a = 173.7, b = 51.8, c = 97.5 Å, β = 122.8°. The crystal contains three molecules in the asymmetric unit (V_M = 2.2 Å^3 Da^{-1}) and has a solvent content of 43.5%. Sixfold noncrystallographic symmetry was identified from self-rotation calculations, assuming the presence of a hexamer in the crystal.

1. Introduction

Circadian rhythms, which reflect the 24 h daily activity cycles present in most organisms, are self-sustained biochemical oscillations. Cyanobacteria are the simplest organisms known to exhibit circadian rhythms. The *KaiABC* circadian-clock gene cluster in *Synechococcus* sp. strain PCC 7942 has been investigated and three main proteins (KaiA, KaiB and KaiC) for the circadian-clock system were found (Ishiura *et al.*, 1998). KaiC is the central protein, forming a pot-shaped homohexamer upon binding ATP molecules (Pattanayek *et al.*, 2004). It has both autophosphorylation and autodephosphorylation activities (Nishiwaki *et al.*, 2000; Xu *et al.*, 2003). KaiA enhances the autophosphorylation activity of KaiC, while KaiB weakens the effect of KaiA (Iwasaki *et al.*, 2002; Williams *et al.*, 2002; Kitayama *et al.*, 2003; Xu *et al.*, 2003). KaiC contains two homologous domains: the N-terminal domain (KaiCI), which is responsible for the rigid ring structure of KaiC obtained by ATP-induced hexamerization, and the C-terminal domain (KaiCII), which is flexible and has both phosphorylation and dephosphorylation activity (Hayashi *et al.*, 2006).

Archea usually inhabit environments with high temperature, strong acidity, high salinity and/or containing poisonous gases (methane *etc.*) such as volcanoes, hot springs and seabeds, and the phenomenon of circadian rhythm has not yet been reported in archea. Interestingly, however, KaiC homologues have been found in some archaea such as *Aeropyrum*, *Archaeoglobus*, *Halobacterium*, *Methanococcus*, *Pyrobaculum*, *Pyrococcus* and *Sulfolobus* (Dvornyk *et al.*, 2003). Most of the KaiC homologues in archea are short and single-domain. The single domains usually match both domains of KaiC, but always have higher similarity to the first domain KaiCI (Dvornyk *et al.*, 2003). The functions of these homologous genes in archea are unknown. Understanding this homologous protein in archea is important in understanding the evolution of circadian rhythms.

In contrast to cyanobacteria, *Pyrococcus horikoshii* OT3 only has KaiC-homologous proteins. It has an operon possessing two genes, PH0186 and PH0187. PH0186 has 35% identity to KaiCI. Like KaiCI, the amino-acid sequence of PH0186 also contains a Walker A motif (P-loop), a Walker B motif and catalytic Glu residues (Hayashi *et al.*, 2003; Fig. 1). In this paper, we report the cloning, expression, puri-



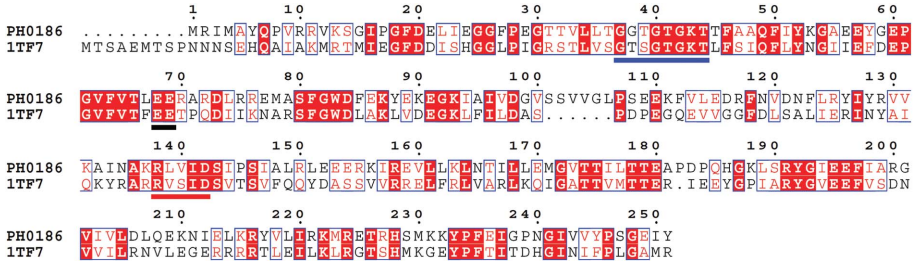


Figure 1
Sequence alignment of PH0186 from *P. horikoshii* and KaiCI from *Synechococcus* sp. strain PCC 7942. The blue, red and black bars indicate the Walker A motif, Walker B motif and catalytic Glu residues, respectively. The sequences were aligned with *ClustalW* (Thompson *et al.*, 1994) and the figure was produced using *ESPript* (Gouet *et al.*, 1999).

fication, crystallization and preliminary crystallographic analysis of selenomethionine-labelled PH0186 protein.

2. Methods and results

2.1. Cloning, expression and purification

The PH0186 (gi:14590121) coding sequence was obtained by polymerase chain reaction (PCR) from genomic DNA of *P. horikoshii* and was subcloned into a pET26b expression vector (Novagen). The expression plasmid was transformed into *Escherichia coli* strain Rosetta (DE3) (Novagen). Selenomethionine (SeMet) labelling was achieved by inhibition of methionine biosynthesis (Doublié, 1997). Bacteria were grown in M9 medium supplemented with 30 mg l^{-1} kanamycin at 310 K until they reached an optical density at 600 nm of 0.3. At this point, solid amino-acid supplements were added to the cultures (100 mg l^{-1} Phe, Thr and Lys, 50 mg l^{-1} Ile, Leu, Val and L-selenomethionine). After 15 min, 1 m*M* isopropyl β-thiogalactopyranose (IPTG) was added in order to induce expression of the cloned protein. Cells were harvested after overnight culturing at 299.5 K and then lysed in 50 m*M* CAPS buffer pH 10 containing 5 m*M* $MgCl_2$, 100 m*M* NaCl and 5 m*M* DTT for sonication. The lysed bacteria were centrifuged at 15 000 rev min^{-1} for 30 min. The supernatant was heated at 353 K for 30 min to denature heat-labile *E. coli* proteins. After centrifugation at 15 000 rev min^{-1} for 30 min, the supernatant was loaded onto a Resource Q anion-exchange column (GE Healthcare) pre-equilibrated with 25 m*M* CAPS buffer pH 10 containing 5 m*M* $MgCl_2$ and 5 m*M* DTT. The protein was eluted using a linear gradient of 0–1 *M* NaCl buffer solution. The eluted protein was then loaded onto a Superdex 200 gel-filtration column (GE Healthcare) pre-equilibrated with 25 m*M* CAPS pH 10 containing 5 m*M* $MgCl_2$, 100 m*M* NaCl and 5 m*M* DTT. The purified protein was concentrated to 3.7 mg ml^{-1} for crystallization.

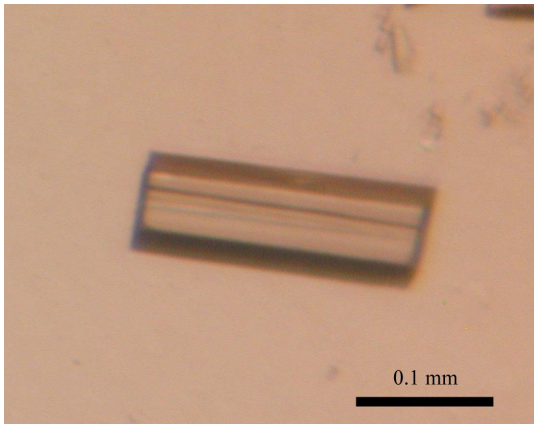


Figure 2
A crystal of PH0186 (SeMet derivative) from *P. horikoshii* obtained by the sitting-drop vapour-diffusion method.

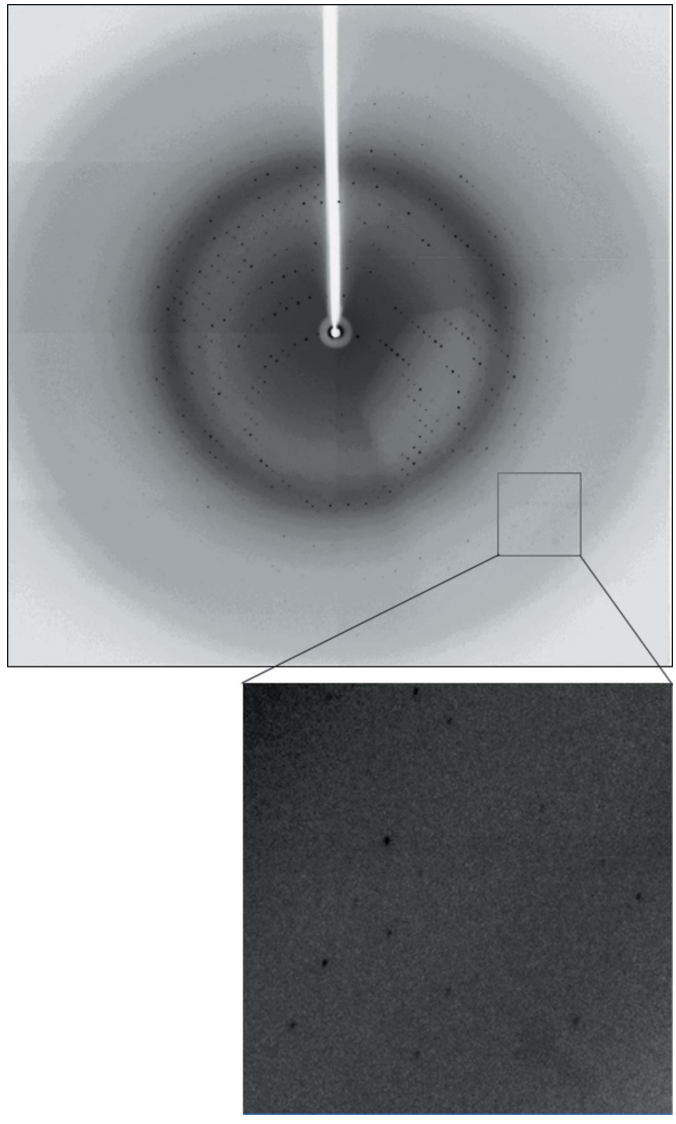


Figure 3
Diffraction pattern of a PH0186 crystal (SeMet derivative). The edge of the image corresponds to a resolution of 1.94 Å. The data were collected on BL38B1 at SPring-8.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

	SeMet data		
	Peak	Edge	Remote
Space group	*C*2		
Unit-cell parameters			
a (Å)	173.66		
b (Å)	51.81		
c (Å)	97.47		
β (°)	122.83		
Wavelength (Å)	0.97905	0.97934	0.99506
Resolution range (Å)	50–2.07 (2.14–2.07)	50–2.25 (2.33–2.25)	50–2.25 (2.33–2.25)
Unique reflections	44784	35062	35186
Redundancy	7.0 (5.1)	3.7 (3.2)	3.6 (3.0)
Completeness (%)	97.7 (84.2)	99.2 (93.6)	97.9 (85.7)
Mean $I/\sigma(I)$	10.1 (2.21)	10.9 (2.41)	10.3 (1.99)
$R_{\rm merge}$† (%)	0.087 (0.391)	0.063 (0.302)	0.064 (0.332)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl, including symmetry-related reflections, and $\langle I(hkl)\rangle$ is its average.

2.2. Crystallization

Crystallization experiments were performed at 293 K using the sitting-drop vapour-diffusion method. Preliminary crystallization trials were carried out using the commercially available sparse-matrix screens Crystal Screens I and II (Hampton Research) and Wizard I and II (Emerald Biostructures). Some rough crystals appeared using a reservoir solution consisting of 15% ethanol, 100 m*M* imidazole pH 8.0 and 200 m*M* $MgCl_2$. After refinement of the crystallization conditions, crystals suitable for X-ray analysis were obtained in 2 d by mixing 1 µl protein solution and 1 µl of a reservoir solution consisting of 9% ethanol, 100 m*M* imidazole pH 7.6 and 200 m*M* $MgCl_2$. Fig. 2 shows a typical crystal (0.2 × 0.05 × 0.05 mm) of PH0186.

2.3. X-ray data collection and processing

A single selenomethionine-labelled crystal was soaked for 1 s in a cryoprotectant solution containing 20%(*v*/*v*) glycerol in the reservoir solution and subsequently flash-cooled in a nitrogen stream at 100 K. Multiwavelength anomalous diffraction (MAD) data sets were collected at synchrotron beamline BL38B1 at SPring-8 (Harima, Japan) using an MSC Jupiter210cs CCD detector. The crystal diffracted to beyond 2.0 Å resolution (Fig. 3). The diffraction data were indexed, integrated and scaled with *HKL*-2000 (Otwinowski & Minor, 1997). The space group of the crystal was determined to be *C*2, with unit-cell parameters $a = 173.7$, $b = 51.8$, $c = 97.5$ Å, $\beta = 122.8°$. To ensure high quality of the data, the resolution was scaled to 2.07 Å for the peak wavelength and 2.25 Å for the edge and remote wavelengths. Statistics of the data collection are summarized in Table 1. The crystals contain three protein molecules per asymmetric unit according to the Matthews coefficient ($V_{\rm M} = 2.2$ Å^3 Da^{-1}; Matthews, 1968) and the solvent content is 43.5%. A self-rotation function was calculated using the program *POLARRFN* as available in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Fig. 4 showed two strongest peaks at $\omega = 90$, $\varphi = 90$ or -90, $\kappa = 60°$, indicating the presence of a noncrystallographic sixfold rotation axis. Because of the crystallographic twofold symmetry in *C*2, the three molecules in the asymmetric unit will form a hexamer by performing the crystallographic symmetry operation; this is consistent with a gel-filtration study, which showed a single peak at almost the molecular weight of a hexamer of PH0186 (data not shown).

To obtain the phase information for structure determination, a multiple anomalous dispersion (MAD) study is under way.

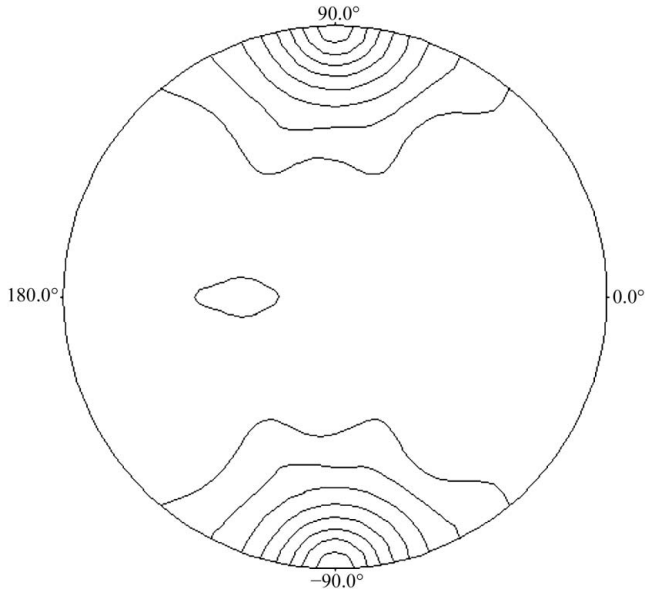


Figure 4
Self-rotation function plot at $\kappa = 60°$. The rotation function was calculated with a radius of integration of 20 Å in the resolution range 20–3 Å. ω (the angle from the pole) is 0 or 180° in the centre and 90° at the edge; φ (the angle around the equator) is as marked on the periphery.

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