Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary crystallographic analysis of the circadian clock protein KaiB from the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1

Ryo Iwase,[a,b]‡ Katsumi Imada,[c,d]*‡ Fumio Hayashi,[a,e]‡ Tatsuya Uzumaki,[a,e] Keiichi Namba[c,d] and Masahiro Ishiura[a,b,e]*

[a]Center for Gene Research, Nagoya University, Furo, Chikusa, Nagoya 464-8602, Japan, [b]Division of Biological Science, Graduate School of Science, Nagoya University, Furo, Chikusa, Nagoya 464-8602, Japan, [c]Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan, [d]Dynamic NanoMachine Project, ICORP, JST, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan, and [e]BRAIN, Nagoya University, Furo, Chikusa, Nagoya 464-8602, Japan

‡ These authors contributed equally to the work.

Correspondence e-mail: kimada@fbs.osaka-u.ac.jp, ishiura@gene.nagoya-u.ac.jp

Received 30 November 2003
Accepted 27 January 2004

KaiB is a component of the circadian clock oscillator in cyanobacteria, which are the simplest organisms that exhibit circadian rhythms. KaiB consists of 108 amino-acid residues and has a molecular weight of 12 025 Da. KaiB and Cys-substituted KaiB mutants from the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1 were expressed as GST-fusion proteins in *Escherichia coli*, purified and crystallized. The crystals of wild-type KaiB belong to the monoclinic space group $P2_1$, with unit-cell parameters $a = 89.6$, $b = 71.2$, $c = 106.8$ Å, $\beta = 100.1^\circ$. While the native crystals diffract to 3.7 Å, osmium derivatives, which show an approximately 4 Å shrinkage in the b axis, diffract to 2.6 Å. The crystals of the singly Cys-substituted mutant T64C with Hg, which show different morphology, diffract to 2.5 Å and belong to the monoclinic space group $P2$, with unit-cell parameters $a = 63.7$, $b = 33.4$, $c = 93.7$ Å, $\beta = 100.1^\circ$. Anomalous difference Patterson maps of the Os- and Hg-derivative crystals had significant peaks in their Harker sections, suggesting that both derivatives are suitable for structure determination.

1. Introduction

Many organisms display autonomous daily cycles in various physiological activities, also known as circadian rhythms, which are an adaptation to the daily cycles in the physical environment on earth such as light and temperature. Previous studies have demonstrated that circadian rhythms are created by an endogenous mechanism known as the circadian clock (Dunlap, 1999).

Cyanobacteria are the simplest organisms that exhibit circadian rhythms. We previously cloned and analyzed the *kai* circadian clock gene cluster in *Synechococcus* sp. strain PCC 7942 (Ishiura *et al.*, 1998). The *kai* cluster consists of three genes, *kaiA*, *kaiB* and *kaiC*, all of which are necessary for circadian oscillations in cyanobacteria. KaiA consists of 283 amino-acid residues with a molecular weight of 32 336 Da, KaiB 108 residues and 12 025 Da and KaiC 518 residues and 57 537 Da. *kaiB* and *kaiC* constitute an operon and *kaiA* is transcribed as an independent gene. Expression of the *kaiBC* operon is negatively regulated by KaiC and positively regulated by KaiA (Ishiura *et al.*, 1998). Interactions between all three Kai proteins were demonstrated *in vitro* and *in vivo* and in the yeast two-hybrid system (Iwasaki *et al.*, 1999). Amino-acid substitution mutations were identified in KaiA, KaiB and KaiC that affect the period length and amplitude of circadian rhythms (Ishiura *et al.*, 1998; Nishiwaki *et al.*, 2000; Taniguchi *et al.*, 2001; Nishimura *et al.*, 2002; Iwasaki *et al.*, 2002), but the roles of individual residues in the mutant proteins have not been elucidated. Recently, the structure of the N-terminal region of KaiA was determined by NMR (Williams *et al.*, 2002) and a hexameric (Mori *et al.*, 2002) or pot-shaped hexameric (Hayashi *et al.*, 2003) structure of KaiC was deduced by electron microscopy or single-particle analysis of electron-microscopic images, respectively.

As part of a larger effort to develop a molecular understanding of the circadian clock machinery, we report here the expression, purification, crystallization and preliminary X-ray analysis of KaiB and Cys-substituted mutants of KaiB from the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1.

2. Materials and methods

2.1. KaiB protein expression and purification

KaiB was expressed as a GST-fusion protein in *Escherichia coli*. A DNA fragment encoding KaiB was cloned into pGEX-6P-1 vector (Amersham Biosciences) using the *Bam*HI–*Xho*I restriction sites. The plasmids were transformed into *E. coli* strain BL21 (Novagen). Cells were grown at 293 K in TB medium (12 g polypeptone, 24 g yeast extract, 4 ml glycerol and 100 ml potassium phosphate buffer per litre) until the culture density reached an OD_{600} of 1.0. KaiB expression was induced for 3 h with isopropyl-1-thio-D-galac-

topyranoside (IPTG) at a final concentration of 0.1 m*M*. Cells were harvested by centrifugation (6600*g* for 10 min), resuspended in 50 m*M* Tris–HCl buffer pH 8.0 containing 50 m*M* NaCl, 1 m*M* EDTA and 1 m*M* DTT and disrupted by sonication. The lysate was centrifuged at 40 000*g* for 30 min to remove cell debris. Clarified supernatant containing GST-KaiB fusion protein was loaded onto a glutathione Sepharose 4B column (Amersham). The column was washed with ten column volumes of 50 m*M* Tris–HCl buffer pH 7.0 containing 150 m*M* NaCl, 1 m*M* EDTA and 1 m*M* DTT. The GST domain was removed from GST-KaiB using proteolytic cleavage by loading PreScission Protease (Amersham) onto the column and incubating at 277 K for 24 h. The protease cleavage produces a KaiB protein that retains five amino acids from the GST domain at its N-terminus. KaiB was eluted with 20 m*M* Tris–HCl buffer pH 7.5 and was purified by ion-exchange chromatography with a MonoQ HR 5/5 column (Amersham). Peak fractions were applied to a Fast Desalting column (Amersham) in 10 m*M* Tris–HCl buffer pH 7.5 containing 10 m*M* NaCl. The eluate was concentrated to 2 mg ml^{-1} by ultrafiltration using a Microcon YM-10 (Millipore) and used for crystallization. The purity of KaiB was assayed by SDS–PAGE and MALDI–TOF mass spectrometry (Voyager-DE/PRO, Applied Biosystems).

2.2. Crystallization

Initial crystallization trials were carried out by the sitting-drop vapour-diffusion method using commercial sparse-matrix screening kits (Crystal Screens I and II, Hampton Research; Wizard I and II, Emerald BioStructures) at 277 and 293 K. Within a week, very thin needle crystals appeared under various conditions containing 10–30%(*w*/*v*) polyethylene glycol (PEG) 3000, PEG 4000 or PEG 8000 at 293 K. The conditions were optimized by varying the buffer pH, precipitant concentration and protein concentration. However, we only obtained clusters of thin needle crystals which were not suitable for X-ray measurement (Fig. 1*a*). The problem was the high nucleation rate. To reduce the nucleation rate, we examined the effects of various organic solvents as an additive, such as 2-propanol, propylene glycol, 2-ethoxyethanol and DMSO. In the presence of DMSO or propylene glycol, small single crystals were grown, but many needle-shaped crystals still appeared in the same drop. We therefore picked up these single crystals for microseeding. The crystals were crushed and seeded into drops with lower precipitant concentration in which no crystals or precipitate had appeared. Nice-looking rod-shaped or thick plate-shaped crystals grew in 1–2 weeks at 293 K. The microseeding procedure requires single crystals, as needle-crystal seeding yielded only thin needle-shaped crystals. After further optimization, crystals suitable for X-ray data collection were produced by the hanging-drop vapour-diffusion method; drops were prepared by mixing 2 µl of 2 mg ml^{-1} KaiB solution and 2 µl of a reservoir solution containing 7.5–10%(*w*/*v*) PEG 3350, 16–21%(*v*/*v*) DMSO and 100 m*M* acetate pH 4.5.

Heavy-atom derivatives were prepared by soaking the crystals in a reservoir solution containing K_2OsCl_6 at 5%(*v*/*v*) saturation. The crystals became slightly coloured after 30 min and were stained dark red purple by the following day. Derivative crystals were typically prepared in 1–2 d.

2.3. Preparation and crystallization of Cys-substituted KaiB mutants

SeMet labelling is a popular and powerful method for phasing. However, this method was not suitable for KaiB, as KaiB contains only one methionine, which is at its N-terminus. Therefore, we introduced a cysteine residue as a binding site for mercury compounds by site-directed mutagenesis. Eight sites for Cys substitution that are likely to be exposed to the solvent and should not affect the folding were selected by solvent-accessibility and secondary-structure prediction (PredictProtein server; http://cubic.bioc.columbia.edu/predictprotein/). Plasmids expressing the mutants were introduced into *E. coli* BL21 (Novagen). Mutant KaiBs were purified by the same procedure as wild-type KaiB, except that DTT was added to all buffers. Although all eight mutants were successfully overexpressed, one of them aggregated during purification and five aggregated after concentration of purified samples. The remaining two, T64C and S81C, were used for crystallization.

Mercury derivatives were prepared by adding 30 m*M* $HgCl_2$ to purified protein solutions to a final concentration of 10 m*M* and incubating for 12 h at 277 K. The solution was loaded onto a PD-10 column (Amersham) and eluted with 10 m*M* Tris–HCl pH 7.5 containing 150 m*M* NaCl to remove excess mercury. The binding of Hg to the protein was confirmed by MALDI–TOF mass spectrometry using a Voyager-DE/PRO.

Hg-derivatized KaiB was concentrated to 2 mg ml^{-1} and used for crystallization in the same way as wild-type KaiB. The solubility of the mutant proteins was sensitive to the salt concentration. Mutants were maintained in solutions containing 150 m*M* NaCl, which was the critical concentration to prevent aggregation.

Crystallization screening for KaiB mutants was initiated around the conditions under which crystals of wild-type KaiB were grown. T64C, S81C and S81C with Hg produced only precipitants. Hg-derivatized T64C (T64C-Hg) formed small single crystals with thin needle clusters. With micro-

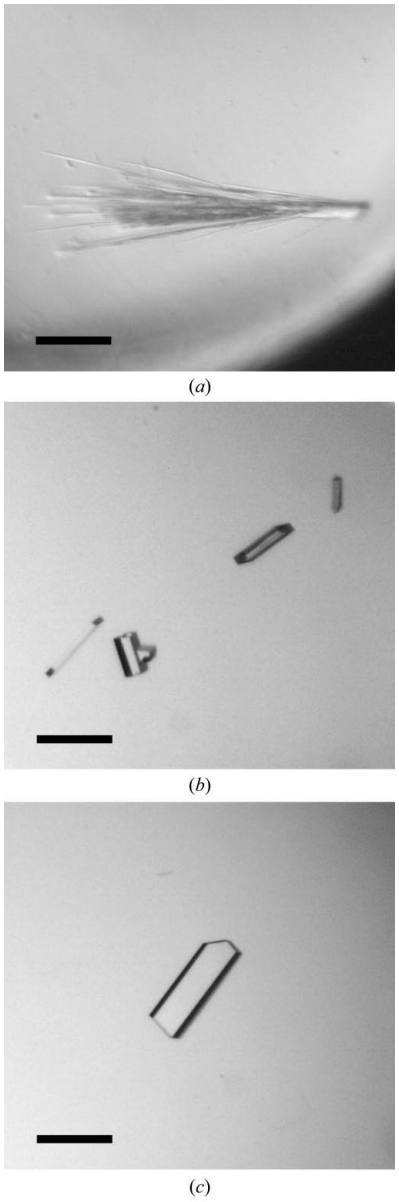

Figure 1
Crystals of KaiB from *T. elongatus*. (*a*) Needle-shaped crystals of KaiB without seeding. (*b*) Typical crystals of mutant T64C with $HgCl_2$ and (*c*) wild-type KaiB. The scale bar indicates 0.1 mm.

seeding, plate-shaped crystals with typical dimensions of 0.1 × 0.1 × 0.03 mm (Fig. 1*b*) were obtained using the hanging-drop vapour-diffusion method. Under optimized conditions, a reservoir solution containing 6–11%(*w*/*v*) PEG 3350, 22–27%(*v*/*v*) DMSO and 100 m*M* acetate buffer pH 4.5 was mixed with an equal volume of protein solution (2 mg ml^{-1}).

2.4. Data collection and processing

Diffraction data were collected at SPring-8 beamlines BL41XU and BL38B1 using MAR CCD (Mar Research) and ADSC Quantum 4R CCD detectors (Area Detector Systems Corporation), respectively. Data were collected at cryogenic temperature. The concentration of DMSO in the crystallization mother liquor was high enough for cryoprotection, which allowed crystals to be directly transferred into liquid nitrogen for freezing and placed in a nitrogen-gas flow at 100 K. Data were indexed, integrated and scaled using the programs *MOSFLM* (Leslie, 1992) and *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

X-ray diffraction measurement was carried out on native crystals of wild-type KaiB with typical dimensions of 0.2 × 0.05 × 0.05 mm (Fig. 1*c*). KaiB crystals belonged to the monoclinic space group $P2_1$, with unit-cell parameters a = 89.6, b = 71.2, c = 106.8 Å, β = 100.1°. While native crystals only diffracted to 3.7 Å, the osmium-derivative crystals gave significantly better diffraction to 2.6 Å resolution. The unit-cell parameters of the derivative crystals were a = 90.1, b = 67.3, c = 105.8 Å, β = 101.3°. The b axis of the derivative crystal was 4 Å shorter than the b axis of the native crystal. When the crystals were soaked in the heavy-atom solution, many cracks appeared immediately and disappeared within 1 h. This observation indicated that the incorporation of the Os atoms in the crystal resulted in some kind of molecular rearrangement and more compact crystal packing.

Matthews coefficient calculations (V_M; Matthews, 1968) indicated six to 13 molecules in the asymmetric unit, corresponding to a solvent content of 71–37%. The self-rotation function calculated using the program *POLARRFN* (Collaborative Computational Project, Number 4, 1994) revealed the presence of at least two twofold non-crystallographic symmetry axes in the crystal *ac* plane. In addition, a very large peak near (0 1/2 0) in the self-Patterson map suggested the presence of a translational symmetry. Because of the complication of possible non-crystallographic symmetry, the number of molecules in the asymmetric unit is ambiguous at this stage of structural analysis.

Table 1
Summary of the data statistics.

Values in parentheses indicate statistics for the last resolution shell.

	KaiB (Os derivative)	T64C (Hg derivative)		
	Peak	Edge	Remote	Peak
Space group	$P2_1$	$P2$		
Unit-cell parameters (Å, °)	a = 90.1, b = 67.3, c = 105.8, β = 101.3	a = 63.7, b = 33.4, c = 93.7, β = 100.1		
Wavelength (Å)	1.1404	1.1408	1.1471	1.0080
Resolution range	56.8–2.8 (2.98–2.8)	56.8–2.7 (2.76–2.7)	56.8–2.8 (3.0–2.8)	48.2–2.5 (2.63–2.5)
Observations	127256	177151	158893	74051
Unique reflections	30755	34308	30718	13914
Completeness (%)	99.7 (99.7)	99.7 (99.7)	99.7 (99.7)	100.0 (100.0)
Redundancy	4.1 (4.2)	5.2 (5.1)	5.2 (5.2)	5.3 (5.4)
$I/\sigma(I)$	4.8 (1.5)	5.1 (2.1)	4.8 (2.4)	6.1 (1.9)
R_{sym}	7.7 (42.4)	5.7 (34.5)	6.1 (40.1)	8.9 (38.1)
R_{ano}	8.4 (24.4)	5.4 (19.8)	3.7 (16.8)	4.6 (17.5)

The T64C-Hg crystals had a different morphology and diffracted to 2.5 Å resolution. The crystals were also monoclinic, but belonged to a different space group, $P2$, with unit-cell parameters a = 63.7, b = 33.4, c = 93.7 Å, β = 100.1°. The Matthews coefficient suggested two to four molecules in the asymmetric unit, with a solvent content of 69–39%. The self-rotation function map suggested the presence of two twofold non-crystallographic symmetry axes in the crystal *ac* plane. No significant peaks were observed in the self-Patterson map. Considering the local symmetry, it is most plausible that there are four molecules in the asymmetric unit.

MAD data collection was attempted for Os-derivative crystals of wild-type KaiB and Hg-derivative crystals of T64C (Table 1). Because of serious radiation damage, only the peak-wavelength data could be collected for T64C-Hg. The anomalous difference Patterson maps of the peak-wavelength data from both derivatives showed significant peaks in their Harker sections. Furthermore, in the dispersive difference Patterson map calculated from the peak- and edge-wavelength data from the Os derivative, significant peaks were observed at the positions of strong peaks found in the anomalous difference Patterson map, suggesting the usefulness of these data for phasing.

We thank Midori Yamane for assistance in crystallization and the staff at SPring-8 beamlines BL41XU and BL38B1 for help in data collection. This research was supported by grants to RI from Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists, grants to MI from the Japanese Ministry of Education, Science and Culture (MEXT), Naito Foundation, PROBRAIN, Future of Japan Society for the Promotion of Science, Japan Space Forum, Aichi Science and Technology Foundation and grants to KI and MI from 'National Project on Protein Structural and Functional Analyses' by MEXT. Division of Biological Science, Graduate School of Science, Nagoya University was supported by a 21st COE grant from MEXT.

References

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Dunlap, J. C. (1999). *Cell*, **96**, 271–290.
Hayashi, F., Suzuki, H., Iwase, R., Uzumaki, T., Miyake, A., Shen, J.-R., Imada, K., Furukawa, Y., Yonekura, K., Namba, K. & Ishiura, M. (2003). *Genes Cells*, **8**, 287–296.
Ishiura, M., Kutsuna, S., Aoki, S., Iwasaki, H., Andersson, C. R., Tanabe, A., Golden, S. S., Johnson, C. H. & Kondo, T. (1998). *Science*, **281**, 1519–1523.
Iwasaki, H., Nishiwaki, T., Kitayama, Y., Nakajima, M. & Kondo, T. (2002). *Proc. Natl Acad. Sci. USA*, **99**, 15788–15793.
Iwasaki, H., Taniguchi, Y., Ishiura, M. & Kondo, T. (1999). *EMBO J.* **18**, 1137–1145.
Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **26**, 27–33.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mori, T., Saveliev, S. V., Xu, Y., Stafford, W. F., Cox, M. M., Inman, R. B. & Johnson, C. H. (2002). *Proc. Natl Acad. Sci. USA*, **99**, 17203–17208.
Nishimura, H., Nakahira, Y., Imai, K., Tsuruhara, A., Kondo, H., Hayashi, H., Hirai, M., Saito, H. & Kondo, T. (2002). *Microbiology*, **148**, 2903–2909.
Nishiwaki, T., Iwasaki, H., Ishiura, M. & Kondo, T. (2000). *Proc. Natl Acad. Sci. USA*, **97**, 495–499.
Taniguchi, Y., Yamaguchi, A., Hijikata, A., Iwasaki, H., Kamagata, K., Ishiura, M., Go, M. & Kondo, T. (2001). *FEBS Lett.* **496**, 86–90.
Williams, S. B., Vakonakis, I., Golden, S. S. & LiWang, A. C. (2002). *Proc. Natl Acad. Sci. USA*, **99**, 15357–15362.