# Structural changes in the cyanobacterial circadian clock machinery revealed by ESR analysis *in vitro*

（藍色細菌生物時計分子装置の構造変化と時間発振）

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**This thesis is based on the following work:**

1. Direct interaction between KaiA and KaiB revealed by a site-directed spin labeling electron spin resonance analysis

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*Genes to Cells*, 2010, 15, 269-280

2. Thermodynamically induced conformational changes of the cyanobacterial circadian clock protein KaiB

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and Masahiro Ishiura

*Applied Magnetic Resonance*, 40:525-534

# 副論文

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(SDSL-ESR 法による KaiA-KaiB 間相互作用の解明)

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## Abbreviation

BSA: bovine serum albumin

CBB: Coomassie Brilliant Blue

DTT: dithiothreitol

ESR: electron spin resonance

GST: glutathione-S-transferase

KaiAc: a KaiA C-terminal clock oscillator domain protein

MSL: mareimide spin label

MTSSL: (1-oxyl-2,2,5,5-tetramethyl-D-pyrroline-3-yl) methyl methanethiosulfonate

NMR: nuclear magnetic resonance

NP-KaiC: non-phosphorylated forms of KaiC

PAGE: polyacrylamide gel electrophoresis

P-KaiC: phosphorylated forms of KaiC

RAP: rhythm-analyzing program

SDS: sodium dodecyl sulfate

SDSL-ESR: site-directed spin labeling electron spin resonance

*Synechococcus*: *Synechococcus* sp. strain PCC 7942

$\tau$: rotational correlation time

TB: terrific broth

**Background**

Circadian rhythms, oscillations with an about 24-h period in metabolic and behavioral activity, are observed from procaryotes to eucaryotes including *Drosophila*, *Neurospora*, *Arabidopsis*, *Chlamydomonas*, and mammals. The rhythms have the following characteristics: (1) persistence in the absence of external cues; (2) resetting by light/dark and low/high temperature signals; and (3) temperature compensation of period length (Bünning, E., 1973). An oscillator located within the cell controls the output pathways that drive the observable rhythms. The phase of the oscillation of the oscillator is reset by input pathways that respond to external time cues such as light and temperature signals (Bünning, E., 1973; Kondo & Ishiura, 1999).

Cyanobacteria are the simplest organisms to exhibit circadian rhythms (Grobbelaar *et al*., 1986). A gene cluster, *kaiABC*, which is essential for the generation of circadian rhythms, has been cloned and analyzed in the cyanobacterium *Synechococcus* sp. strain PCC 7942 (hereafter *Synechococcus*) (Ishiura *et al*., 1998). The cluster consists of two operons, the *kaiA* gene, whose product enhances *kaiBC* expression, and the *kaiBC* operon, which is repressed by *kaiC* gene product (Ishiura *et al*., 1998) (Fig. 1).

The phosphorylation level of KaiC oscillated with a 24-h period in cyanobacterial cells (Iwasaki *et al*., 2002). KaiC has autophosphorylation (Nishiwaki *et al*., 2000; Hayashi *et al*., 2004; Uzumaki *et al*., 2004) and ATPase activities (Terauchi *et al*., 2007; Murakami *et al*., 2008), and both the activities

are enhanced by KaiA (Iwasaki *et al*., 2002; Williams *et al*., 2002; Hayashi *et al*., 2004; Uzumaki *et al*., 2004). KaiB is believed to attenuate these enhancing effects of KaiA (Kitayama *et al*., 2003). Both the N-terminal and C-terminal domains of KaiC have extremely weak ATPase activity (Murakami *et al*., 2008). The ATPase activity of KaiC is temperature-compensated (Tearauchi *et al*., 2007; Murakami *et al*., 2008), and a mutant lacking two phosphorylation sites does not show the temperature-compensation (Murakami *et al*., 2008). Oscillations in the phosphorylation level (Nakajima *et al*., 2005) and ATPase activity (Terauchi *et al*., 2007) of KaiC can be reconstituted *in vitro* in the presence of ATP from only three Kai proteins, KaiA, KaiB and KaiC, indicating that the circadian clock machinery comprising KaiA, KaiB and KaiC generates circadian oscillation in the presence of ATP.

KaiA is a homodimer as revealed by its crystal structure (Garces *et al*., 2004; Uzumaki *et al*., 2004; Ye *et al*., 2004). Its subunit is composed of three functional domains, the N-terminal amplitude-amplifier domain, the central period-adjuster domain and the C-terminal clock-oscillator domain (Uzumaki *et al*., 2004). KaiB has an unusual homotetrameric structure comprising two asymmetrical dimers, as revealed by crystallography (Hitomi *et al*., 2005; Iwase *et al*., 2005; Pattanayek *et al*., 2008). The tetramer has a positively charged cleft flanked by two negatively charged ridges (Iwase *et al*., 2005), and the cleft may play a role in the binding of ligands (Iwase *et al*., 2005). KaiB's C-terminal tail forming the ridges plays a role in sustaining robust rhythms of bioluminescence

*in vivo* (Iwase *et al*., 2005). KaiC forms a hexamer in the presence of ATP, and the hexamer has a pot-shaped structure composed of six identical dumbbell-shaped subunits (Hayashi *et al*., 2003), as revealed by a single particle analysis of electron microscopic images, and the spherical regions of the structure correspond to the N-terminal and C-terminal domains of the subunit (Hayashi *et al*., 2003). This structure has been confirmed by the crystal structure of KaiC hexamer (Pattanayek *et al*., 2004; Pattanayek *et al*., 2009). Although each Kai protein's structure has been determined, the atomic structure of Kai protein complexes has not yet. The structure of various Kai protein complexes and dynamics of their formation during circadian oscillation should be solved to understand the molecular mechanism by which the circadian clock machinery generates oscillations at the atomic level.

Associations among KaiA, KaiB, and KaiC have been demonstrated *in vivo* and *in vitro*. Associations among the Kai proteins in cyanobacterial cells have been revealed by immunoprecipitation analyses (Iwasaki *et al*., 1999; Hayashi *et al*., unpublished). Interactions between KaiA and KaiC were studied in detail *in vitro* using highly purified protein preparations (Uzumaki *et al.*, 2004; Hayashi *et al*., 2004). An equimolar complex of KaiA and KaiC is formed temporarily in the presence of ATP (Hayashi *et al*., 2004) *via* the interaction between KaiC's C-terminal domain (Hayashi *et al.*, 2006) and KaiA's C-terminal clock-oscillator domain (Uzumaki *et al*., 2004; Kim *et al*., 2008). Associations between KaiB and KaiC have been revealed by pull-down assay (Kageyama *et*

*al*., 2006), and KaiB is suggested to bind to the C-terminal domain of KaiC from the results of small-angle X-ray scattering analysis (Akiyama *et al*., 2008). In fact, KaiB and KaiC (or the C-terminal domain of KaiC) form a stable complex as revealed by native polyacrylamide gel electrophoresis (PAGE) (Pattanayek *et al*., 2008). Weak interaction between KaiA and KaiB has been demonstrated using the yeast two-hybrid system and immunoblotting (Iwasaki *et al*., 1999), but direct interaction remains to be detected. Interactions between KaiA and KaiB could not be detected by surface plasmon resonance analysis (Hayashi *et al*., unpublished).

The cyanobacterial clock-related genes such as the input pathway period-extender gene *pex* (Kutsuna *et al*., 1998; Takai *et al*., 2006a) (Fig. 1), and the output pathway genes *sasA* (Iwasaki *et al*., 2000), *rpaA* (Takai *et al*., 2006b) and *labA* (Taniguchi *et al*., 2007) also have been identified (Fig. 1). Pex is a DNA binding protein and a dimer as revealed by crystal structure (Arita *et al*., 2007; Kurosawa *et al*., 2009). Pex may be a repressor that suppresses *kaiA* expression (Kutsuna *et al*., 2007; Kurosawa *et al*., 2009) (Fig. 1). Mutational analysis showed that a 5-bp cis-element (AGAGA) in the *kaiA* upstream region is essential to the negative regulation of *kaiA* by Pex (Kutsuna *et al*., 2007). SasA is a histidine kinase, which interacts with KaiC (Iwasaki *et al*., 2000; Takai *et al*., 2006b) (Fig. 1). RpaA is an OmpR-type DNA-binding response regulator, which interacts with SasA (Takai *et al*., 2006b) (Fig. 1). Both SasA and RpaA are important output factors that regulate the transcription of clock-controlled genes

including *kaiBC* (Fig. 1). In the presence of KaiC and ATP, a phosphotransfer reaction from SasA to RpaA occurs *in vitro* (Takai *et al*., 2006b). The *labA* gene was identified as a gene required for the negative feedback regulation of KaiC (Taniguchi *et al*., 2007). LabA is likely to be involved in the repression of circadian *kaiBC* expression through RpaA (Fig. 1), mainly during subjective early night when the phosphorylation level of KaiC level is high (Taniguchi *et al*., 2007).

Electron spin resonance (ESR) spectroscopy is a powerful tool for analyses of protein structure and function, as well as the dynamics and relative orientation of the protein components of protein complexes. Because the majority of proteins do not contain magnetic species, an extrinsic probe called a spin label is introduced at a specific site (usually a cystein residue (Cys)) of the protein. Spin labels are nitroxide derivatives carrying a stable unpaired electron and a functional group for specific attachment to the protein. Nitroxides produce a three-line hyperfine pattern due to the unpaired electron on the nitrogen atom of the NO group. When it is introduced into the Cys of proteins, we can know the environment in the vicinity of the spin labels from the ESR spectra of the labeled-proteins by analyzing the spectra and calculating the correlation time $\tau$ (mobility) of spin labels from the ESR spectra.

Here, I analyzed the structural changes of Kai proteins during their interactions using a site-directed spin labeling (SDSL)-ESR method. I labeled each Cys of mutant KaiB proteins from the thermophillic cyanobacterium

*Thermosynechococcus elongatus*, which grows optimally at 57 °C (Yamaoka *et al*., 1978), performed ESR analyses. *T. elongatus* proteins were chosen for this study because they are highly stable. Furthermore, efficient procedures for gene transfer and manipulation and the real-time monitoring of circadian bioluminescence rhythms in *T. elongatus* have been established (Onai *et al*., 2004). I found that KaiA and KaiB interact with each other directly, and that this interaction occurs at temperatures higher than 30 °C. This is the first report of direct stoichiometric interaction between KaiA and KaiB. I determined the interaction sites of both KaiA and KaiB. I demonstrated the temperature dependence of the mobility of the spin labels introduced at the 64th residue. I compared the calculated correlation times $\tau$ of five sites in KaiB, two located on the second $\alpha$ helix on the dimer-dimer interface of the KaiB molecule (Iwase *et al*., 2005) and three on the loop on the negatively charged ridges (Iwase *et al*., 2005), before and after incubation at 40 °C for 24 h. The results showed that the mobility of the spin labels around the dimer-dimer interface of the KaiB tetramer molecule, but not on the negatively charged ridges, changes on incubation.

Fig. 1

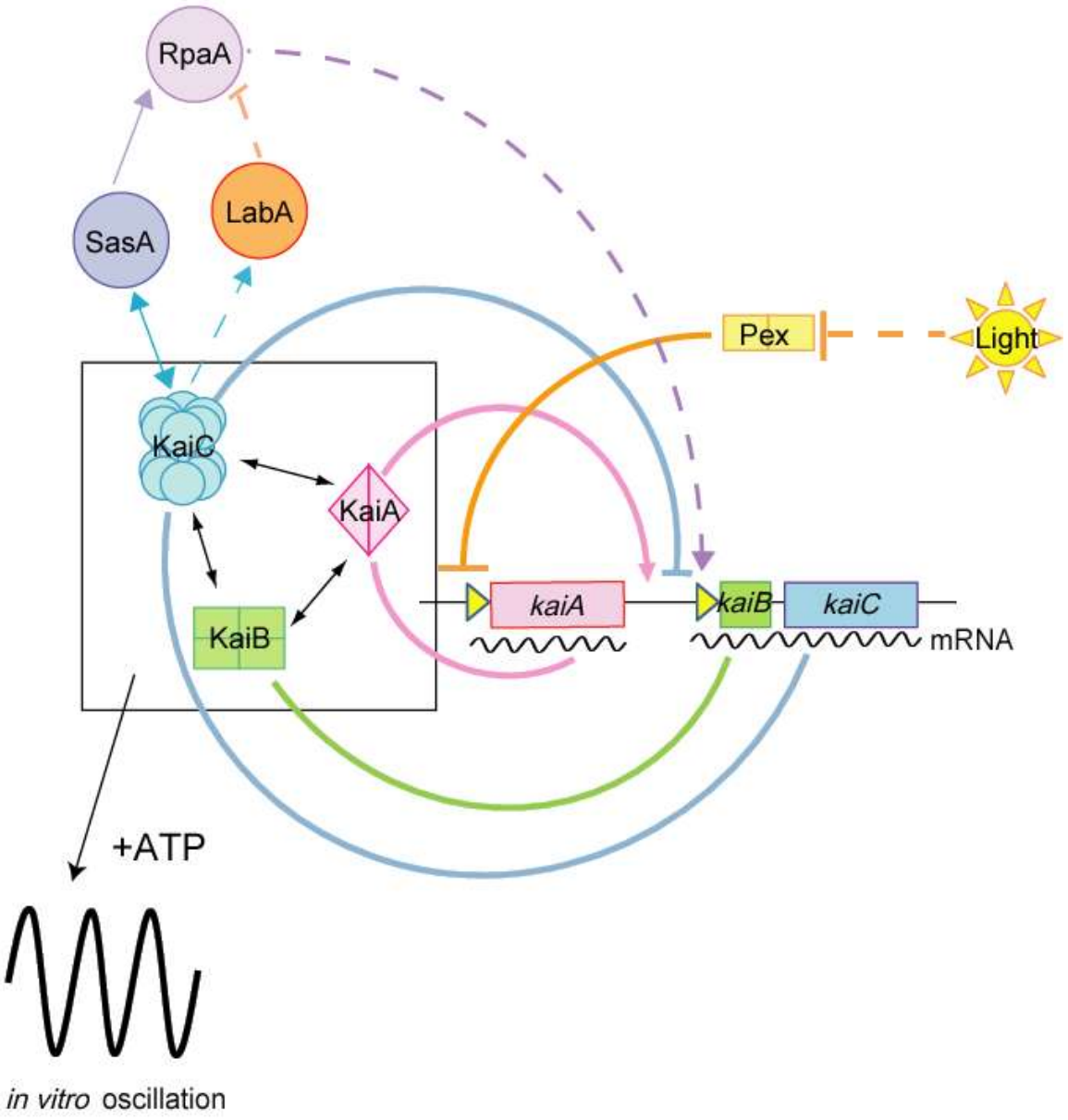

RpaA
LabA
SasA
Pex
Light
KaiC
KaiA
KaiB
kaiA
kaiB
kaiC
mRNA
+ATP
in vitro oscillation

Fig. 1. A model of the cyanobacterial circadian clock system including the oscillator and the input and output pathways.

## Chapter 1

Direct interaction between KaiA and KaiB

revealed by a site-directed spin labeling electron spin resonance analysis


### Abstract

In cyanobacteria, three clock proteins, KaiA, KaiB, and KaiC, play essential roles in generating circadian oscillations. The interactions of these proteins change during the circadian cycle. Here, I demonstrated direct interaction between KaiA and KaiB using electron spin resonance (ESR) spectroscopy. I prepared cystein residue (Cys)-substituted mutants of *Thermosynechococcus elongatus* KaiB, labeled specifically their Cys with spin labels, and measured the ESR spectra of the labeled KaiB. I found that KaiB labeled at the 64th residue showed spectral changes in the presence of KaiA, but not in the presence of KaiC or bovine serum albumin as a negative control. KaiB labeled at the 101st residue showed no such spectral changes even in the presence of KaiA. The results suggest that KaiB interacts with KaiA in the vicinity of the 64th residue of KaiB. Further analysis demonstrated that the C-terminal clock-oscillator domain of KaiA is responsible for this interaction.

## Introduction

Circadian rhythms, biological oscillations with a period of 24-hour in various metabolic and behavioral activities, are observed ubiquitously from procaryotes to eucaryotes (Bünning, E., 1973). Cyanobacteria are the simplest organisms that exhibit circadian rhythms (Grobbelaar *et al*., 1986). The clock machinery itself is composed of only three clock proteins called KaiA, KaiB, and KaiC. KaiC has an autophosphorylation activity (Nishiwaki *et al*., 2000; Hayashi *et al*., 2004; Uzumaki *et al*., 2004), which is enhanced through interaction with KaiA (Iwasaki *et al*., 2002; Williams *et al*., 2002; Hayashi *et al*., 2004; Uzumaki *et al*., 2004). KaiB is believed to attenuate the enhancing effect of KaiA (Kitayama *et al*., 2003). The phosphorylation level of KaiC oscillates not only in cyanobacterial cells (Iwasaki *et al*., 2002) but also in an ATP-dependent *in vitro* clock system composed of KaiA, KaiB and KaiC (Nakajima *et al*., 2005). The three clock proteins interact each other, and the size and stoichiometry of Kai protein complex may change during clock oscillation. The interaction between KaiA and KaiC has been reported previously (Uzumaki *et al.*, 2004; Hayashi *et al*., 2004). KaiA C-terminal domain interacts with KaiC C-terminal domain (Hayashi *et al*., 2004). The interaction between KaiB and KaiC has also been reported previously (Kageyama *et al*., 2006, Akiyama *et al*., 2008; Pattanayek *et al*., 2008). KaiC C-terminal domain interacts with KaiB (Pattanyek *et al*., 2008). No direct stoichiometric interaction between KaiA and KaiB had been detected.

Here, I demonstrated the interaction between KaiA and KaiB by the site-directed spin labeling (SDSL) electron spin resonance (ESR) method, identified the interaction sites on KaiA and KaiB, and determined its stoichiometry.

## Materials and methods

*Preparation of the wild-type and mutant KaiA, KaiB, and KaiC proteins*

I produced recombinant Kai proteins using *Escherichia coli* as a host, as described previously with modifications (Hayashi *et al*., 2004). The plasmids that express the wild-type and mutant forms of KaiA (pTekaiA expressing wild-type KaiA and pTekaiAc expressing a KaiA C-terminal clock oscillator domain protein (KaiAc) containing amino acid residues 174 to 283 of KaiA; the N-terminal residue of KaiAc is numbered 174), KaiB (pTekaiB expressing wild-type KaiB and pTekaiB$_{T64C}$ expressing a mutant KaiB with a Cys-substitution at the 64th residue, threonine (Thr) (Thr64)), and KaiC (pTekaiC) derived from *T. elongatus* were described previously (Uzumaki *et al*., 2004; Hayashi *et al*., 2004; Iwase *et al*., 2005). Other plasmids for the production of mutant forms of *T. elongatus* KaiA (pTekaiAc$_{H270A}$ carrying the *kaiA* gene with an alanine (Ala)-substitution mutation at the 270th residue, histidine (His) (His270) and pTekaiAc$_{C272A}$ carrying *kaiA* with an Ala-substitution mutation at the 272nd residue, Cys (Cys272)) and KaiB (pTekaiB$_{A101C}$ carrying *kaiB* with a Cys-substitution mutation at the 101st residue, Ala (Ala101)) were constructed by a procedure described previously (Hayashi *et al*., 2004). *E. coli* strain BL21 cells carrying pTekaiA, pTekaiAc, pTekaiAc$_{H270A}$, pTekaiAc$_{C272A}$, pTekaiB, pTekaiB$_{T64C}$, pTekaiB$_{A101C}$, and pTekaiC, respectively, were grown in Terrific Broth (TB) supplemented with 50 μg/ml ampicillin. Cells were harvested by centrifugation, resuspended in 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 1 mM dithiothreitol (DTT) and 50

mM NaCl, and disrupted by sonication. Cleared lysates were prepared by centrifugation, and glutathione-S-transferase (GST)-fused forms of KaiA, KaiB, and KaiC were prepared from the lysates using Glutathione Sepharose 4B (GE Healthcare, Buckinghamshire, UK) and cleaved by a PreScission Protease (GE Healthcare). The digests were dialyzed at 4 °C for 4 h against 1 L of 20 mM Tris-HCl buffer (pH 7.5 for KaiB and KaiC, pH 8.5 for KaiA) containing 1 mM DTT to remove glutathione. Then, they were further dialyzed at 4 °C for 12 h against 1 L of 20 mM MES-NaOH buffer (pH 6.5 for KaiB) or 20 mM Tris-HCl buffer (pH 8.5 for KaiA, pH 7.5 for KaiC) containing 1 mM DTT and 50 mM NaCl. The dialysates were applied to a Hiload 26/10 Q Sepharose HP column (GE Healthcare) to purify the wild-type and mutant KaiB and KaiC proteins or to a Hitrap DEAE FF column (GE Healthcare) to purify the wild-type and mutant KaiA proteins, and the proteins were further purified by ion-exchange chromatography on a MonoQ HR 5/5 column (GE Healthcare). We estimated protein concentrations using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., California, U. S. A.) and bovine serum albumin (BSA) as a standard, as described previously (Hayashi *et al*., 2004).

*Preparation of spin-labeled KaiB*

I introduced spin labels into each unique Cys of mutant KaiB proteins with a Cys-substitution mutation ($KaiB_{T64C}$ and $KaiB_{A101C}$) (Figs. 1A and 1B). Just before labeling, DTT was removed from solutions containing the mutant KaiB

proteins by gel filtration chromatography on a HiTrap Desalting column (GE Healthcare) equilibrated with 20 mM MES-NaOH buffer (pH 6.5) containing 200 mM NaCl. Fractions containing the mutant KaiB proteins were collected and concentrated to a concentration of 2.0 mg/ml using Amicon 5k (Millipore, MA, U. S. A.). The proteins were incubated with a 100-fold molar excess of (1-oxyl-2,2,5,5-tetramethyl-D-pyrroline-3-yl) methyl methanethiosulfonate (MTSSL) or maleimide spin label (MSL) in the MES-NaOH (pH 6.5) buffer at 20 °C for 2 h. Then, unreacted free MTSSL or MSL was removed from the reaction mixtures by gel filtration chromatography on a HiTrap Desalting column equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl. Fractions containing the proteins were collected and concentrated using Amicon 5k to a protein concentration of 1.5 mg/ml. I estimated the efficiency of spin labeling by integrating the spectra of spin-labeled proteins and found that each subunit of the Cys-substituted KaiB proteins was almost completely labeled with MSL and MTSSL under my conditions.

*Assay for phosphorylation of KaiC*

KaiC hexamer (0.5 μM) was incubated with both 0.75 μM KaiA dimer and 0.75 μM MSL-KaiB$_{T64C}$ tetramer at 40 °C in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM ATP, 5 mM $MgCl_2$ and 150 mM NaCl. Then, 15-μl aliquots of the reaction mixtures were collected every 3 h and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) on

12.5 % gels (acrylamide: bisacrylamide= 144: 1), and then the gels were stained with Coomassie Brilliant Blue (CBB). KaiC shows triplet bands on SDS-PAGE (Hayashi *et al*., 2006). The upper two bands correspond to the phosphorylated forms of KaiC (P-KaiC), whereas the lowest band corresponds to the non-phosphorylated forms of KaiC (NP-KaiC). The ratio of P-KaiC to total KaiC (the phosphorylation level of KaiC) was calculated by densitometry using Lane Analyzer (ATTO) and CS Analyzer (ATTO). Circadian oscillations were simulated by rhythm-analyzing program (RAP) (Okamoto *et al*., 2005).

*ESR measurements*

Twenty micromolar (in monomer) of spin-labeled KaiB (MTSSL-labeled $KaiB_{T64C}$ (MTSSL-$KaiB_{T64C}$), MTSSL-labeled $KaiB_{A101C}$ (MTSSL-$KaiB_{A101C}$), and MSL-labeled $KaiB_{T64C}$ (MSL-$KaiB_{T64C}$) was mixed with 5 μM of either KaiA dimer (including the mutant KaiA proteins), KaiC hexamer, or BSA in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM ATP, 5 mM $MgCl_2$ and 150 mM NaCl, and incubated at 40 ˚C. Then, 20-μl aliquots of the reaction mixtures were injected into a 80-μl ESR sample capillary tube. I measured their ESR spectra at 4 ˚C for 15 min, using a Bruker ESP-300E spectrometer (Bruker BioSpin, Ettlingen, Germany) or a Varian E-109 ESR spectrometer (Varian, Palo Alto, U.S.A.). I defined signal intensity as the sum of the three peak heights, $h_{-1}$, $h_0$, and $h_1$, of ESR spectra as shown in Fig. 3A.

*Calculation of the rotational correlation time $\tau$ of spin labels*

Flexibility of molecular motion is related quantitatively in ESR to the rotational correlation time $\tau$ of nitroxide spin-labeled molecules. The general theory for a fast rotation model and derivation of $\tau$ expression in nitroxide spin-labels was established by McConnell (McConnell, 1956) and in more detail by Kivelson (Kivelson, 1960). $\tau$ was calculated by the following equation in the region between $\tau$ = $10^{-11}$ and $10^{-9}$ s:

$$\tau = 6.5 \times 10^{-10} W_0 \left(\sqrt{\frac{h_0}{h_{-1}}} - 1\right),$$

where $W_0$ is the width peak-to-peak of the central peak, and $h_0$ and $h_{-1}$ are the heights of the mid- and high-field peaks on a first-derivative absorption spectrum, respectively (see Fig. 3A for $h_0$ and $h_{-1}$).

For a slow rotation model in the region between $\tau$=$10^{-9}$ and $10^{-6}$ s, $\tau$ was calculated (Mason and Freed, 1974):

$$\tau = a\left(1 - \frac{A_Z'}{A_Z}\right)^b,$$

where $A_Z$ and $A_Z$' are hyperfine constants for the rigid limited value and experimental value, respectively. The rigid limited value was evaluated with the ESR spectra obtained at -276 ˚C. The parameters *a* and *b* are given by the calculated diffusion model.

*Calculation of the time constant $t_c$ for reactions between MTSSL-KaiB$_{T64C}$ and KaiA*

The signal intensity of MTSSL-KaiB$_{T64C}$ in the absence or presence of

KaiA, KaiC or BSA was plotted against time *t*, and the curves were fitted using the following equation:

$$y = A(1-\exp(-t/t_c))+B,$$

where *A* and *B* are constants, $t_c$ is a time constant, and *y* is signal intensity. By using this equation, I calculated the value of $t_c$.

*Assay for the formation of a stable complex between KaiB$_{T64C}$ and KaiA by forming an inter-protein disulfide bond*

Reaction mixtures containing MTSSL-KaiB$_{T64C}$ and KaiAc were incubated at 40 ˚C as described above. Then, 10-μl aliquots of the reaction mixtures were subjected to SDS-PAGE on 15 % gels (acrylamide: bisacrylamide = 37.5:1), and visualized by staining with CBB. I estimated the amount of complex formed between KaiB$_{T64C}$ and KaiAc by densitometry using a Lane Analyzer (ATTO, Tokyo, Japan) and a CS Analyzer (ATTO).

*Immunoblot analysis*

I identified a complex between KaiB$_{T64C}$ and KaiAc by SDS-PAGE followed by immunoblotting. Reaction mixtures containing MTSSL-KaiB$_{T64C}$ (or KaiB$_{T64C}$ or wild-type KaiB) and/or KaiAc were incubated at 40 ˚C for 6 h as described above and then at 90 ˚C for 5 min in the absence or presence of 10 mM DTT. Five microliter aliquots of the reaction mixtures were subjected to SDS-PAGE, blotted onto Hybond ECL nitrocellulose membranes (GE

Healthcare), and visualized using Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, MA, U. S. A.) and an rabbit anti-KaiA antiserum (diluted to 1/2000) or an anti-KaiB antiserum (diluted to 1/2000), as described previously (Hayashi *et al*., 2004). A donkey anti-rabbit Ig antibody (GE Healthcare) was used as a secondary antibody.

*Identification of free MTSSL released from MTSSL-KaiB$_{T64C}$ after the reaction between MTSSL-KaiB$_{T64C}$ and KaiA*

Reaction mixtures containing MTSSL-KaiB$_{T64C}$ and KaiA were incubated at 40 °C for 12 h as described above, then released spin labels were separated from the reaction mixtures by gel filtration chromatography on a HiTrap Desalting column equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. Fractions containing spin labels were collected and measured ESR spectra as described above.

*Estimation of the molar ratio of KaiB$_{T64C}$ to KaiAc in a complex between KaiB$_{T64C}$ and KaiAc by gel filtration chromatography*

Reaction mixtures containing MTSSL-KaiB$_{T64C}$ and KaiAc were incubated at 40 ˚C for 6 h as described above and then applied to a Superdex 75/HR 10/30 column (GE Healthcare) equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl at 4 ˚C. After gel filtration chromatography, fractions containing a complex between KaiB$_{T64C}$ and KaiAc were subjected to

SDS-PAGE on 15 % gels and visualized by staining with CBB. I determined the amounts of $KaiB_{T64C}$ and KaiAc in the complex by densitometry to estimate the molar ratio of $KaiB_{T64C}$ to KaiAc.

## Result

*Circadian oscillation of the phosphorylation level of KaiC in an in vitro KaiABC clock system*

Oscillation in the phosphorylation level of KaiC occurred *in vitro* in reaction mixtures containing three Kai proteins at a molar ratio of 3: 3: 2 (KaiA 2mer: KaiB 4mer: KaiC 6mer) in the presence of ATP at 40 °C, as reported in an *in vitro Synechococcus* KaiABC system (Nakajima *et al*., 2005). The oscillation occurred even when MSL-KaiB$_{T64C}$ was used in replace of wild-type KaiB, although the period of oscillation observed in MSL-KaiB$_{T64C}$ was 1.5 h longer than that in wild-type KaiB (Fig. 2). This indicates that MSL-KaiB$_{T64C}$ is able to generate circadian oscillation as well as wild-type KaiB.

*ESR spectra of spin-labeled KaiB*

I introduced a Cys into Thr64 and Ala101 of KaiB, respectively, by site-directed mutagenesis (Figs. 1A and 1B). The ESR spectra of free MTSSL and free MSL comprised only three sharp peaks of almost equal height reflecting the mobility of the free spin labels (Fig. 3A). The peaks in the ESR spectra of MTSSL-KaiB$_{T64C}$ (Fig. 3B, black) and MSL-KaiB$_{T64C}$ (Fig. 3J, black), by contrast, were broadened, indicating the mobility of the spin labels to be partially limited by covalent bonding to KaiB$_{T64C}$.

The ESR spectra of MTSSL-KaiB$_{T64C}$ and MTSSL-KaiB$_{A101C}$ did not change significantly during incubation at 40 ˚C for 6 h (Figs. 3B and 3C). The

spectral differences between MTSSL-KaiB$_{T64C}$ and MTSSL-KaiB$_{A101C}$ are related to rotational correlation time, $\tau$ (Keith *et. al*., 1970). The $\tau$ value of MTSSL-KaiB$_{T64C}$ was about 121 ns at 4 ˚C, assuming slow rotation and a Brownian diffusion model with a 3-gauss linewidth. The $\tau$ value of MTSSL-KaiB$_{A101C}$ was about 1.8 ns at 4 ˚C, assuming a fast rotation model. Thus, MTSSL's mobility in KaiB$_{T64C}$ was about 70 times lower than that in KaiB$_{A101C}$. This result is consistent with the fact that the 64th residue is located inside of the KaiB molecule, whereas the 101st residue is located outside (Iwase *et al*., 2005).

*ESR spectral changes of MTSSL-KaiB in the presence of KaiA*

MTSSL-KaiB$_{T64C}$ showed spectral changes when incubated with KaiA at 40 ˚C (Fig. 3D). The signal intensity gradually increased and had almost reached a plateau at 6 h (Fig. 4A). Because these spectral changes were not observed when MTSSL-KaiB$_{T64C}$ was incubated with either KaiC (Fig. 3E) or BSA (Fig. 3F), they are likely to have resulted from the specific interaction of MTSSL-KaiB$_{T64C}$ with KaiA. Furthermore, MTSSL-KaiB$_{A101C}$ showed no such spectral changes even in the presence of KaiA (Fig. 3G). Therefore, it seems that KaiA approached the MTSSL connected to the 64th Cys (Cys64) of KaiB$_{T64C}$ during its interaction with KaiB, and the interaction changed the environment around the spin label. Neither KaiC nor BSA could interact with the MTSSL-labeled Cys64 of MTSSL-KaiB$_{T64C}$. Furthermore, the MTSSL-labeled Cys101 of MTSSL-KaiB$_{A101C}$

could not interact with KaiA.

*Kinetics of the interaction of MTSSL-KaiB$_{T64C}$ with KaiA*

I measured the spectral changes of MTSSL-KaiB$_{T64C}$ in the presence of various amounts of KaiA. The reaction rate for increases in signal intensity rose rapidly as the concentration of KaiA dimer increased up to 2 μM (Fig. 5A). I calculated the Michaelis constant ($K_m$) for the KaiA dimer in the reaction of MTSSL-KaiB$_{T64C}$ with KaiA to be 0.30 ± 0.05 μM ($n$=3) (Fig. 5B).

*Temperature-dependence of the spectral changes of MTSSL-KaiB$_{T64C}$*

I examined the effects of reaction temperature on the spectral changes of MTSSL-KaiB$_{T64C}$ in the presence of KaiA. MTSSL-KaiB$_{T64C}$ scarcely reacted with KaiA at 4 ˚C or 10 ˚C, as demonstrated by the ESR spectra (Fig. 4A). The reaction rate for increases in signal intensity rose abruptly at 30 ˚C and continued to increase linearly as the temperature increased (Fig. 4A). This suggests that some changes in structure or conformation required for the reaction of MTSSL-KaiB$_{T64C}$ with KaiA, occurred with a sharp boundary at around 30 ˚C (Fig. 4B). I calculated the Arrhenius activation energy ($E_a$) for the reaction and obtained values of 577 kJ/mol from 4 ˚C to 30 ˚C and 91.2 kJ/mol from 30 ˚C to 50 ˚C.

*Structural or conformational changes of KaiB induced by temperature changes*

To clarify the changes of KaiB induced by changes in temperature, I used another spin label, MSL, in place of MTSSL, and measured ESR spectra of MSL-KaiB$_{T64C}$. The spectra of MSL-KaiB$_{T64C}$ did not change significantly on incubation at 4 ˚C for 6 h (Fig. 3J). However, when MSL-KaiB$_{T64C}$ was incubated at 40 ˚C for 6 h, its ESR spectrum greatly changed and its signal intensity also greatly increased (Fig. 3K, see the red spectrum). This indicates that the environment surrounding the MSL connected to the Cys64 of KaiB changed on incubation at 40 ˚C. This phenomenon did not occur in MTSSL-KaiB$_{T64C}$. MSL, which has a bulky five-member ring (Fig. 1D), may be susceptible to minor structural or conformational changes in KaiB$_{T64C}$, whereas MTSSL, which has a narrowside chain than MSL (Fig. 1C), may be less susceptible to such changes. This result suggests that changes in structure or conformation around the Cys64 of KaiB$_{T64C}$ occur at 40 ˚C, but not at 4 ˚C. I will describe this in detail in Chapter 2.

*KaiB-interaction site of KaiA*

To clarify the site of KaiA that interacts with KaiB, I examined the effects of KaiA mutations on the reaction of MTSSL-KaiB$_{T64C}$ with KaiA. First, I measured the spectral changes of MTSSL-KaiB$_{T64C}$ in the presence of KaiAc, a C-terminal domain protein of KaiA (Uzumaki *et al*., 2004). A time constant for increases in the signal intensity of MTSSL-KaiB$_{T64C}$ in the presence of KaiAc was within the margin of error in the presence of wild-type KaiA (Table 1). This

suggests that the site of interaction with KaiB is located in the C-terminal domain of KaiA.

Next, I measured the spectral changes of MTSSL-KaiB$_{T64C}$ in the presence of two amino acid residue-substitution mutants of KaiAc, KaiAc$_{C272A}$ and KaiAc$_{H270A}$ (Figs. 1E and 1F), and found the time constant for increases in signal intensity to be greatly increased by the mutations (Table 1). This suggests the Cys272 and His270 of KaiAc to be involved in the interaction with MTSSL-KaiB$_{T64C}$. Cys272 is the only Cys of KaiAc (Uzumaki *et al*., 2004), and His270 is a functionally essential residue of KaiA required for interaction with KaiC and the generation of circadian oscillations (Uzumaki *et al*., 2004).

*Release of free MTSSL from MTSSL-KaiB$_{T64C}$ in the presence of KaiA*

By subtracting the ESR spectra of reaction mixtures containing MTSSL-KaiB$_{T64C}$ and KaiA incubated at 40 ˚C for 6 h from those of the reaction mixtures before incubation, I obtained three sharp peaks, corresponding to the ESR spectrum of highly mobile free MTSSL (Fig. 3H). This suggests that the MTSSL bound covalently to the Cys64 of KaiB$_{T64C}$ *via* a disulfide bond was released by the interaction with KaiA.

To confirm the release of free MTSSL from MTSSL-KaiB$_{T64C}$ on interaction with KaiA, I separated the released spin labels from the reaction mixtures by gel filtration chromatography, and then measured the ESR spectra of the fractions (low-molecular weight fractions) containing free labels. Spectra

with three sharp peaks of equal height were obtained, confirming the existence of free MTSSL in the fractions (Fig. 3I).

*Formation of a stable complex via a disulfide bond after reactions between MTSSL-KaiB$_{T64C}$ and KaiAc*

To examine the possible formation of a stable complex between KaiB$_{T64C}$ and KaiAc during a 6-h incubation at 40 ˚C, I analyzed the products of the reaction between KaiB (MTSSL-KaiB$_{T64C}$, KaiB$_{T64C}$, or wild-type KaiB) and KaiAc by SDS-PAGE (Figs. 6A-C). When MTSSL-KaiB$_{T64C}$ was incubated with KaiAc, a new band corresponding to a KaiB-KaiAc complex was recognized by both the anti-KaiB antiserum and the anti-KaiA antiserum (Figs. 6D and 6E). When the reaction products were treated with 10 mM DTT before SDS-PAGE, this band disappeared (Figs. 6D and 6E). Therefore, this result indicates a temporary complex comprising KaiB$_{T64C}$ and KaiAc covalently connected by a disulfide bond (Fig. 6F).

Because Cys272 is the only Cys of KaiAc, it should be responsible for this disulfide bond. A mutant KaiAc carrying no Cys, KaiAc$_{C272A}$, showed similar spectral changes although its time constant was extremely high (namely, its reaction rate was extremely low) (Table 1), suggesting other reactive residues, probably located around the Cys, to be involved in the release of free MTSSL from MTSSL-KaiB$_{T64C.}$ However, because the contribution of those residues was very minor, it was not considered here.

To determine the stoichiometry of the complex comprising $KaiB_{T64C}$ and KaiAc, I measured the amounts of $KaiB_{T64C}$ and KaiAc in a $KaiB_{T64C}$-KaiAc complex by gel filtration chromatography followed by SDS-PAGE and densitometry (Fig. 7A). In our experimental conditions, the complex contained 9.2 ± 0.3 pmol of KaiAc monomer and 11.4 ± 0.4 pmol of $KaiB_{T64C}$ monomer (*n*=3) (Fig. 7B), suggesting that one subunit of $KaiB_{T64C}$ bound to one subunit of KaiAc.

**Discussion**

Despite results suggesting interaction between KaiA and KaiB (Iwasaki *et al*., 1999), no direct stoichiometric interaction had been detected probably because KaiA and KaiB interact only temporarily. Here, I clearly demonstrated their interaction by using SDSL-ESR. In MTSSL-KaiB$_{T64C}$, MTSSL connected covalently to the Cys64 of KaiB$_{T64C}$ *via* a disulfide bond (Fig. 1C). When MTSSL-KaiB$_{T64C}$ was incubated with KaiA at 40 ˚C, the disulfide bond was replaced with one between KaiB$_{T64C}$ and KaiA, resulting in a stable KaiB$_{T64C}$-KaiA complex (Fig. 6B) and the concomitant release of free MTSSL (Figs. 3H and 3I). A similar disulfide-exchange reaction has been reported between phospholamban and sarcoplasmic reticulum $Ca^{2+}$-ATPase (Kirby *et al*., 2004).

This phenomenon occurred in MTSSL-KaiB$_{T64C}$ but not MTSSL-KaiB$_{A101C}$ (Fig. 3G). Thus, the 64th residue, not the 101st residue, of KaiB should be located near the KaiA-interaction site. The Cys272 of KaiA's C-terminal domain (the only Cys of KaiAc) is responsible for this reaction (Table 1). Therefore, the 64th residue of KaiB and the Cys272 of KaiA should closely approach each other during the formation of a temporal KaiB-KaiA complex. Based on the crystal structure of the KaiB tetramer, the four 64th residues are located inside of the molecule (Iwase *et al*., 2005) although they can be seen from outside. On one surface of the molecule (defined as the upper surface), there is a positively charged cleft, which is covered with two negatively charged

ridges (Iwase *et al*., 2005). Two of the 64th residues of the KaiB tetramer are located near the positively charged cleft, which is the functional site of KaiB required for generating circadian rhythms *in vivo* (Iwase *et al*., 2005). Although these two residues occur near the cleft, the 64th residue itself is not essential (Iwase *et al*., 2005). Previously, Iwase *et al*. have proposed that the positively charged cleft may be exposed for ligand binding by moving the negatively charged ridge (Iwase *et al*., 2005). On the other side of the molecule (defined as the lower surface), there are no distinguishable substructures. Therefore, Iwase *et al*. hypothesize that KaiB interacts with KaiA and KaiC on the upper surface (Iwase *et al*., 2005). I believe that KaiB's structure or conformation changes during interactions with KaiA (and KaiC). The two Cys272 of the KaiA dimer are also located inside of the molecule (Uzumaki *et al*., 2004). Therefore, not only KaiB but also KaiA probably changes its structure to interact directly with the other.

The rate of the reaction between MTSSL-KaiB$_{T64C}$ and KaiA increased sharply at around 30 ˚C (Fig. 4A). There was a large difference in $E_a$ between 4 ˚C to 30 ˚C and 30 ˚C to 50 ˚C. Because the $E_a$ at 4 ˚C to 30 ˚C was 577 kJ/mol, the reactions between MTSSL-KaiB$_{T64C}$ and KaiA that occurred at temperatures higher than 30 ˚C scarcely occurred at temperatures lower than 30 ˚C. The structure or conformation of KaiB may have changed at temperatures higher than 30 ˚C as schematized in Fig. 8. When MSL-KaiB$_{T64C}$ was incubated at 40 ˚C, the signal intensity increased (Fig. 3K). This suggests that the environment

surrounding the spin label changed at 40 ˚C, and supports our hypothesis. *T. elongatus* cells grow optimally at 57 ˚C (Yamaoka *et al*., 1978), and although circadian bioluminescence rhythms *in vivo* are observed even at 30 ˚C, their amplitude is very small (Onai *et al*., 2004). This is also consistent with the present finding.

I determined the stoichiometry of a $KaiB_{T64C}$-KaiA complex to be one subunit of KaiB to one subunit of KaiA (Figs. 7A and 7B). Because KaiA is a stable homodimer (Garces *et al*., 2004; Uzumaki *et al*., 2004; Ye *et al*., 2004), two subunits of KaiB should bind to one molecule of KaiA dimer. If KaiB is also a stable homotetramer (Hitomi *et al*., 2005; Iwase *et al*., 2005; Pattanayek *et al*., 2008), one molecule of KaiB tetramer binds to two molecules of KaiA dimer. Based on the available atomic structure of KaiA (Garces *et al*., 2004; Uzumaki *et al*., 2004; Ye *et al*., 2004) and KaiB (Hitomi *et al*., 2005; Iwase *et al*., 2005; Pattanayek *et al*., 2008), it is not easy to explain the stoichiometry of the $KaiB_{T64C}$-KaiA complex determined here. It is possible that large changes in the structure of KaiB and/or KaiA including oligomerization must occur in order for these interactions to occur.

KaiA interacted with the MTSSL of MTSSL-$KaiB_{T64C}$ *via* its Cys272, which is located near its His270, a functionally essential residue required for both the generation of circadian rhythms *in vivo* and interaction with KaiC *in vitro* (Uzumaki *et al*., 2004). Thus, KaiB may compete with KaiC to interact with KaiA. KaiB may inhibit the ATPase (Terauchi *et al*., 2007) and autokinase activities of

KaiC *via* direct binding to KaiC (Pattanayek *et al*., 2008) or binding to KaiA or both. The value of $K_m$ for the interaction between KaiA and KaiB determined here was 0.30 μM. Circadian oscillations *in vitro* occur at concentrations of Kai proteins ranging from 0.35 μM to 5.0 μM (Kageyama *et al*., 2006; Mutoh *et al*., unpublished). Thus, the KaiB-KaiA interaction reported here occurred in a similar concentration range to that reported previously, suggesting its physiological significance.

In this study, I determined one of the sites responsible for the interaction between KaiA and KaiB. By mapping these sites more precisely not only on KaiB but also on KaiA, I can simulate the structural or conformational changes in KaiB and KaiA required for interaction. A similar approach may also be useful for the analysis of interactions between KaiA and KaiC, between KaiB and KaiC, and among KaiA, KaiB and KaiC during circadian oscillations. An X-ray crystallographic analysis of Kai protein complexes and an ESR analysis of distance changes during interactions among Kai proteins are necessary.

Fig. 1

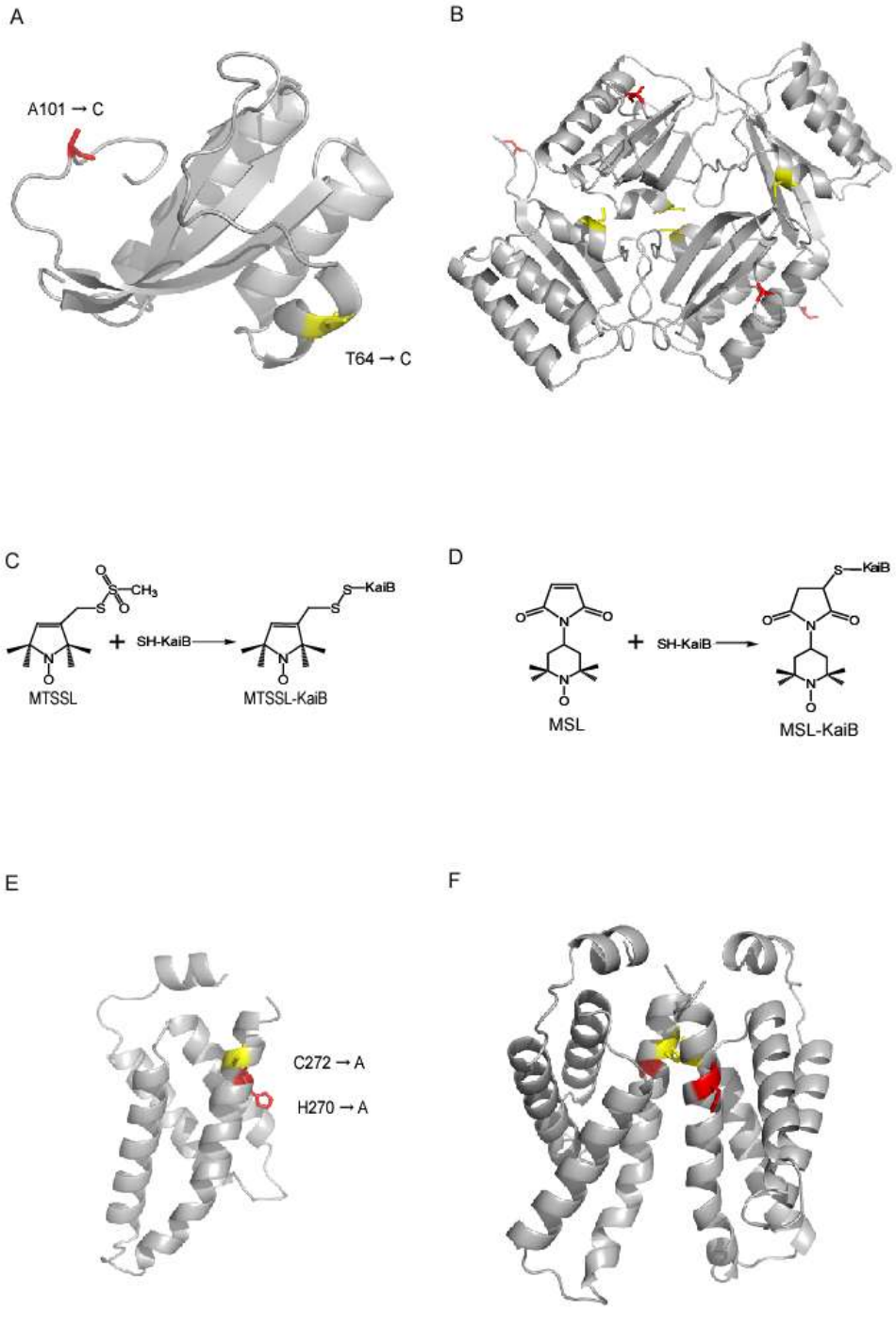

A
A101 → C
T64 → C
B
C
MTSSL
SH-KaiB
MTSSL-KaiB
D
MSL
SH-KaiB
MSL-KaiB
E
C272 → A
H270 → A
F

Fig. 1. Sites of the Cys introduced by site-directed mutagenesis shown in the crystal structure of the *T. elongatus* KaiB monomer and tetramer, a scheme for the spin labeling of the Cys, and the site of the Ala-substitutions introduced into *T. elongatus* KaiAc by site-directed mutagenesis. $KaiB_{T64C}$ and $KaiB_{A101C}$ have the labeling site mutations T64C (yellow) and A101C (red), respectively (A, monomer; B, tetramer). Each Cys of the KaiB mutants was reacted with MTSSL (C) or MSL (D). MTSSL is connected to the Cys of proteins by a disulfide bond whereas MSL is connected to the residues by a C-S bond. Therefore, the former can be released by reduction with DTT, but not the latter. The sites of the Ala-substitution mutations introduced into *T. elongatus* KaiAc, His270 and Cys272, are shown in red and yellow, respectively (E, monomer; F, dimer).

Fig. 2

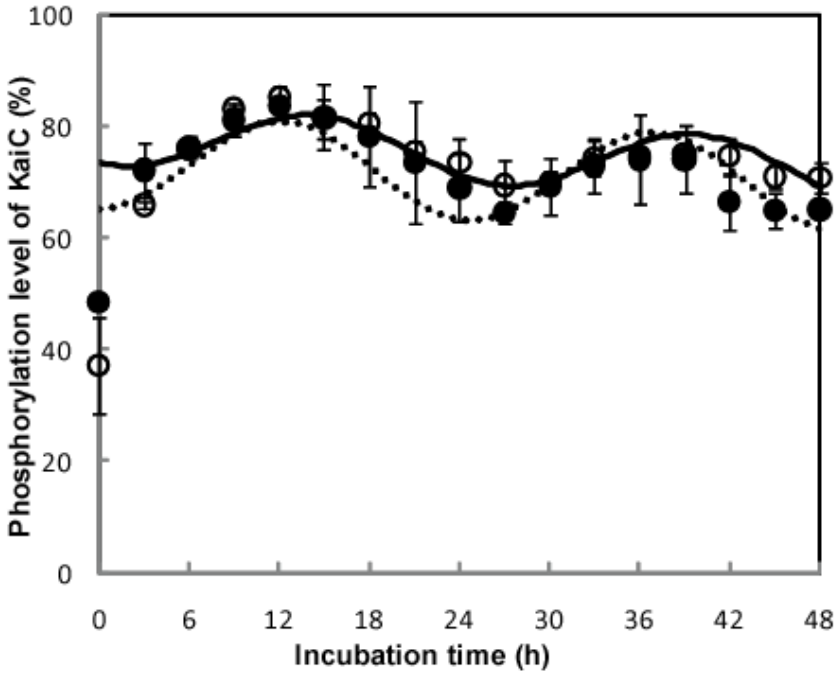

Phosphorylation level of KaiC (%)
100
80
60
40
20
0
0
6
12
18
24
30
36
42
48
Incubation time (h)

Fig. 2 Circadian oscillation of the phosphorylation level of KaiC in an *in vitro* KaiABC system using MSL-KaiB$_{T64C}$ in place of wild-type KaiB. Reaction mixtures containing KaiA (0.75 μM in dimer), KaiB (wild-type KaiB (closed circle, dotted line) or MSL-KaiB$_{T64C}$ (open circle, solid line)) (0.75 μM in tetramer) and KaiC (0.5 μM in hexamer) were incubated in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM ATP, 5 mM $MgCl_2$, and 150 mM NaCl at 40 °C. The ratio of phosphorylated KaiC to total KaiC (phosphorylation level of KaiC) was assayed by SDS-PAGE followed by densitometry. Standard deviation was calculated from triplicate measurements.

Fig. 3

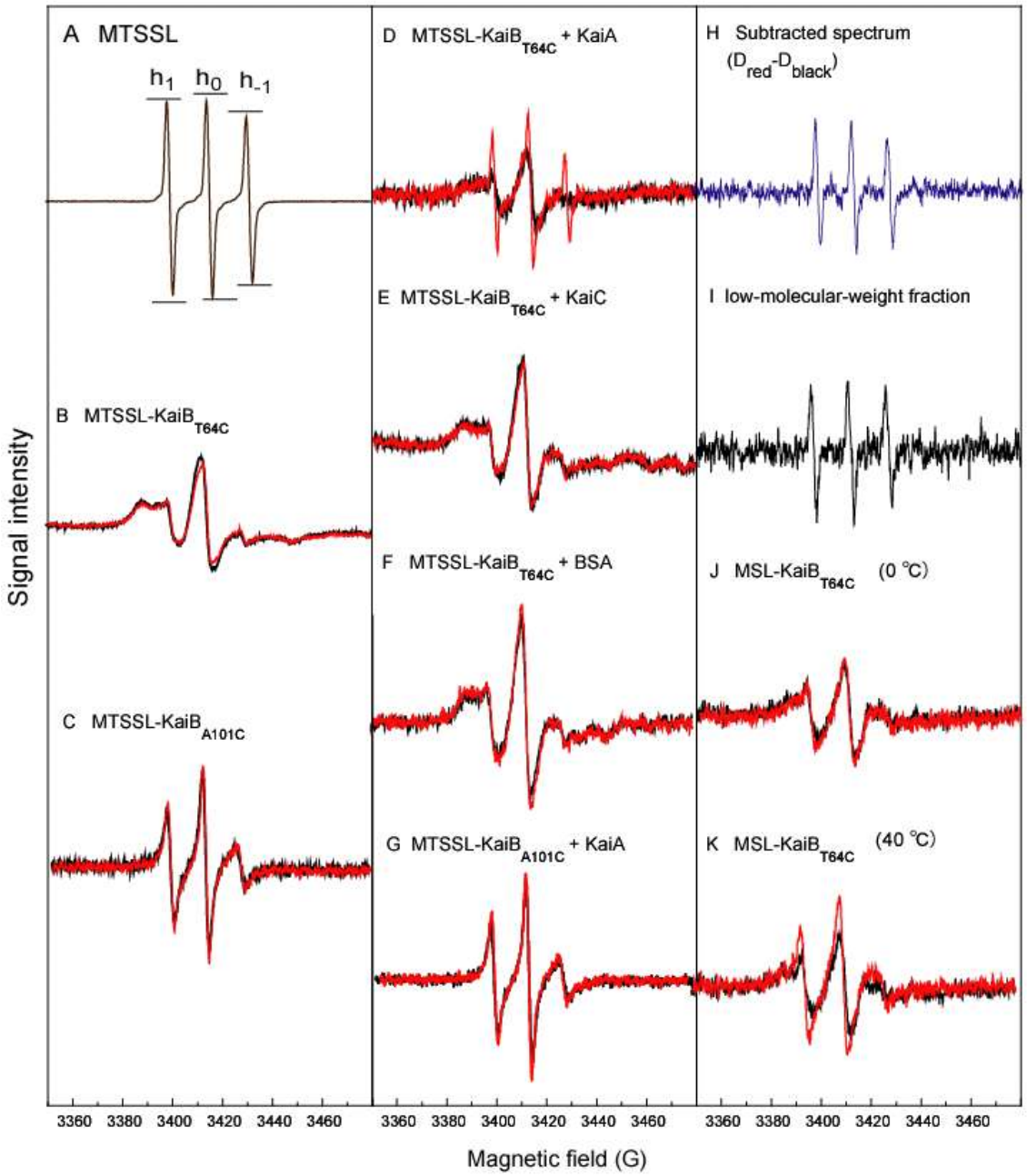
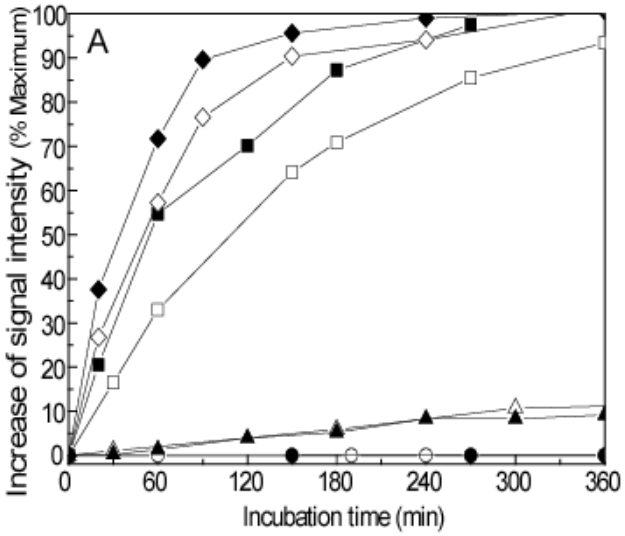

A MTSSL
h1 h0 h-1
B MTSSL-KaiB T64C
C MTSSL-KaiB A101C
D MTSSL-KaiB T64C + KaiA
E MTSSL-KaiB T64C + KaiC
F MTSSL-KaiB T64C + BSA
G MTSSL-KaiB A101C + KaiA
H Subtracted spectrum (Dred-Dblack)
I low-molecular-weight fraction
J MSL-KaiB T64C (0 ℃)
K MSL-KaiB T64C (40 ℃)
Signal intensity
3360 3380 3400 3420 3440 3460
Magnetic field (G)

Fig. 3. ESR spectra of free MTSSL, MTSSL-KaiB$_{T64C}$, MTSSL-KaiB$_{A101C}$, MSL-KaiB$_{T64C}$, and those of the reaction mixtures containing MTSSL-KaiB$_{T64C}$ and either KaiA or KaiC or BSA. The ESR spectra of spin-labeled KaiB proteins alone and those of reaction mixtures containing 20 μM (in monomer) of the labeled KaiB proteins and either 5 μM KaiA dimer or 5 μM KaiC hexamer or 5 μM BSA before and after incubation were measured in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM ATP, 5 mM $MgCl_2$, and 150 mM NaCl under the following conditions: temperature, 4 ˚C; microwave frequency, 9.45 GHz; microwave power, 2 mW; modulation frequency, 100 kHz; modulation amplitude, 1 G. ESR spectra: free MTSSL (A); MTSSL-KaiB$_{T64C}$ (B) and MTSSL-KaiB$_{A101C}$ (C) before (black) and after (red) incubation at 40 ˚C for 6 h; the reaction mixtures of MTSSL-KaiB$_{T64C}$ and KaiA (D), MTSSL-KaiB$_{T64C}$ and KaiC (E), MTSSL-KaiB$_{T64C}$ and BSA (F), and MTSSL-KaiB$_{A101C}$ and KaiA (G) before (black) and after (red) incubation at 40 ˚C for 3 h (the red samples shown in F were incubated for 1.5 h); the difference spectrum obtained by subtracting spectrum D in black from spectrum D in red (H); the spectrum of the low-molecular-weight fractions containing the released spin labels (I); MSL-KaiB$_{T64C}$ before (black) and after (red) incubation at 4 ˚C (J) and 40 ˚C (K) for 6 h.

Fig. 4


A
Increase of signal intensity (% Maximum)
Incubation time (min)
0
10
20
30
40
50
60
70
80
90
100
0
60
120
180
240
300
360

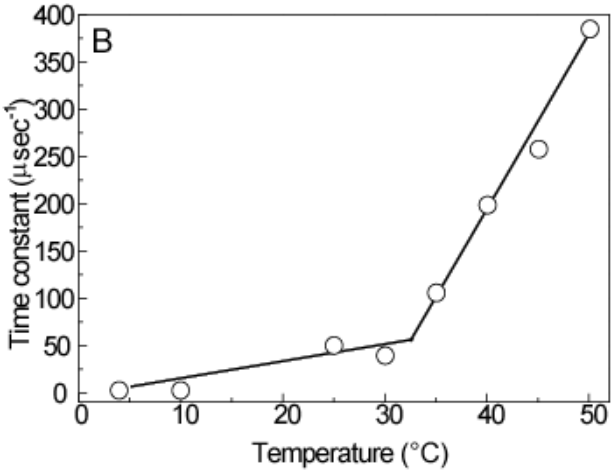

B
Time constant (μsec-1)
Temperature (°C)
0
50
100
150
200
250
300
350
400
0
10
20
30
40
50

Fig. 4. Time course of increases in signal intensity and temperature-dependence of the time constant for increases in signal intensity. Reaction mixtures containing MTSSL-KaiB$_{T64C}$ and KaiA were incubated at 4 ˚C (open circles), 10 ˚C (closed circles), 25 ˚C (open triangles), 30 ˚C (closed triangles), 35 ˚C (open squares), 40 ˚C (closed squares), 45 ˚C (open diamonds), and 50 ˚C (closed diamonds), and then, 20-μl aliquots of the reaction mixtures were subjected to ESR measurements. Other conditions were the same as described in the legend for Fig. 3. (A) Time course of increases in signal intensity. Values of the sum of the three peak-to-peak heights ($h_1$, $h_0$, and $h_{-1}$ shown in Fig. 3A) were plotted. (B) Temperature-dependence of the time constant for increases in signal intensity. A typical plot from three independent experiments is shown.

Fig. 5

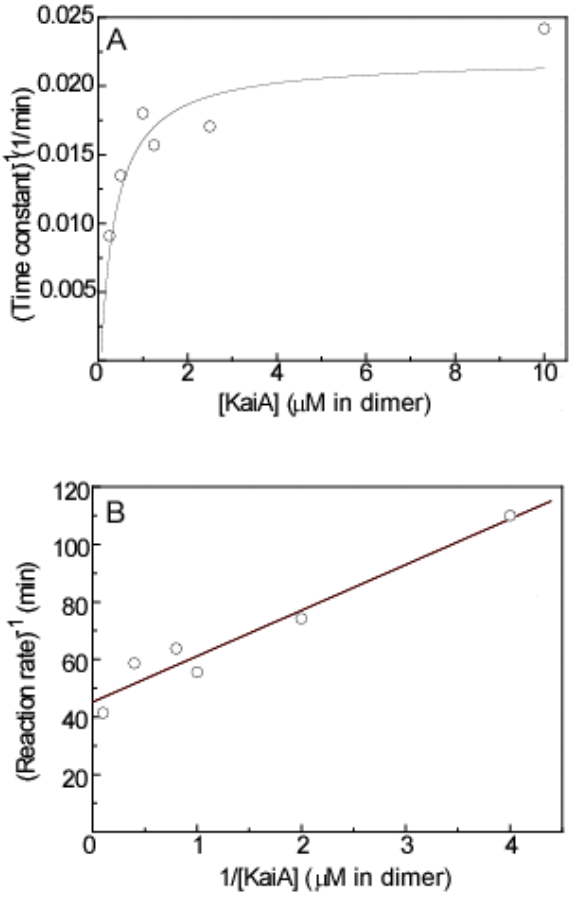

A
(Time constant)$^{-1}$ (1/min)
0.025
0.020
0.015
0.010
0.005
0
2
4
6
8
10
[KaiA] (μM in dimer)
B
(Reaction rate)$^{-1}$ (min)
120
100
80
60
40
20
0
1
2
3
4
1/[KaiA] (μM in dimer)

Fig. 5. KaiA concentration-dependency of increases in signal intensity of MTSSL-KaiB$_{T64C}$ and its reciplocal plot. Reaction mixtures containing MTSSL-KaiB$_{T64C}$ and various amounts of KaiA were incubated at 40 ˚C for 6 h. Other conditions were the same as described in the legend for Fig. 3. A typical plot from three independent experiments is shown.

Fig. 6

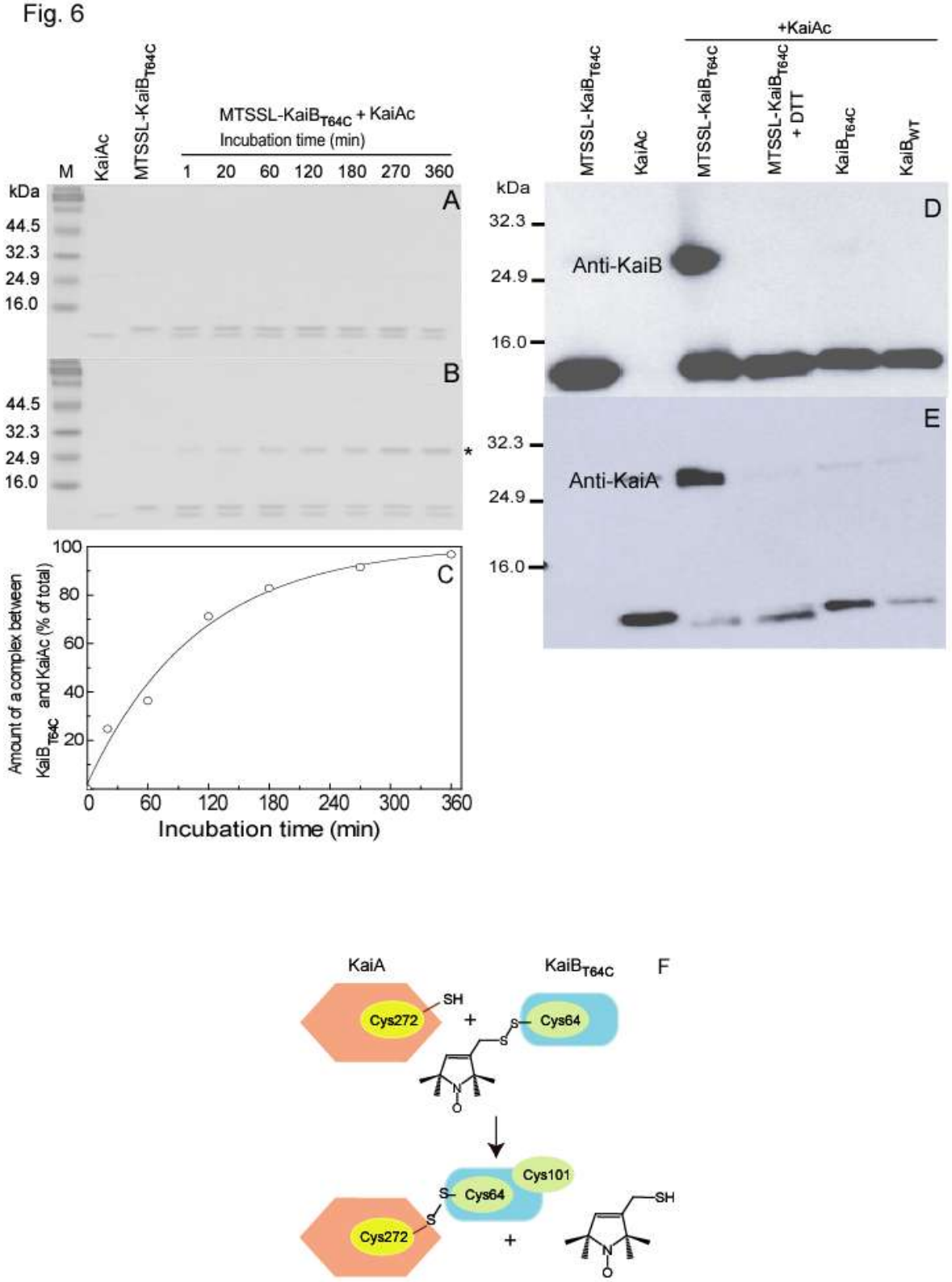

M
KaiAc
MTSSL-KaiB$_{T64C}$
MTSSL-KaiB$_{T64C}$ + KaiAc
Incubation time (min)
1 20 60 120 180 270 360
kDa
44.5
32.3
24.9
16.0
A
B
*
C
Amount of a complex between KaiB$_{T64C}$ and KaiAc (% of total)
100
80
60
40
20
0 60 120 180 240 300 360
Incubation time (min)
+KaiAc
MTSSL-KaiB$_{T64C}$
KaiAc
MTSSL-KaiB$_{T64C}$
MTSSL-KaiB$_{T64C}$ + DTT
KaiB$_{T64C}$
KaiB$_{WT}$
kDa
32.3
24.9
16.0
D
Anti-KaiB
E
Anti-KaiA
F
KaiA
KaiB$_{T64C}$
Cys272
SH
+
Cys64
Cys101
Cys64
Cys272
SH
+

Fig. 6. Formation of a stable complex between $KaiB_{T64C}$ and KaiAc *via* an inter-protein disulfide bond. Reaction mixtures containing MTSSL-$KaiB_{T64C}$ and KaiAc were incubated at 40 ˚C for 6 h, then at 90 ˚C for 5 min in the presence or absence of 10 mM DTT, and subjected to SDS-PAGE. Samples treated with DTT (A) or not treated (B). Other conditions were the same as described in the legend for Fig. 3. The asterisk indicates a complex between $KaiB_{T64C}$ and KaiAc. (C) Amount of $KaiB_{T64C}$-KaiAc complex estimated by densitometry. (D) Immunoblots of the reaction products of MTSSL-$KaiB_{T64C}$ with KaiAc after SDS-PAGE. Reaction mixtures were incubated at 40 ˚C for 6 h and then at 90 ˚C for 5 min in the absence or presence of DTT, subjected to SDS-PAGE, blotted to nitrocellulose membranes, and then reacted with an anti-KaiB antiserum (D) or an anti-KaiA antiserum (E) to visualize KaiA and KaiB, respectively. Other conditions were the same as described in (A) and (B). Lanes are numbered from left to right. Lane 1, MTSSL-$KaiB_{T64C}$; lane 2, KaiAc; lane 3, MTSSL-$KaiB_{T64C}$ + KaiAc; lane 4, MTSSL-$KaiB_{T64C}$ + KaiAc, treated with DTT; lane 5, $KaiB_{T64C}$ + KaiAc; lane 6, wild-type KaiB + KaiAc. (F) Disulfide bond-exchanging reactions between MTSSL-$KaiB_{T64C}$ and KaiAc (KaiA). The disulfide-exchange reactions result in the formation of a stable $KaiB_{T64C}$-KaiAc complex connected covalently by a disulfide bond between the Cys64 of $KaiB_{T64C}$ and the Cys272 of KaiAc and the concomitant release of free MTSSL.

Fig. 7

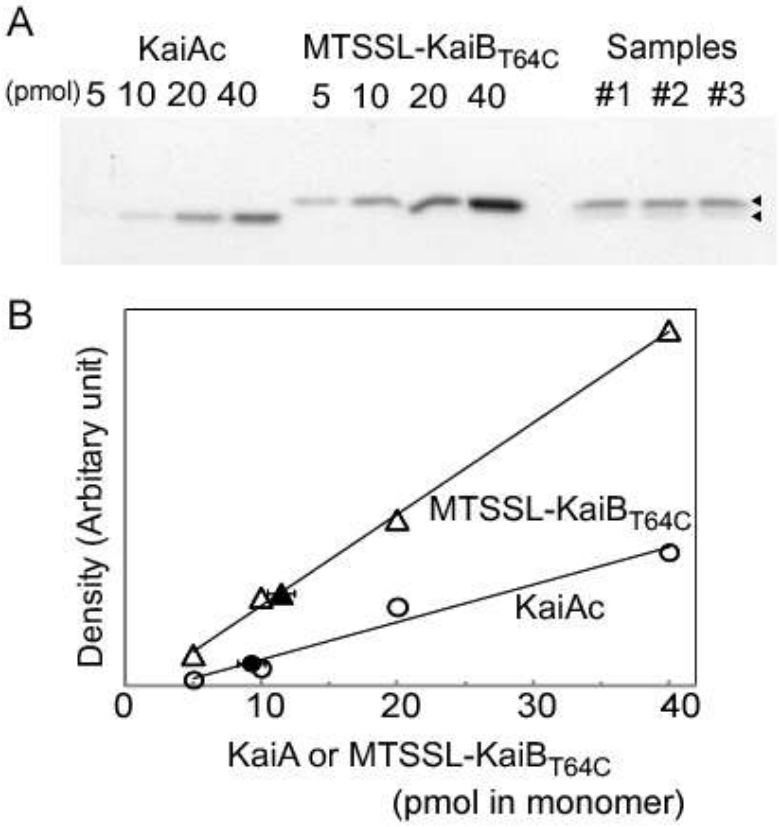

A
KaiAc
MTSSL-KaiB$_{T64C}$
Samples
(pmol) 5 10 20 40
5 10 20 40
#1 #2 #3
B
Density (Arbitary unit)
MTSSL-KaiB$_{T64C}$
KaiAc
0
10
20
30
40
KaiA or MTSSL-KaiB$_{T64C}$
(pmol in monomer)

Fig. 7. Estimation of the molar ratio of $KaiB_{T64C}$ to KaiAc in the $KaiB_{T64C}$-KaiAc complex. Lanes are numbered from left to right. (A) SDS-PAGE to calibrate the amounts of MTSSL-$KaiB_{T64C}$ and KaiAc, and the amounts of $KaiB_{T64C}$ and KaiAc in the $KaiB_{T64C}$-KaiAc complex. Reaction mixtures (500 μl) containing MTSSL-$KaiB_{T64C}$ and KaiAc were incubated at 40 ˚C for 6 h and applied to a Superdex 75 column (GE Healthcare). After gel filtration chromatography, samples containing the $KaiB_{T64C}$-KaiA complex were subjected to SDS-PAGE on 15 % gels and visualized by staining with CBB. Standard amounts of KaiAc and MTSSL-$KaiB_{T64C}$ were also applied to the SDS-PAGE gels. Other conditions were the same as described in the legend for Fig. 3. Lanes 1 to 4, 5 to 40 pmol KaiAc; lanes 5 to 8, 5 to 40 pmol MTSSL-$KaiB_{T64C}$; lanes 9 to 11, triplicate samples from the fractions of gel filtration chromatography containing the $KaiB_{T64C}$-KaiAc complex. (B) Calibration curves. Symbols: open triangles, MTSSL-$KaiB_{T64C}$; open circles, KaiAc; closed triangle and circle, $KaiB_{T64C}$ and KaiAc in the complex, respectively. A typical plot from triplicate experiments is shown.

Fig. 8

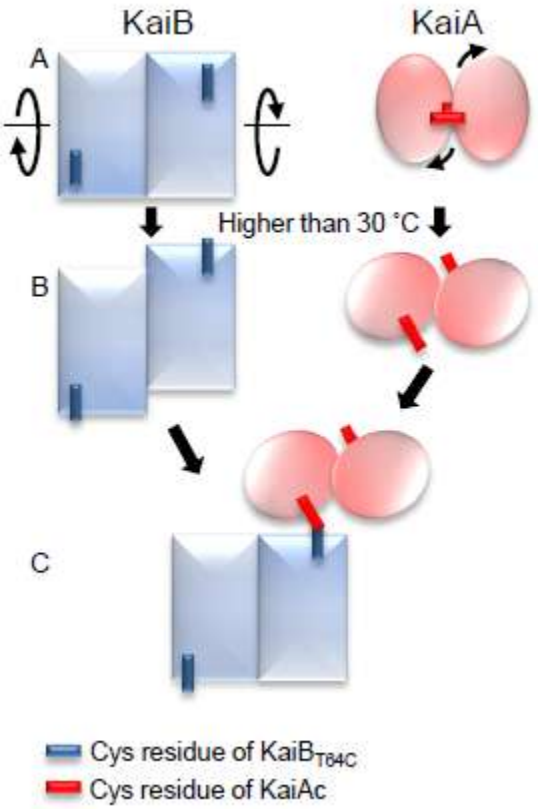

KaiB
KaiA
A
Higher than 30 °C
B
C
Cys residue of KaiB$_{T64C}$
Cys residue of KaiAc

Fig. 8. Model for the structural or conformational changes required for interaction between KaiB and KaiA. KaiA and KaiB do not interact at low temperatures (A). At higher temperatures, both KaiA and KaiB undergo changes in structure or conformation (B) in order to interact, and during this temporary interaction, the Cys272 of KaiA and the 64th residue of KaiB closely approach each other (C).

Table 1. Time constant of reactions between MTSSL-KaiB$_{T64C}$ and wild-type and mutant KaiA proteins.

| KaiA proteins | Time constant (min) (*n*=3) |
|---|---|
| KaiA (WT) | 86.2 ± 13.7 |
| KaiAc | 96.4 ± 23.2 |
| KaiAc$_{C272A}$ | -* |
| KaiAc$_{H270A}$ | 367 ± 55.8 |

*The ESR spectra of MTSSL-KaiB$_{T64C}$ changed little in the presence of KaiAc$_{C272A}$.

Table 1. Reaction mixtures containing MTSSL-KaiB$_{T64C}$ and KaiA (wild-type KaiA, KaiC, KaiAc$_{C272A}$, and KaiAc$_{H270A}$) were incubated at 40 ˚C for 6 h. Time constant was calculated as described in Materials and Methods and shown as the mean ± SD (*n*=3). Other conditions were the same as described in the legend for Fig. 3.

## Chapter 2

Thermodynamically induced conformational changes of

cyanobacterial circadian clock protein KaiB


### Abstract

Site-directed spin labeling electron spin resonance (ESR) was applied to investigate the local environments of the cyanobacterial circadian clock protein KaiB. I prepared five cysteine residue (Cys)-substituted mutants of KaiB, labeled with maleimide spin label (MSL). By comparing the ESR spectra of KaiBs carrying MSL at different positions (threonine64 (T64), lysine67 (K67), tyrosine94 (Y94), glycine98 (G98), and alanine101 (A101)), local conformational changes were identified. The ESR spectra of MSL-KaiB$_{T64C}$ and MSL-KaiB$_{K67C}$ showed the relatively slow motion of MSL of $\tau$ = 79 and 59 ns at 4 °C, respectively. The spectra of MSL-KaiB$_{Y94C}$, MSL-KaiB$_{G98C}$, and MSL-KaiB$_{A101C}$ showed relatively fast motion of $\tau$ = 8.0, 4.1 and 3.1 ns at 4 °C, respectively. The differences in the $\tau$ value can be explained by the local environments of the position in the KaiB molecule where MSL was introduced. When labeled KaiBs were incubated at 40 °C for 24 h, all of their ESR spectra changed, which can be explained by the structural relaxation of KaiB.

## Introduction

The cyanobacterial circadian clock machinery is composed of three clock proteins called KaiA, KaiB and KaiC. The three clock proteins are thought to play different roles in generating circadian oscillations. KaiC has autophosphorylation activity (Nishiwaki *et al*., 2000; Hayashi *et al*., 2004; Uzumaki *et al*., 2004) and very weak ATPase activity (Terauchi *et al*., 2007; Murakami *et al*., 2008), which are both enhanced by KaiA (Iwasaki *et al*., 2002; Williams *et al*., 2002; Uzumaki *et al*., 2004). KaiB interacts with KaiA, as revealed by electron spin resonance (ESR) spectroscopy (Mutoh *et al*., 2010), and with KaiC, as revealed by native polyacrylamide gel electrophoresis (PAGE) (Pattanayek *et al*., 2008). KaiB is believed to attenuate the enhancing effects of KaiA on KaiC (Williams *et al*., 2002; Kitayama *et al*., 2003). The functions of KaiB, however, remain to be elucidated.

KaiB has an unusual homotetrameric structure comprising two asymmetrical dimers, as revealed by crystallography (Hitomi *et al*., 2005; Iwase *et al*., 2005; Pattanayek *et al*., 2008). The tetramer has a positively charged cleft flanked by two negatively charged ridges (Iwase *et al*., 2005), and the cleft may play a role in the binding of ligands (Iwase *et al*., 2005). The cleft, where the functionally important residues of KaiB are concentrated, may play a role in the binding of ligands (Iwase *et al*., 2005). Previously, we proposed that the positively charged cleft is exposed to ligands through the movements of the negatively charged ridges (Iwase *et al*., 2005) because the cleft is covered by

the ridges in the crystal structure (Iwase *et al*., 2005). Recently, I found that the 64th residue, a threonine (Thr), is one of the KaiA-interacting sites of KaiB (Mutoh *et al*., 2010). This residue is located near functionally important residues (Iwase *et al*., 2005). Recently, I demonstrated that the conformation or structure of KaiB around the 64th residue changes upon interaction with KaiA, depending on temperature (Mutoh *et al*., 2010). I have proposed that the structural changes of KaiB and/or KaiA that occur at temperatures higher than 30 °C induce the interaction between KaiA and KaiB (Mutoh *et al*., 2010). However, the changes of KaiB, KaiA, or both remain to be determined.

In this study, I analyzed the local mobility of the KaiB molecule at various temperatures by site-directed spin labeling (SDSL)-ESR. I prepared five cysteine residue (Cys)-substituted mutants of KaiB, labeled with maleimide spin label (MSL). By comparing the ESR spectra of KaiBs carrying MSL at different positions (Thr64, lysine (Lys) 67, tyrosine (Tyr) 94, glycine (Gly) 98, and alanine (Ala) 101) before and after incubation for 24 h, I identified local conformational changes. The results showed that the mobility of the spin labels around the dimer-dimer interface of the KaiB tetramer molecule (Thr64 and Lys67), but not on the negatively charged ridges (Tyr94, Gly98 and Ala101), changed on incubation.

## Materials and methods

*Preparation of mutant KaiB proteins*

I produced recombinant Kai proteins derived from *T. elongatus* using *Escherichia coli* as a host, as described previously with modifications (Hayashi *et al*., 2004). The plasmids expressing two Cys-substituted KaiBs, $KaiB_{T64C}$ and $KaiB_{A101C}$ ($pTekaiB_{T64C}$ and $pTekaiB_{A101C}$), have been described previously (see Chapter 1). Other plasmids for the production of new Cys-substituted KaiBs, $KaiB_{K67C}$, $KaiB_{Y94C}$ and $KaiB_{G98C}$ ($pTekaiB_{K67C}$, $pTekaiB_{Y94C}$ and $pTekaiB_{G98C}$ carrying the *kaiB* gene with a Cys-substitution at Lys67 (K67), Tyr94 (Y94), or Gly98 (G98), respectively), were constructed by PCR-mediated *in vitro* mutagenesis as described previously (Hayashi *et al*., 2004). Kai proteins were purified as reported previously (See Chapter 1).

*Spin labeling of KaiB*

I introduced MSL into each unique Cys of the mutant KaiBs ($KaiB_{T64C}$, $KaiB_{K67C}$, $KaiB_{Y94C}$, $KaiB_{G98C}$ and $KaiB_{A101C}$) as described previously (See Chapter 1).

*Assay for the activity of labeled KaiBs to decrease the phosphorylation level of KaiC*

Reaction mixtures containing 0.5 μM KaiC hexamer and 0.5 μM KaiA dimer in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM ATP, 5 mM $MgCl_2$ and

150 mM NaCl were incubated at 40 °C for 12 h to elevate the phosphorylation level of KaiC. After incubation, 0.5 μM (in tetramer) labeled KaiBs were added to the reaction mixtures, and then the reaction mixtures were further incubated at 40 °C. Then, 15-μl aliquots of the reaction mixtures were collected every 6 h, subjected to SDS-PAGE on 12.5 % gels (acrylamide: bisacrylamide= 144: 1), and then the gels were stained with Coomassie Brilliant Blue (CBB). The ratio of P-KaiC to total KaiC (the phosphorylation level of KaiC) was calculated by densitometry using Lane Analyzer (ATTO, Tokyo, Japan) and CS Analyzer (ATTO).

*ESR measurements*

First, 10 μM (in tetramer) of MSL-labeled KaiBs (MSL-KaiB$_{T64C}$, MSL-KaiB$_{K67C}$, MSL-KaiB$_{Y94C}$, MSL-KaiB$_{G98C}$ and MSL-KaiB$_{A101C}$) were incubated at various temperatures (4, 10, 20, 30, 35, 40, and 50 °C) for 24 h in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM ATP, 5 mM $MgCl_2$ and 150 mM NaCl using block incubators (BI-516S; ASTEC, Japan). Then, 20-μl aliquots of the reaction mixtures were injected into 80-μl ESR sample capillary tubes, and ESR spectra were measured at 4 °C for 8 min using a Bruker ESP-300E spectrometer (Bruker BioSpin, Germany) with a standard resonator (ER4102).

*Calculation of the rotational correlation time $\tau$ of spin labels*

The ESR line shapes of the spin labels in solution are generally

explained by the different correlation time $\tau$. In the case of fast motion ($10^{-11}$ ns < $\tau$ < $10^{-9}$ ns), the correlation time was derived from the following equation (McConnell, 1956; Kivelson, 1960 ):

$$\tau = 6.5 \times 10^{-10} W_0 \left(\sqrt{\frac{h_0}{h_{-1}}} - 1\right),$$

where $W_0$ is the width peak-to-peak of the central peak, and $h_0$ and $h_{-1}$ are the heights of the mid- and high-field peaks, respectively, on a first-derivative absorption spectrum (see Fig. 3A for $h_0$ and $h_{-1}$). In the case of slow motion ($10^{-11}$ ns < $\tau$ < $10^{-9}$ ns), the correlation time was derived from the following equation (Mason and Freed, 1974):

$$\tau = a\left(1 - \frac{A_Z'}{A_Z}\right)^b,$$

where $A_Z$ and $A_Z'$ are hyperfine constants for the rigid limited value and the experimental value, respectively. The rigid limited value was evaluated from the ESR spectra obtained at -276 ˚C. The parameters *a* and *b* are given by the calculated diffusion model.

## Results

*Decreasing of the phosphorylation level of KaiC by KaiB*

The phosphorylation level of KaiC once elevated by KaiA can be decreased by KaiB in reaction mixtures containing KaiA, KaiB, KaiC and ATP (Kageyama *et al*., 2006). This decrease occurred even when MSL-KaiB$_{K67C}$, MSL-KaiB$_{Y94C}$, MSL-KaiB$_{G98C}$ and MSL-KaiB$_{A101C}$ were used in place of wild-type KaiB (Fig. 2), indicating that all the MSL-labeled KaiBs examined as well as wild-type KaiB have activity to decrease the phosphorylation level of KaiC elevated by KaiA.

*ESR spectral changes of MSL-KaiBs*

The ESR spectra of free MSL showed three peaks separated by 17 G, arising from nitrogen nuclei spin (Fig. 3A). The ESR spectra of the MSL introduced into KaiBs showed anisotropic line shapes (black lines in Figs. 3B-F), indicating that the mobility of the spin labels introduced into KaiBs was partially restricted.

Thr64 is located on the second $\alpha$ helix of KaiB (Iwase *et al*., 2005) (Figs. 1A and B) and is not exposed to the surface of the molecule; Lys67 is also located on the second $\alpha$ helix (Iwase *et al*., 2005) in the hollow (Figs. 1A-C). These locations restrict the mobility of the spin labels introduced into these residues, with the result that MSL-KaiB$_{T64C}$ and MSL-KaiB$_{K67C}$ have ESR spectra characteristic of slow motion components (black lines in Figs. 3B and 3C), with $\tau$

values calculated to be 79 and 59 ns, respectively. The ESR spectra of both MSL-KaiB$_{T64C}$ and MSL-KaiB$_{K67C}$ showed two sets of hyperfine components, reflecting fast and slow motion, of the spin labels, respectively, which are ascribed to two different local environments in each MSL-labeled KaiB.

Without incubation at 40 °C, the ESR spectra of MSL-KaiB$_{Y94C}$, MSL-KaiB$_{G98C}$ and MSL-KaiB$_{A101C}$ were characterized by a relatively fast motion of the spin labels (black lines in Fig. 3D-F). The $\tau$ values of MSL-KaiB$_{Y94C}$ (Fig. 3D), MSL-KaiB$_{G98C}$ (Fig. 3E) and MSL-KaiB$_{A101C}$ (Fig. 3F) were calculated to be 8.0, 4.1 and 3.1 ns, respectively. The relatively fast motion is ascribed to the position of the introduced spin labels in the KaiB molecule, because Tyr94 is located at the bottom of the negatively charged ridges of the molecule, and Gly98 and Ala101 are located on the loop on the ridge, and the side chains of these amino residues are exposed (Fig. 1A).

On incubation at 40 °C for 24 h, all ESR spectra of the labeled KaiBs changed (gray lines in Figs. 3B-F), although the magnitude of these changes depended on the position of the spin label in the KaiB molecule. Notably, MSL-KaiB$_{T64C}$ showed large spectral changes (gray line in Fig. 3B). The $\tau$ value of MSL-KaiB$_{T64C}$ decreased from 79 to 1.9 ns upon incubation at 40 °C for 24 h, and the slow motion peak of its ESR spectra completely disappeared. The mobility of the spin labels introduced into Lys67 changed slightly, and a slight decreasing of the fast motion peak was detected (gray line in Fig. 3C). The $\tau$ values of MSL-KaiB$_{Y94C}$ (Fig. 3D), MSL-KaiB$_{G98C}$ (Fig. 3E) and MSL-KaiB$_{A101C}$

(Fig. 3F) were calculated to be 8.0, 4.1 and 3.1 ns prior to incubation and 4.6, 3.3 and 2.2 ns on 24 h at 40 °C, respectively. Thus, the $\tau$ values did not change significantly after the incubation.

MSL-KaiB$_{T64C}$ has activity to generate circadian oscillation in the *in vitro* KaiABC clock machinery (Chapter 1). The tetrameric structure of MSL-KaiB$_{T64C}$ was confirmed by gel filtration chromatography. All the spin-labeled KaiBs used here also showed activity to decrease the phosphorylation level of KaiC elevated by KaiA (Fig. 2). Therefore, my observations are likely to be physiologically significant. The temperature-dependent spectral changes reported here could not be reversed even after incubation at 4 °C for 24 h.

*Temperature dependence of ESR spectral changes of MSL-KaiB$_{T64C}$*

To clarify the temperature dependence of the ESR spectral changes of MSL-KaiB$_{T64C}$, I measured the spectral changes of MSL-KaiB$_{T64C}$ during incubation at various temperatures (4, 10, 20, 30, 35, 40, and 50 °C) for 24 h (Fig. 4A). I defined signal intensity as the sum of the three peak heights $h_{-1}$, $h_0$, and $h_1$ in the ESR spectra (Fig. 3A). The Arrhenius plot for increases in signal intensity was linear (Fig. 4B), and the activation energy (*Ea*) for the mobility change of the spin labels introduced into the 64th residue was calculated to be 54 kJ/mol.

**Discussion**

After incubation at temperatures higher than 30 °C for 24 h, the slow motion of the ESR spectra of MSL-KaiB$_{T64C}$ disappeared component completely, with only a fast motion component remaining. Because the 64th residue is located on the dimer-dimer interface of the KaiB molecule (Iwase *et al*., 2005), amino acid residues of different subunits exist around the 64th residue. Therefore, the mobility of the MSL introduced into the 64th residue is probably restricted by these residues. The disappearance of the slow motion peaks in the spectra of MSL-KaiB$_{T64C}$ could be caused by the relaxation of the dimer-dimer interaction (structure) to resolve the steric hindrance (Figs. 5A and 5B).

The activation energy for the mobility of the spin labels introduced into the 64th residue was 54 kJ/mol. Previously, I reported that the interaction between KaiA and KaiB occur at temperatures higher than 30 °C and that there is a large difference in the *Ea* values between 4 and 30 °C and between 35 and 50 °C (Mutoh *et al*., 2010). In contrast, the Arrhenius plot for the conformational changes of MSL-KaiB$_{T64C}$ was monophasic (Fig. 4B), and there was no difference in *Ea* from 4 to 50 °C. Therefore, the temperature-dependent interaction between KaiA and KaiB can be explained partly by the temperature-dependent conformational changes of KaiB described herein, but other unknown factors should be considered. *T. elongatus* cells grow optimally at 57 °C (Yamaoka *et al*., 1978), but these cells show circadian rhythms with a temperature-compensated period at temperatures between 30 and 60 °C (Onai

*et al*., 2004). The temperature-dependent conformational changes of KaiB may be involved in the temperature-compensation mechanism of the clock machinery.

The $\tau$ values of MSL-KaiB$_{T64C}$ and MSL-KaiB$_{K67C}$ became smaller upon incubation at 40 °C for 24 h, suggesting that the local conformation of KaiB around the introduced spin labels became free from steric hindrance as the result of loosening of the dimer-dimer interface of KaiB molecule (Fig. 5B). The 64th residue of KaiB is located near the active site (Iwase *et al*., 2005). The 67th residue is one of the functional residues of KaiB and lies in the positively charged hollow (Iwase *et al*., 2005). Both of these residues are located on the dimer-dimer interface of the KaiB molecule (Iwase *et al*., 2005). Iwase *et al*. have proposed that the positively charged cleft surrounded by the negatively charged ridges plays a role in the binding of ligands and the biological function of KaiB because several functionally important residues are concentrated in the positively charged cleft (Iwase *et al*., 2005). Therefore, the ESR spectral changes of MSL-KaiB$_{T64C}$ and MSL-KaiB$_{K67C}$ suggest that the local conformational changes around the positively charged cleft in the KaiB molecule, which cause the relaxation of the dimer-dimer interface of the KaiB molecule that is required for the interaction with other Kai proteins, could be induced by incubation at 40 °C (Fig. 5B).

Gly98 and Ala101 are located on the negatively charged ridges and are exposed to the surface of the KaiB molecule. Each ridge consists of a loop and,

therefore, can move like a hinge (Iwase *et al*., 2005). The $\tau$ values of MSL-KaiB$_{G98C}$ and MSL-KaiB$_{A101C}$ did not change significantly on incubation at 40 °C because the negatively charged ridges can move freely at either 4 or 40 °C. Tyr94 is located at the bottom of the negatively charged ridges of the KaiB molecule. Therefore, the environment in the vicinity of Tyr94 may not change drastically during incubation at 40 °C.

Previously, I reported the ESR spectra of two spin labels, MSL and MTSSL, introduced into the 64th residue of KaiB (Mutoh *et al*., 2010). The spectra of MTSSL-KaiB$_{T64C}$ did not change on incubation at 40 °C (See Chapter1, Fig. 3B), but those of MSL-KaiB$_{T64C}$ did. This difference may be the result of the difference in the chemical structures of MSL and MTSSL. MSL contains a bulky five-member ring (Fig. 1D), whereas MTSSL contains a narrowside chain (Fig. 1E). MSL may be easily affected by steric hindrance from the amino acid residues surrounding the spin labels and may be sensitive to even modest changes in the vicinity of the spin labels. The appropriate choice of the spin label could allow us to approach to the local structure of Kai proteins.

Fig. 1

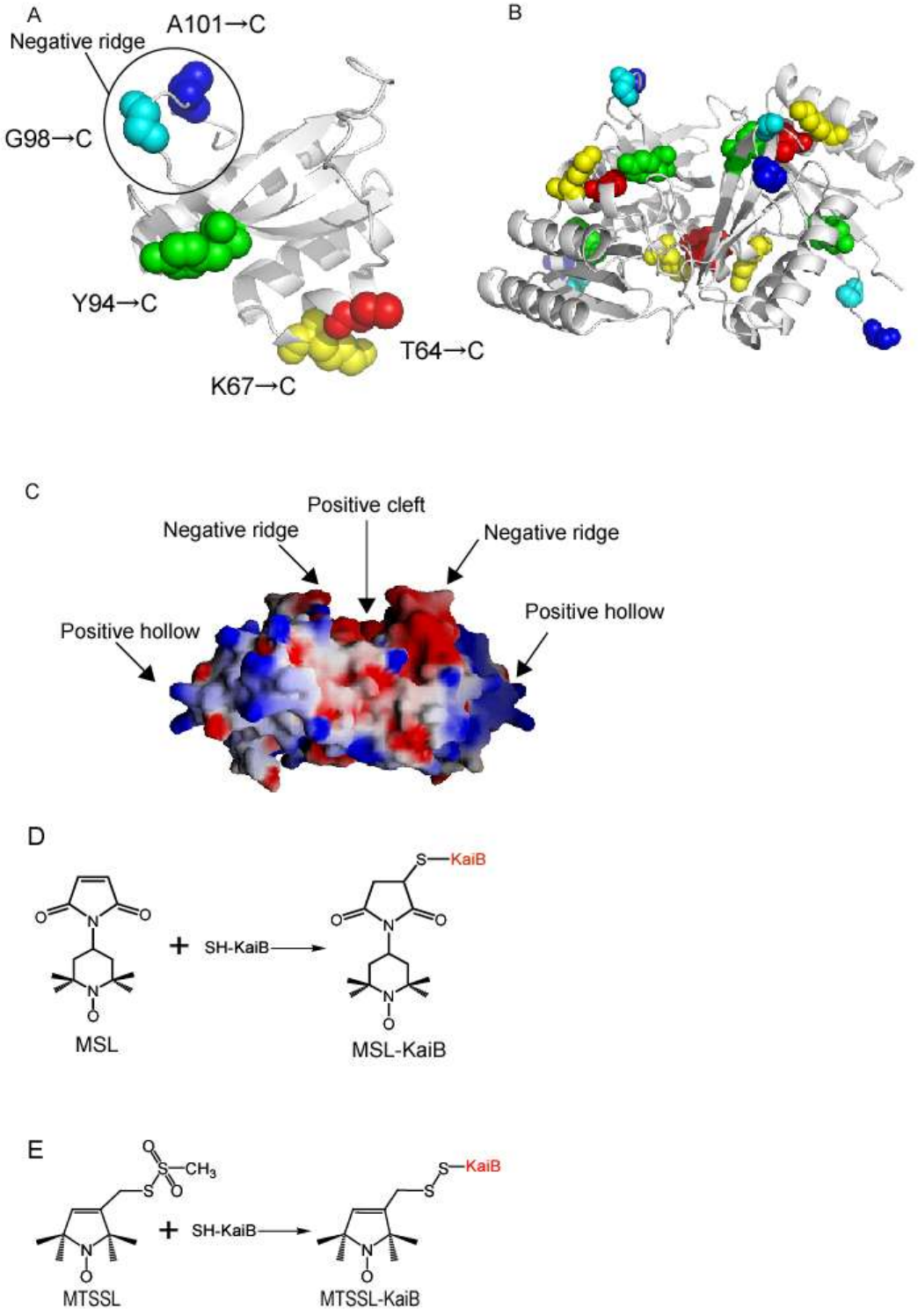

A
Negative ridge
A101→C
G98→C
Y94→C
T64→C
K67→C
B
C
Positive cleft
Negative ridge
Negative ridge
Positive hollow
Positive hollow
D
S—KaiB
SH-KaiB
MSL
MSL-KaiB
E
CH3
SH-KaiB
MTSSL
MTSSL-KaiB

Fig. 1. Site of the Cys residues introduced by site-directed mutagenesis shown in the crystal structure of the *T. elongatus* KaiB monomer (A) and tetramer (B), and electrostatic potential of the surface of the KaiB molecule (electrostatic surface potential is color-coded: blue, positive; red, negative) (C). A scheme for spin labeling with MSL (D) and MTSSL (E). $KaiB_{T64C}$, $KaiB_{K67C}$, $KaiB_{Y94C}$, $KaiB_{G98C}$ and $KaiB_{A101C}$ contained the following Cys substitutions for spin labeling: T64C (red), K67C (yellow), Y94C (green), G98C (cyan) and A101C (blue), respectively. Each Cys residue of the mutant KaiBs was labeled with MSL (D).

Fig. 2

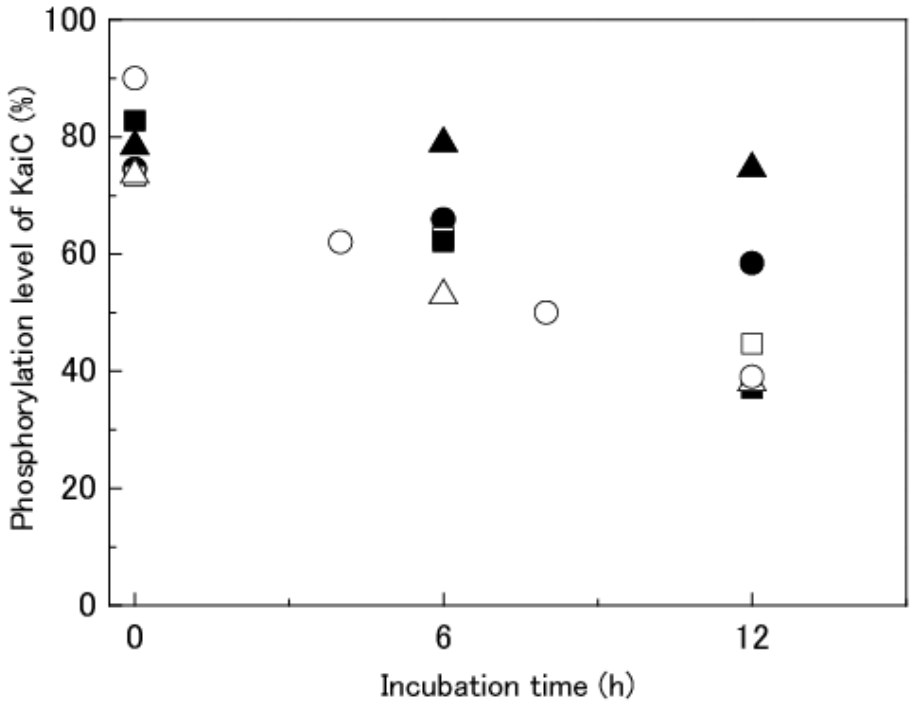

100
80
60
40
20
0
Phosphorylation level of KaiC (%)
0
6
12
Incubation time (h)

Fig. 2. Decreases in the phosphorylation level of KaiC induced by spin-labeled KaiBs. Reaction mixtures containing KaiC (0.5 μM in hexamer) and KaiA (0.5 μM in dimer) in 20 mM Tris-HCl (pH 7.5) containing 1 mM ATP, 5 mM $MgCl_2$ and 150 mM NaCl were incubated at 40 °C for 12 h. Then, wild-type KaiB (open circle) or spin-labeled KaiBs, MSL-$KaiB_{K67C}$ (closed circle), MSL-$KaiB_{Y94C}$ (open square), MSL-$KaiB_{G98C}$ (closed square) and MSL-$KaiB_{A101C}$ (open triangle), were separately added to the reaction mixtures, and then the reaction mixtures were further incubated at 40 °C for the periods indicated. The reaction without KaiB (closed triangle) was also shown. Other conditions were the same as described in the legend for Fig. 2 in Chapter 1.

Fig.3

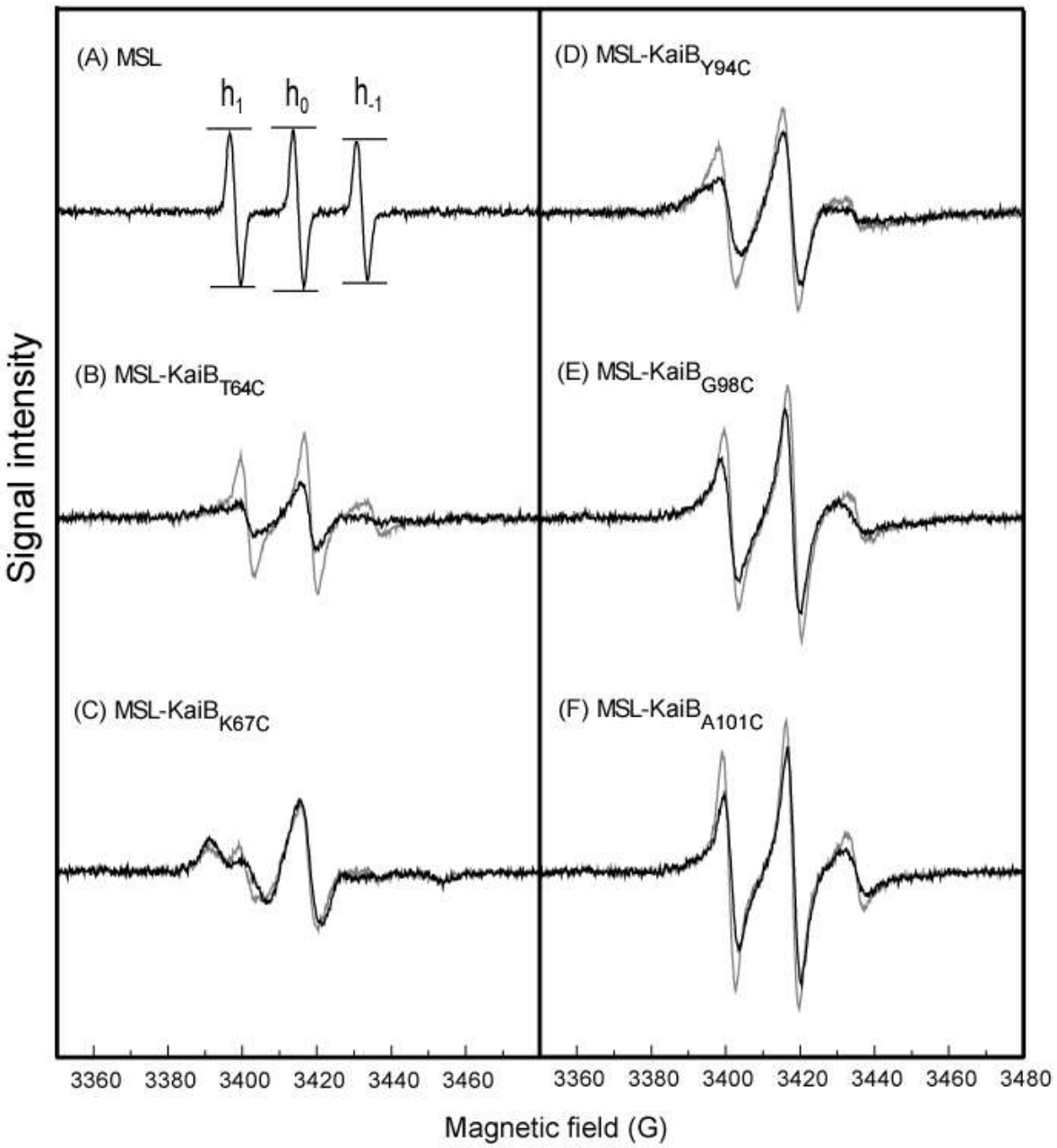

(A) MSL
h1
h0
h-1
(D) MSL-KaiBY94C
(B) MSL-KaiBT64C
(E) MSL-KaiBG98C
(C) MSL-KaiBK67C
(F) MSL-KaiBA101C
Signal intensity
3360 3380 3400 3420 3440 3460
3360 3380 3400 3420 3440 3460 3480
Magnetic field (G)

Fig. 3. ESR spectra of (A) free MSL, (B) MSL-KaiB$_{T64C}$, (C) MSL-KaiB$_{K67C}$, (D) MSL-KaiB$_{Y94C}$, (E) MSL-KaiB$_{G98C}$ and (F) MSL-KaiB$_{A101C}$ before (black) and after (gray) incubation at 40 °C for 24 h. Measurement conditions: temperature, 4 °C; microwave frequency, 9.75 GHz; microwave power, 2 mW; modulation frequency, 100 kHz; and modulation amplitude, 1 G.

Fig. 4

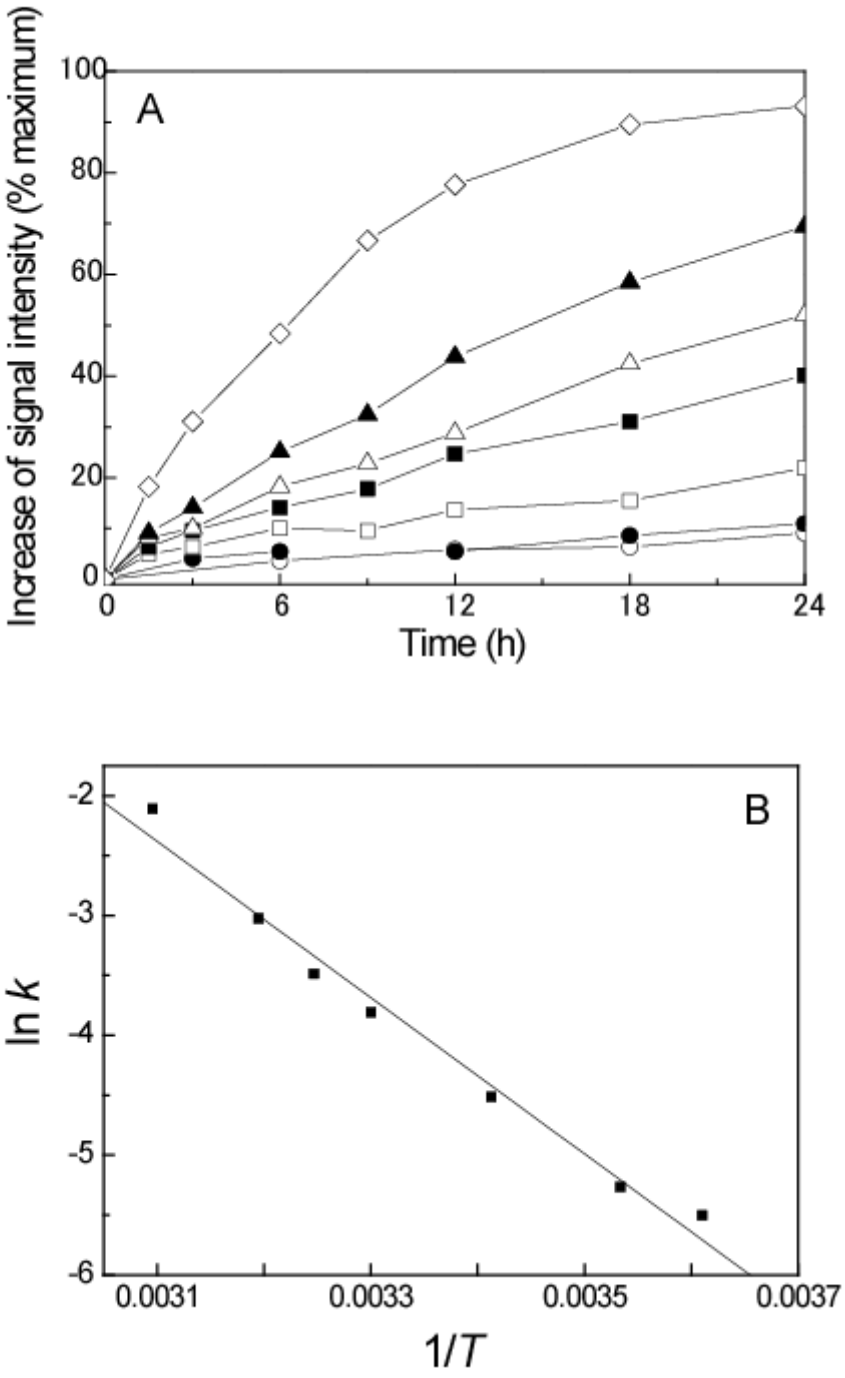

A
Increase of signal intensity (% maximum)
100
80
60
40
20
0
0
6
12
18
24
Time (h)
B
ln k
-2
-3
-4
-5
-6
0.0031
0.0033
0.0035
0.0037
1/T

Fig. 4. Time course of the increases in the signal intensities of the ESR spectra of MSL-KaiB$_{T64C}$ incubated at 4 °C (open circle), 10 °C (closed circle), 20 °C (open squares), 30 °C (closed squares), 35 °C (opem triangles), 40 °C (closed triangles), 50 °C (open diamonds) (A), and an Arrhenius plot (B). The rate constants *k* were calculated from the traces shown in panel A.

Fig. 5

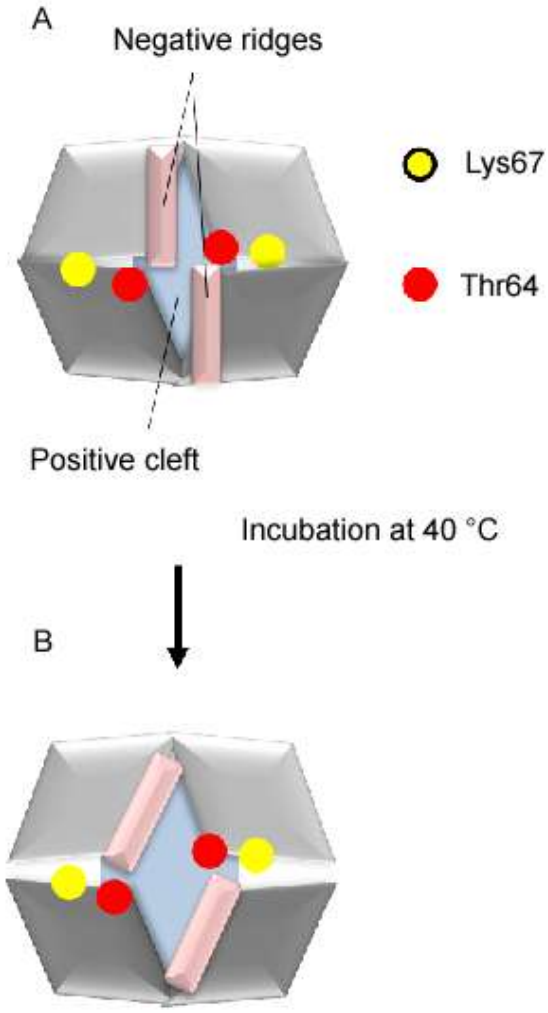

A
Negative ridges
Lys67
Thr64
Positive cleft
Incubation at 40 °C
B

Fig. 5. Model for the conformational changes of KaiB induced by incubation at 40 °C. Before incubation (A), and after incubation (B).

## General discussion

The cyanobacterial circadian clock machinery is composed of three Kai proteins, KaiA, KaiB and KaiC. These proteins interact each other to form complexes, and the stoichiometry of the complexes may change during clock oscillation. Interactions between KaiA and KaiC (Iwasaki *et al*., 2002; Williams *et al*., 2002; Hayashi *et al*., 2004; Uzumaki *et al*., 2004; Hayashi *et al*., 2006) and those between KaiB and KaiC (Kageyama *et al*., 2006, Akiyama *et al*., 2008; Pattanayek *et al*., 2008) are well known. On the other hand, interaction between KaiA and KaiB has remained to be detected.

There are many approaches to the molecular mechanism of clock machinery at the atomic level, such as X-ray crystal structure analysis, electron microscopy analysis, small-angle X-ray scattering analysis, nuclear magnetic resonance (NMR) and ESR. Although the crystal structures of the three Kai proteins have been solved (KaiA: Garces *et al*., 2004; Uzumaki *et al*., 2004; Ye *et al*., 2004; KaiB: Hitomi *et al*., 2005; Iwase *et al*., 2005; KaiC: Pattanayek *et al*., 2008; Pattanayek *et al*., 2004; Pattanayek *et al*., 2009), the structures of Kai protein complexes remain to be solved. It is not easy to stabilize the state of the complexes, because they exist in many states and the states may change during crystalization. The global structural changes of the complexes can be observed by electron microscopy analysis and small-angle X-ray scattering analysis, but the interaction sites of the complexes can not be identified by these analyses because their resolution is low. Although NMR analysis is a very useful tool to

analyze the structures and interactions of proteins at the atomic level, it can not be applied to high molecular weight proteins including protein complexes. Furthermore, NMR analysis needs very high concentration of protein samples, and therefore, it is difficult to perform NMR analysis under physiological conditions.

ESR analysis is also a useful tool for studying protein structure and function at the atomic level, and it is suitable for studying the conformational changes of proteins and identifying their interaction sites in proteins at the atomic level. This method covers only a narrow range in the vicinity of spin labels. Therefore it is not suitable for studying the global structural changes of proteins.

In further study, it is the key to solve at the atomic level how Kai proteins associate each other and dissociate from Kai protein complexes and how the conformational changes of each Kai protein in the complexes occur during clock oscillation. Now, I am surveying the KaiA- and KaiC- interaction sites on KaiB and also measuring the distance between spin labels on KaiB to simulate the conformational changes of KaiB during oscillation. I am also trying to crystallize the protein complexes using mutant proteins, where the state of the proteins would be stabilized. I would like to clarify how three Kai proteins tick tack.

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**Acknowledgements**

I appreciate Prof. Masahiro Ishiura for helpful advice and encouragement. I also appreciate Associate Prof. Hiroyuki Mino for technical support, helpful advice and discussions. I thank Dr. Reiko Murakami, Dr. Tatsuya Uzumaki, Satoko Ogawa and Kumiko Tanaka for technical support and members in Ishiura's lab. for kind support. I also thank Drs. K. Namba and K. Imada of Osaka University for the critical reading of this manuscript. The research was supported by a Global Center of Excellence grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), the Sasakawa Scientific Research Grant from The Japan Science Society and Research Fellowships of the Japan Society for the Promotion of Science (JSPS) for Young Scientists.