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Stoichiometric interactions between cyanobacterial clock proteins KaiA and KaiC

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Abstract

We determined the stoichiometry of KaiA–KaiC interactions. Using immunoblotting and two-dimensional Native- and SDS–PAGE (2DNS-PAGE) analysis, we demonstrated that the reaction products of KaiA–KaiC interactions in the presence of ATP consisted of only phosphorylated KaiC whereas in the presence of the unhydrolyzable analogue 5′-adenylylimidodiphosphate (AMPPNP) they consisted of KaiA and KaiC. In the presence of ATP, the *KE* (molar ratio of KaiA dimer to KaiC hexamer giving half saturation in the enhancement of KaiC phosphorylation) was 0.25, and IAsys affinity biosensor analysis demonstrated that 1 molecule of KaiA dimer interacted with 1 molecule of KaiC hexamer. In the presence of AMPPNP, the ratio of KaiA dimer to KaiC hexamer in KaiA–KaiC complexes was determined to be 2 by 2DNS-PAGE, Native-PAGE/Scatchard plot, and IAsys analyses. These results suggest that 2 molecules of KaiA dimer can interact with 1 molecule of KaiC hexamer, and that interactions of at least 1 molecule of KaiA dimer with 1 molecule of KaiC hexamer are enough to enhance the phosphorylation of KaiC by KaiA at an almost saturated level.

Keywords: Cyanobacteria; Clock; KaiA; KaiC; Interaction; Stoichiometry; Phosphorylation; IAsys

Circadian rhythms—oscillations in metabolic and behavioral activities with a period of about 24 h—are ubiquitous in nature. Cyanobacteria are the simplest organisms that exhibit circadian rhythms. We previously cloned and analyzed the *kaiABC* circadian clock gene cluster in the cyanobacterium *Synechococcus* sp. strain PCC 7942 (hereafter called *Synechococcus*) and found it to consist of two operons—*kaiA*, which enhances *kaiBC* promoter activity, and *kaiBC*, which is repressed by *kaiC* itself [1]. KaiA, KaiB, and KaiC proteins interact in any combination [2], and KaiC also interacts with SasA, a sensory histidine kinase that enhances *kaiBC* promoter activity [3]. KaiC is phosphorylated by KaiC itself and the phosphorylation is enhanced by KaiA [4–7]. KaiC has a dumbbell-shaped hexagonal pot-like structure composed of six identical subunits [8]. KaiA is composed of three structural and functional domains—the N-terminal, the central, and the C-terminal domains. The C-terminal domain (the C-terminal clock oscillator domain) is essential for the interaction with KaiC and the enhancement of KaiC phosphorylation in vitro and the generation of clock oscillations in vivo [7]. The X-ray crystal structure of the C-terminal domain of KaiA demonstrated a lens-like dimer structure with two concavities [7], and residue His_{270}, located on the center of each concavity, is important for the interaction with KaiC, the enhancement of KaiC phosphorylation, and the residue is essential for the generation of clock oscillations [7].

Here, we show stoichiometric interactions between KaiA and KaiC and their kinetic parameters.

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Materials and methods

Plasmid construction. The coding region of the *Thermosynechococcus elongatus kaiA* gene was amplified by the polymerase chain reaction (PCR) with two primers, 5′-CGCGGATCCGTGGCGCAG TCAACCGCACTCACAATTT-3′ (the *Bam*HI site is underlined) and 5′-CCGCTCGAGTTACACCTCCCGCGGAATG GAGCGACGAT A-3′ (the *Xho*I site is underlined), and the genomic DNA as a template, and was inserted into pGEX-6P-1 (Amersham Biosciences) at the unique *Bam*HI and *Xho*I sites. The resulting plasmid (p*Te*KaiA) was introduced into and propagated in *Escherichia coli* DH5α and BL21. *E. coli* cells were grown in Luria–Bertani broth (LB) and Terrific Broth (TB) supplemented with 50 μg/ml ampicillin and on LB plates containing 1.5% agar.

Production of KaiA and KaiC in E. coli and their purification. *E. coli* strain BL21 cells carrying *pTe*KaiA were grown in TB supplemented with 50 μg/ml ampicillin. They were harvested by centrifugation, resuspended in 50 mM Tris–HCl buffer (pH 8.0) containing 50 mM NaCl, 1 mM EDTA, and 5 mM dithiothreitol (DTT), and disrupted by sonication [8]. Cleared lysates were prepared by centrifugation and applied to a glutathione–Sepharose-4B (Amersham) column. GST–KaiA fusion protein was eluted with a buffer containing 10 mM reduced glutathione. To cleave GST–KaiA, 4 U PreScission Protease (Amersham) per mg GST-KaiA was added to a pooled fraction containing GST–KaiA, and the mixture was dialyzed twice at 4 °C for 2 h against 0.25 L 10 mM Tris–HCl buffer (pH 8.0) containing 50 mM NaCl, 0.1 mM EDTA, and 1 mM DTT. The dialysate was applied to a Hiload 26/10 Q Sepharose HP column, and KaiA was eluted with a linear gradient of 50–300 mM NaCl using an ÄKTA prime (Amersham). We determined the molecular weights of a KaiA subunit and native KaiA to be 32 and 60 kDa, respectively, by both SDS–PAGE (Fig. 1A) and gel-filtration chromatography on a Superdex 75 HR 10/30 (Fig. 1B), suggesting that KaiA is a homodimer. We confirmed this by chemical cross-linking experiments using 10% Weber–Osborn gels as described previously [8] with minor modifications (Fig. 1C).

KaiC was produced in *E. coli* and purified as described previously [8]. Briefly, GST–KaiC fusion protein produced in *E. coli* cells as a soluble protein was trapped with glutathione–Sepharose-4B. KaiC was cut out from the GST–KaiC by PreScission Protease, purified by ion exchange chromatography on a MonoQ HR5/5 column (Amersham), and subjected to gel filtration chromatography on a Superdex200 HR10/30 column (Amersham). It was eluted as a single symmetric peak at a position corresponding to a globular protein of 60 kDa by gel filtration chromatography on a Superdex200 HR10/30 column (data not shown).

We determined protein concentration by spectrometry after applying Coomassie brilliant blue (CBB; Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad)), using bovine serum albumin (BSA) as a standard. SDS–PAGE was carried out either on mini-gels using a dually cooled mini-slab gel electrophoresis device (BIO CRAFT) as described previously [8] or on PhastGel Gradient 8–25% gels with PhastGel Buffer Strips SDS (Amersham) using a PhastSystem (Amersham). Native-PAGE was carried out on PhastGel Gradient 8–25% gels and PhastGel Buffer Strips Native (Amersham) using a PhastSystem. Mini-gels were stained with Simply Blue SafeStain (Invitrogen). PhastGel Gradient 8–25% gels were stained with 0.1% PhastGel Blue R (Amersham) solution in 30% methanol and 10% acetic acid.

Assay for phosphorylation of KaiC. KaiC monomer (44 pmol) was incubated at 50 °C for 2 h with 10 mM [γ-^{32}P]ATP (0.33 pCi/mM, Amersham) in 30 μl of 20 mM Tris–HCl buffer (pH 7.5) containing 5 mM $MgCl_2$. Fifteen microliter aliquots of the reaction mixture were electrophoresed on 7.5% SDS–polyacrylamide gels. KaiC bands and radioactivity incorporated into the bands were visualized by staining with Simply Blue SafeStain and by BAS2000, respectively. [α-^{32}P]ATP (0.33 pCi/mM, Amersham) and [^{35}S]ATPγ S (0.33 pCi/mM, Amersham) were also used in place of [γ-^{32}P]ATP.

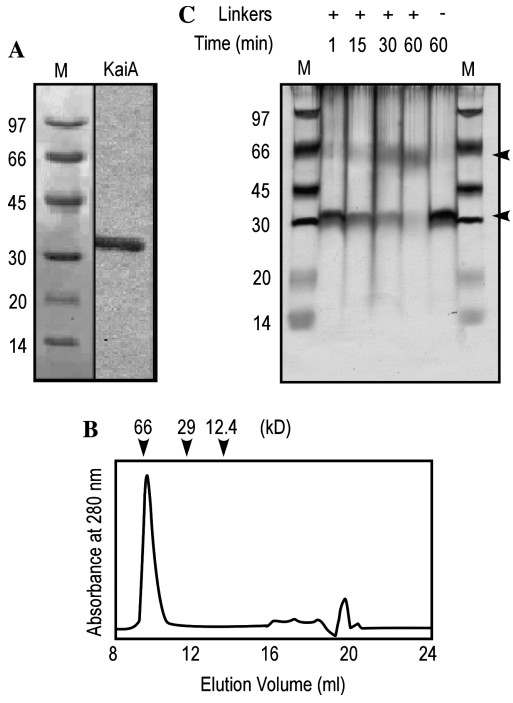

Fig. 1. Size determination of native and denatured KaiAs. (A) KaiA and molecular weight markers (M) electrophoresed on a 10% SDS–polyacrylamide gel. (B) Elution profile of KaiA by gel filtration chromatography on a Superdex75 HR 10/30 column. BSA (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa) were used as size markers. (C) Chemical crosslinking of KaiA. The arrow heads indicate bands corresponding to monomer and dimer.

Protein phosphatase treatment. To dephosphorylate KaiC, we incubated KaiC monomer (43.8 pmol) with 200 U λ-protein phosphatase (λ-PPase, New England Biolabs)—a protein phosphatase with activity towards phosphoserine, phosphothreonine, and phosphotyrosine residues in protein—at 30 °C for 30 min in 10 μl of 50 mM Tris–HCl buffer (pH 7.5) containing 2 mM $MnCl_2$, 0.1 mM EDTA, 5 mM DTT, and 0.01% Brij-35. To confirm dephosphorylation, we subjected 5-μl aliquots to SDS–PAGE and stained the gels with Simply Blue SafeStain.

Assay for the enhancement of KaiC phosphorylation by KaiA. KaiC monomer (7.3 pmol in hexamer) was mixed with 43.8 pmol of KaiA (in dimer) and incubated in the presence of 1 mM ATP in 30 μl of 20 mM Tris–HCl buffer (pH 7.5) containing 5 mM $MgCl_2$ at 50 °C for various times (Fig. 3A). KaiC was also mixed with various amounts of KaiA and incubated for 2 h similarly, except for the amount of KaiA added and incubation time (Fig. 3B). Fifteen microliter aliquots of the reaction mixtures were electrophoresed on SDS–polyacrylamide gels and the gels were stained with Simply Blue SafeStain as described above.

Immunoblot analysis of KaiA–KaiC complexes. KaiA dimer and KaiC monomer (30 pmol each) were mixed and incubated at 25 °C for 1 h in the presence of 1 mM ATP or 1 mM AMPPNP in 10 μl of 20 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT. Four microliters of aliquots of the reaction mixture were subjected to Native-PAGE. Proteins on the gels were visualized by PhastGel Blue R staining or blotted onto polyvinylidene difluoride (PVDF) membranes (Amersham). The PVDF membranes were agitated in Tris-buffered saline (TBS) containing 150 mM NaCl in 25 mM Tris–HCl (pH 7.5) supplemented with 5% nonfat milk (Wako) at room temperature for 30 min, and then incubated at 25 °C for 1 h with rabbit

anti-KaiA antiserum or rabbit anti-KaiC antiserum diluted to 1/5000 with TBS supplemented with 5% nonfat milk. Each membrane was washed with TBS and incubated at 25 °C for 1 h with goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (anti-rabbit IgG (H&L) (AP-linked), New England Biolabs) diluted to 1/10,000 with TBS supplemented with 5% non-fat milk. Immunoblots were visualized with CDPstar Chemiluminescence Reagent (Perkin–Elmer) and Hyperfilm ECL films (Amersham).

Two-dimensional Native- and SDS–polyacrylamide gel electrophoresis (2DNS-PAGE). To analyze the components of the bands separated by Native-PAGE, we devised a two-dimensional technique of Native-PAGE followed by SDS–PAGE using a PhastSystem. KaiA dimer and KaiC monomer (30 pmol each) were mixed and incubated at 25 °C for 1 h in 10 μl reaction buffer containing 1 mM ATP or 1 mM AMPPNP, 5 mM $MgCl_2$, 1 mM DTT, and 150 mM NaCl in 20 mM Tris–HCl buffer (pH 7.5), and the reaction products were subjected to Native-PAGE. Each lane was cut out as a narrow slab with scissors and soaked for 5 min at room temperature in SDS-equilibrium buffer containing 2.5% (w/v) SDS, 1% (w/v) DTT, and 0.01% (w/v) bromphenol blue in 50 mM Tris–HCl buffer (pH 7.0). The narrow gel slab was laid on the starting end of a PhastGel Gradient 8–25% gel equilibrated with PhastGel Buffer Strips SDS, and the proteins were electrophoresed. The gels were stained with PhastGel Blue R.

Scatchard plot analysis of KaiA–KaiC complexes. We determined the amount of KaiA bound to KaiC by Native-PAGE. We prepared KaiC hexamer from monomeric KaiC by incubating the latter overnight at 4 °C in reaction buffer containing 1 mM AMPPNP, 5 mM $MgCl_2$, and 150 mM NaCl in 20 mM Tris–HCl buffer (pH 7.5). KaiC hexamer (60 pmol) and various amounts of KaiA dimer were mixed in 16 μl reaction mixture containing 1 mM AMPPNP, 5 mM $MgCl_2$, and 1 mM DTT in 20 mM Tris–HCl buffer (pH 7.5) and incubated at 25 °C for 1 h. After incubation, 4-μl aliquots of the reaction mixtures were subjected to Native-PAGE and the gels were stained with PhastGel Blue R. The amounts of KaiA and KaiC were determined by densitometry. We calculated the molar ratio of KaiA dimer bound to KaiC hexamer and apparent K_D value by Scatchard plot analysis of the Native-PAGE data.

KaiA–KaiC interaction analysis by IAsys affinity biosensor analysis. We analyzed KaiA–KaiC interactions at 25 °C using the IAsys Plus (ThermoLabosystems). KaiC hexamer prepared as described above was dialyzed against running buffer containing 1 mM ATP or 1 mM AMPPNP, and 5 mM $MgCl_2$ in 20 mM Pipes–NaOH buffer (pH 7.5), and then KaiC hexamer was immobilized on a carboxylate cuvette that had been activated by 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce) and 50 mM *N*-hydroxysuccinimide (Pierce) and equilibrated with 10 mM sodium phosphate buffer (pH 7.5) containing 1 mM AMPPNP or 1 mM ATP and 5 mM $MgCl_2$. The carboxylate cuvette carrying KaiC hexamer was washed with the running buffer and blocked with 1 mg/ml BSA. Both the binding of various concentrations of KaiA dimer to the immobilized KaiC hexamer and the dissociation of bound KaiA from the KaiC were monitored continuously. Kinetic analyses were carried out using FASTPlot (ThermoLabosystems) and Grafit (Erithacus Software).

Results

Phosphorylation of KaiC and its enhancement by KaiA

KaiC prepared from GST–KaiC produced in *E. coli* showed a doublet band on SDS–polyacrylamide gels (Fig. 2A), and the upper band usually consisted of 25–40% of the total KaiC. When the purified KaiC monomer was treated with 20,000 U/ml λ-PPase at 30 °C for 30 min, the upper band disappeared (Fig. 2A). Contrary,

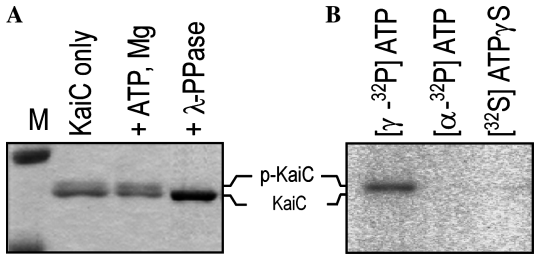

Fig. 2. SDS–PAGE of KaiC incubated with ATP or λ-PPase. (A) Stained with Simply Blue SafeStain. (B) Autoradiography by BAS2000.

when KaiC monomer was incubated with 1 mM ATP at 50 °C for 2 h, the relative amount of the upper band increased from 27% to 46% with a concomitant decrease in the relative amount of the lower band (Figs. 2A and 3A). When KaiC monomer was incubated with [γ-^{32}P]ATP and subjected to SDS–PAGE, ^{32}P-radioactivity was incorporated into the upper band of KaiC, whereas, when [α-^{32}P]ATP and [^{35}S]ATPγS were used in place of [γ-^{32}P]ATP (Fig. 2B), no radioactivity was incorporated into the KaiC bands. These results indicate that the upper band was phosphorylated KaiC (p-KaiC) and that KaiC phosphorylates KaiC.

When KaiC was incubated with 1 mM ATP at 50 °C for various times, the relative amount of p-KaiC increased gradually from 27% at 0 h to 46% at 2 h (Fig. 3A). The relative amount of p-KaiC in the presence of 43.8 pmol KaiA (in dimer) was 1.7 times the amount in its absence (Figs. 3A and B). The enhancement of phosphorylation increased with the amount of KaiA added, and it reached 76% saturation at a molar KaiA dimer/KaiC hexamer ratio of 1 (Fig. 3C). When we defined *KE* as the molar ratio of KaiA dimer to KaiC hexamer that gives half saturation in the enhancement of KaiC phosphorylation by KaiA, the value was 0.25 (Figs. 3C and D), suggesting that interaction of 1 molecule KaiA dimer with 1 molecule KaiC hexamer enhances the phosphorylation of KaiC at almost the saturation level. It should be noted that the relative amounts of p-KaiC never reached 100%—neither by longer incubation nor by further increases in the amount of KaiA added.

KaiA–KaiC complexes formed in the presence of AMPPNP

KaiA dimer migrated far from the origin on Native-polyacrylamide gels, whereas KaiC hexamer migrated only a short distance (Fig. 4A). KaiC was detected as a single band on Native-polyacrylamide gels although it had been detected as a doublet band on SDS–polyacrylamide gels, as described above. The probable reason is that the percentages of p-KaiC and KaiC subunits included in the KaiC hexamers were almost constant. When KaiA and KaiC were mixed and incubated in the

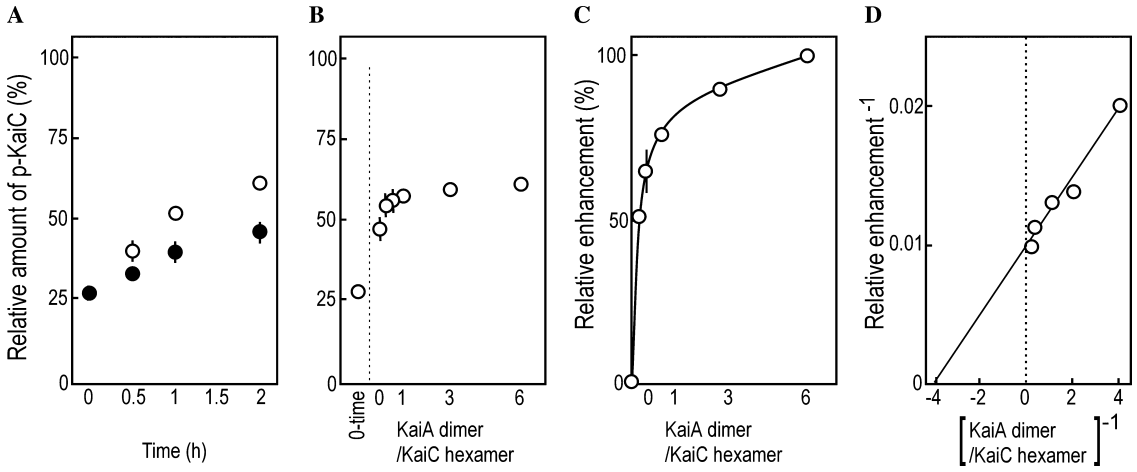

Fig. 3. Enhancement of the phosphorylation of KaiC by KaiA. (A) Time course of phosphorylation. KaiC monomer (43.8 pmol) was incubated in the presence of 1 mM ATP at 50 °C for the various times indicated with (○) or without (●) 43.8 pmol KaiA dimer. (B) KaiA dose-dependence of the enhancement of KaiC phosphorylation. KaiC monomer was incubated in the presence of ATP for 2 h with the various amounts of KaiA dimer indicated. Other conditions were the same as described for (A). (C) The data of (B) replotted. The relative enhancement of the phosphorylation of KaiC by KaiA at zero or an infinite amount of KaiA was defined as 0 or 100%, respectively. The real value of the latter enhancement was estimated by the reciprocal plot shown in (D). (D) Reciprocal plot analysis of (C) data. (A–C) Mean values of triplicate assays (bars represent SD). (D) Data from a typical experiment.

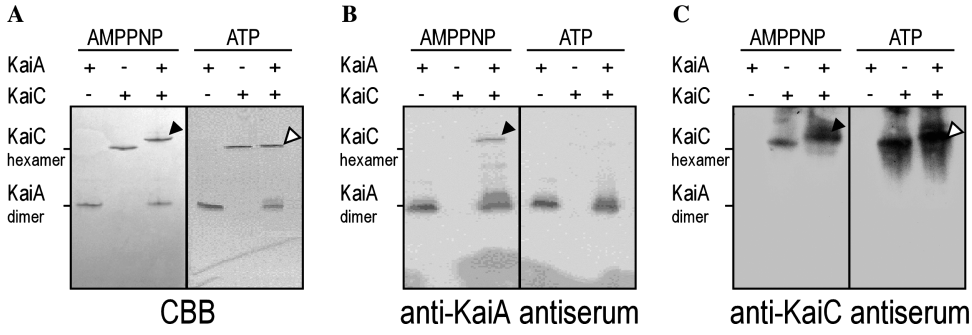

Fig. 4. Native-PAGE and immunoblot analysis of the reaction products of KaiA–KaiC interactions in the presence of ATP or AMPPNP. KaiA dimer was incubated with KaiC monomer (30 pmol each) at 25 °C for 1 h in the presence of 1 mM ATP or 1 mM AMPPNP. (A) PhastGel Blue R staining of native gels. (B) Immunoblots with anti-KaiA antiserum. (C) Immunoblots with anti-KaiC antiserum. The open and closed arrowheads indicate the reaction products of KaiA–KaiC interactions in the presence of ATP and AMPPNP, respectively.

presence of 1 mM ATP or 1 mM AMPPNP and the products subjected to Native-PAGE, a new band appeared corresponding to the reaction products of KaiA–KaiC interactions (Fig. 4A). The new band detected in the presence of ATP was slightly faster than the new band detected in the presence of AMPPNP (Fig. 4A), although the mobilities of KaiA dimer and KaiC hexamer were the same under both conditions (Fig. 4A).

We determined the components of the reaction products of KaiA–KaiC interactions in the presence of ATP and AMPPNP by immunoblot analysis using anti-KaiA and anti-KaiC antisera (Figs. 4B and C). The reaction product in the presence of ATP consisted of only p-KaiC, whereas in the presence of AMPPNP it consisted of KaiA and KaiC. These results suggest that the reaction products formed in the presence of ATP and AMPPNP were, respectively, p-KaiC and KaiA–KaiC complexes.

2DNS-PAGE analysis of KaiA–KaiC complexes formed in the presence of AMPPNP

When the reaction product formed in the presence of ATP was subjected to 2DNS-PAGE, the corresponding band formed only a KaiC band (Fig. 5A). When the KaiA–KaiC reaction product formed in the presence of AMPPNP was subjected to 2DNS-PAGE, however, the KaiA–KaiC complex band separated into KaiA and KaiC (Fig. 5B). These results are consistent with the results obtained by immunoblot analysis (Fig. 4).

Using the results of 2DNS-PAGE, we estimated the amount of KaiA dimer and KaiC hexamer in the KaiA–KaiC complex band on the SDS–polyacrylamide gel to be 2.3 ± 0.2 and 1.1 ± 0.1 pmol, respectively (Fig. 5B). These results indicate that the KaiA–KaiC complex formed in the presence of AMPPNP consisted of 2 molecules of KaiA dimer and 1 molecule of KaiC hexamer (Table 1).

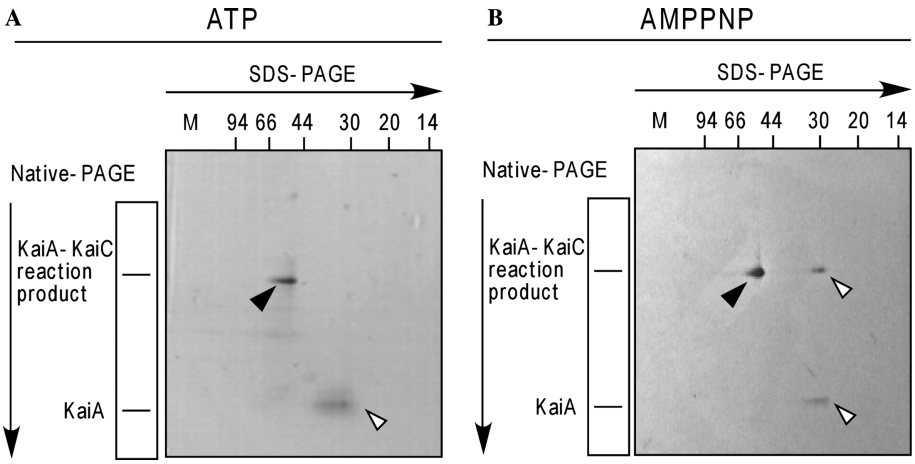

Fig. 5. 2DNS-PAGE analyses of the reaction products of KaiA–KaiC interactions. (A) Reaction products in the presence of ATP. (B) Reaction products in the presence of AMPPNP. The open and closed arrowheads indicate KaiA and KaiC subunits, respectively. Conditions were the same as described in the legend for Fig. 4.

Table 1
Summary of parameters in KaiA–KaiC interactions

Experiments	Stoichiometry KaiA dimer/KaiC hexamer	K_D (nM)	*kass* ($M^{-1}\,s^{-1}$)	*Kdis* (s^{-1})	Number of experiments
2DNS–PAGE (AMPPNP)[a]	2.1 ± 0.2	—	—	—	5
Native–PAGE/Scatchard plot (AMPPNP)[b]	1.9 ± 0.1	152 ± 26	—	—	3
IAsys (AMPPNP)[c]	2.0 ± 0.3	914 ± 80	$2.0 \pm 0.1 \times 10^4$	$1.8 \pm 0.1 \times 10^{-2}$	2
IAsys (ATP)[c]	0.9 ± 0.5	1340 ± 154	$3.0 \pm 0.2 \times 10^4$	$4.1 \pm 0.3 \times 10^{-2}$	2
Enhancement of KaiC phosphorylation (ATP)	—	0.25[d]	—	—	3

Values represent means ± SD.
[a] Calculated by the 2DNS–PAGE analysis shown in Fig. 5.
[b] Calculated by the Scatchard plot analysis shown in Fig. 6.
[c] Calculated by the IAsys analysis shown in Fig. 7 .
[d] *KE*, the molar ratio of KaiA dimer to KaiC hexamer that gives half saturation of the enhancement.

Scatchard plot analysis of KaiA–KaiC complex

We analyzed the KaiA–KaiC complexes formed in the presence of AMPPNP by Native-PAGE (Fig. 6A) and determined the stoichiometry of KaiA and KaiC by Scatchard plot analyses (Fig. 6B). We calculated the amount of KaiA dimer bound to 15 pmol KaiC hexamer to be 28.2 ± 1.5 pmol, suggesting that 2 molecules of KaiA dimer bound to 1 molecule of KaiC hexamer (Table 1). This was consistent with the 2DNS-PAGE analysis. By Scatchard plot analysis, the apparent K_D value for the KaiA–KaiC complex was 152 ± 26 nM (Table 1).

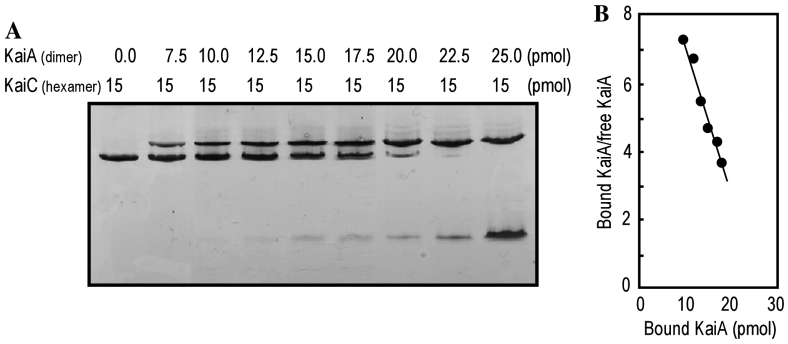

Fig. 6. Determination of the stoichiometry and dissociation constant in KaiA–KaiC interaction in the presence of 1 mM AMPPNP by Native-PAGE and Scatchard plot analyses. (A) Native-PAGE. Gel was stained with PhastGel Blue R. (B) Scatchard plot.

The IAsys assay for KaiA–KaiC interactions

Using an IAsys assay, we analyzed KaiA–KaiC interactions in real time in the presence of 1 mM ATP or 1 mM AMPPNP (Fig. 7). The values of the apparent association rate constant (*kass*) of KaiA dimer to immobilized KaiC hexamer in the presence of ATP and AMPPNP were $3.0 \times 10^4\ M^{-1}\ s^{-1}$ and $2.0 \times 10^4\ M^{-1}\ s^{-1}$, respectively (Table 1). While the dissociation of KaiA from the immobilized KaiC was completed in the presence of ATP, it was not completed in the presence of AMPPNP. The values of apparent dissociation rate constant (*kdis*) in the presence of ATP and AMPPNP were $4.1 \times 10^{-2}\ s^{-1}$ and $1.8 \times 10^{-2}\ s^{-1}$, respectively (Table 1). These values gave apparent equilibrium dissociation constants (K_D) of 1340 nM in the presence of ATP and 914 nM in the presence of AMPPNP (Table 1). The K_D value in the presence of AMPPNP calculated by IAsys assay was 6 times the value obtained by Native-PAGE/Scatchard plot (Table 1). The immobilization of the KaiC hexamer required for the IAsys assay might have caused the difference.

The ratio of KaiA dimer to KaiC hexamer in KaiA–KaiC interactions in the presence of ATP was estimated by IAsys analysis to be 1. In the presence of AMPPNP, the ratio was estimated to be 2, indicating that 2 molecules of KaiA dimer bound to 1 molecule of KaiC hexamer. Thus, result by IAsys analysis was consistent with those by Native-PAGE/Scatchard plot analysis and 2DNS-PAGE analysis (Table 1).

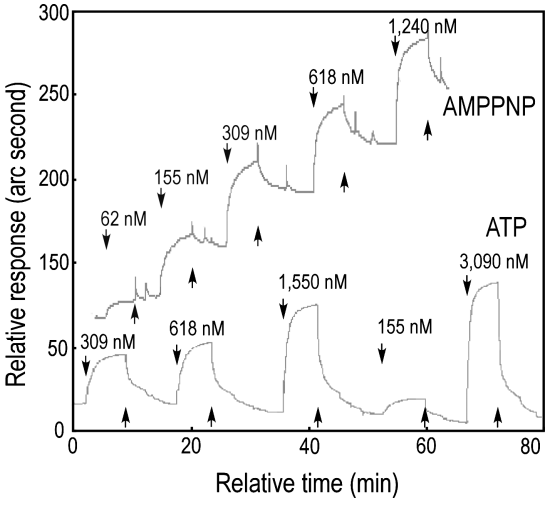

Fig. 7. KaiA–KaiC interactions analyzed with an IAsys affinity biosensor. KaiC hexamerized in the presence of 1 mM ATP or 1 mM AMPPNP was immobilized on a carboxylate cuvette. The various concentrations of KaiA dimer indicated were injected into a cuvette equilibrated with a running buffer containing 1 mM ATP or 1 mM AMPPNP and 5 mM $MgCl_2$ (downward arrow), and signals showing the association of KaiA dimer to the immobilized KaiC were monitored. Then the cuvette was washed twice with running buffer (upward arrow), removing free KaiA, and the signals show the dissociation of bound KaiA dimer from the KaiA–KaiC complex.

Discussion

When KaiA and KaiC were mixed and incubated in the presence of AMPPNP, KaiA–KaiC complexes were detected by immunoblotting, 2DNS-PAGE, and IAsys assays (Figs. 4–7). In the presence of ATP, the formation of KaiA–KaiC complexes was detected by the IAsys assay (Fig. 7), but stable KaiA–KaiC complexes were not detected by Native-PAGE or 2DNS-PAGE, although p-KaiC was (Figs. 4 and 5). We propose the following scheme:

(a) KaiC + ATP (or AMPPNP) + KaiA ↔ (b) KaiC · ATP (or AMPPNP) · KaiA → (c) p-KaiC · ADP · KaiA ↔ (d) p-KaiC + ADP + KaiA → (e) Pi + KaiC + ADP + KaiA.

When KaiC, ATP, and KaiA are mixed (a), a tertiary complex of KaiC, ATP, and KaiA is formed (b). The γ-phosphate group of ATP is transferred to KaiC, and p-KaiC and ADP are formed in the complex (c). Then p-KaiC, ADP, and KaiA dissociate from the complex (d). In the presence of AMPPNP, the step from (b) to (c) is inhibited. We assume the formation of state (e), although we have not yet confirmed it. When we compared the apparent *kass*, *kdis*, and K_D values in the reaction of (a) to (b), the apparent *kass* and K_D values in the presence of ATP determined by IAsys assays were 1.5 and 1.4 times the value in the presence of AMPPNP, respectively (Table 1). Thus, both the formation rate of the KaiA–KaiC complexes and the affinity of KaiA–KaiC interactions did not differ much in the presence of ATP and in the presence of AMPPNP. The apparent *kdis* value in the presence of AMPPNP was 56% lower than the value in the presence of ATP (Table 1), suggesting that the slower dissociation rate favors the formation of stable KaiA–KaiC complexes, including AMPPNP complexes (KaiC · AMPPNP · KaiA).

Systems in which a triphosphate nucleotide hydrolyzing protein is partnered with an activating protein, such as *E. coli* DnaK and DnaJ [9,10] and Cdc42 and Cdc42 GTPase activating protein (Cdc42GAP) [11], are well known. DnaK and DnaJ are members of the DnaK/DnaJ/GrpE molecular chaperone system, which mediates protein folding, protein translocation, protein assembly/disassembly, and repair of unfolded proteins [12,13]. DnaJ interacts with DnaK in the presence of ATP and enhances the ATP hydrolysis rate of DnaK up to >1000-fold [10]. Cdc42, a member of the Rho subfamily, is involved in a wide variety of cellular processes, such as cell cycle progression and apoptosis [14,15], and Cdc42GAP is a critical regulator for hydrolysis of GTP by Cdc42 [16]. In the activating mechanism of GTP hydrolysis [11], a conserved Arg residue on Cdc42GAP is key. The GTP hydrolysis rate of Cdc42 is enhanced 3-fold by its interaction with Cdc42GAP [17].

By assuming that (1) KaiC hexamer but not monomer had ATP hydrolyzing activity, (2) one phosphate

group was incorporated into each KaiC subunit, generating the upper band on SDS–polyacrylamide gels (Fig. 2A), and (3) the ATP hydrolysis rate was almost constant for 30 min (Fig. 3A), we calculated the ATP hydrolysis rate of KaiC to be $2 \times 10^{-4}\,s^{-1}$. The ATP hydrolysis rate of KaiC is comparable to that of DnaK in the absence of DnaJ and GrpE ($6 \times 10^{-4}\,s^{-1}$) [10], and the rates are very small. Even in the presence of 6-fold molar excess amounts of KaiA dimer to KaiC hexamer, which gave almost saturation in the enhancement of KaiC phosphorylation, the phosphorylation rate of KaiC was calculated to be $3 \times 10^{-4}\,s^{-1}$. It is possible that another protein that enhances ADP exchange, such as GrpE, may be involved in the phosphorylation reaction of KaiC enhanced by KaiA. GrpE interacts with DnaK holding ADP-Pi, which is a reaction product of ATP hydrolysis, and enhances the removal of the ADP-Pi from DnaK [18]. ATP hydrolysis activity of DnaK is enhanced up to 200-fold by its interaction with GrpE [19]. Dbl [16,20,21] is a guanine nucleotide exchange factor for Cdc42, which catalyzes the exchange of GDP for GTP. Both the DnaK/DnaJ/GrpE molecular chaperone system and the Cdc42/Cdc42GAP/Dbl Cdc42 cycle system are composed of a triphosphate nucleotide hydrolysis protein (DnaK and Cdc42), a hydrolysis rate-activating protein (DnaJ and Cdc42GAP), and a diphosphate nucleotide removal/exchange protein (GrpE and Dbl) [10,20]. It is possible that the clock molecular machinery in cyanobacteria is composed of an ATP hydrolysis protein (KaiC), a hydrolysis rate-activating protein (KaiA), and an ADP removal/exchange protein (unknown).

How many KaiA dimers interact with the KaiC hexamer, which has twelve ATP binding sites [8]? IAsys analysis demonstrated that in the presence of ATP, 1 molecule of KaiA dimer interacted with 1 molecule of KaiC hexamer (Fig. 7 and Table 1) and the efficiency of KaiA enhancement of KaiC phosphorylation was almost saturated (Fig. 3 and Table 1). On the other hand, all three analyses described here—2DNS-PAGE, Native-PAGE/Scatchard plot, and IAsys—demonstrated that in the presence of AMPPNP, 2 molecules of KaiA dimer interact with 1 molecule of KaiC hexamer (Figs. 5–7 and Table 1). These results suggest that 2 molecules of KaiA dimer can interact with 1 molecule of KaiC hexamer, and that interactions of at least 1 molecule of KaiA dimer with 1 molecule of KaiC hexamer are enough to enhance the phosphorylation of KaiC at an almost saturated level.

If we use previously reported data on the number of KaiA and KaiC molecules in a cell [22] and assume the size of a cyanobacterium to be $100\,\mu m^3$ ($\phi = 3.98\,\mu m$, $l = 10\,\mu m$), the concentrations of KaiA dimer and KaiC hexamer are approximately 1.6–3.2 μM and 16–40 μM, respectively. Compared with the apparent K_D value (1.3 μM) for KaiA–KaiC interactions, the concentration of KaiA dimer in vivo (1.6–3.2 μM) suggests that a slight fluctuation in KaiA concentration affects the level of p-KaiC. We suppose a cyclic conversion of KaiC states (named here KaiC cycles), including p-KaiC, p-KaiC · ADP, KaiC · ATP, and KaiC without nucleotides, in the clock machinery. The KaiC cycle regulated by KaiA and a hypothetical ADP exchange protein may be crucial for generating circadian oscillations.

We previously determined the interaction site of KaiA involved in KaiA–KaiC complexes at the atomic level [7], but the interaction site of KaiC involved in KaiA–KaiC complex formation remains to be solved. The phosphorylation site of KaiC also remains to be determined. X-ray crystal structures of each KaiC state in the KaiC cycle should be solved for in-depth understanding of the circadian clock machinery in cyanobacteria.

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