Nucleotide binding and autophosphorylation of the clock protein KaiC as a circadian timing process of cyanobacteria

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A negative feedback control of *kaiC* expression by KaiC protein has been proposed to generate a basic oscillation of the circadian clock in the cyanobacterium *Synechococcus* sp. PCC 7942. KaiC has two P loops or Walker's motif As, that are potential ATP-/GTP-binding motifs and DXXG motifs conserved in various GTP-binding proteins. Herein, we demonstrate that *in vitro* KaiC binds ATP and, with lower affinity, GTP. Point mutation by site-directed mutagenesis of P loop 1 completely nullified the circadian rhythm of *kaiBC* expression and markedly reduced ATP-binding activity. Moreover, KaiC can be autophosphorylated *in vitro*. These results suggest that the nucleotide-binding activity of KaiC plays important roles in the generation of circadian oscillation in cyanobacteria.

Circadian rhythms, biological oscillations with 24-h periodicity, are observed ubiquitously among eukaryotes and cyanobacteria. An endogenous oscillator named the circadian clock temporally regulates various biological activities to match them with daily environmental alterations (1). To elucidate the molecular mechanism of the circadian clock, several clock genes and clock-related genes have been cloned and analyzed in cyanobacteria, *Neurospora*, *Drosophila*, *Arabidopsis*, and mammals (2).

Cyanobacteria are the simplest organisms known to have the circadian clock. An essential clock gene cluster *kaiABC* was cloned from the cyanobacterium *Synechococcus* sp. strain PCC 7942 (3). Various circadian phenotypes can arise from mutations in any of the *kai* genes. In particular, 14 distinct clock mutations including those for period length and arrhythmia were mapped to *kaiC*. Experiments on the promoters of *kai* genes suggest that KaiC suppresses its own (*kaiBC*) expression in a basic negative feedback loop, and KaiA enhances *kaiBC* expression to make the system oscillate (3). Moreover, the KaiA, KaiB, and KaiC proteins directly associate in all possible combinations in the yeast two-hybrid system, *in vitro*, and in cyanobacterial cells, and a long-period allele, *kaiA1*, markedly enhances KaiA–KaiB interaction (4). These observations suggest that physical interactions among the Kai proteins are crucial to the circadian timing mechanism.

Although the biochemical functions of the Kai proteins remain unknown, the amino acid sequence of KaiC contains two ATP-/GTP-binding motifs (P loops or Walker's motif As) whose consensus is GXXXXGKT/S (X represents any amino acid) in the tandem duplicated domains of KaiC (CI and CII domains) (Fig. 1*A*; refs. 3–6). In addition, two DXXG motifs that are highly conserved in the GTPase superfamily (7, 8) are found in the CI domain. These motifs could provide clues to the biochemical function of KaiC in circadian rhythm generation. In this report, we show that KaiC binds ATP and, to a lesser extent, GTP *in vitro*, and disruption of the P loop in the CI domain causes arrhythmia and a marked reduction in ATP-binding activity. Moreover, we have identified autophosphorylation activity of KaiC *in vitro*. These observations strongly suggest that the ATP-binding activity of KaiC is important for the generation of circadian oscillation in *Synechococcus*.

Materials and Methods

Bacterial Strains, Media, Cultures, and Manipulation of DNA. Because the expression of the *kaiBC* operon was assumed to be the key process of the cyanobacteria oscillator (3), we used a reporter strain of *Synechococcus*, NUC39 (4), that carried a bacterial luciferase gene set *luxAB* fused to a promoter of *kaiBC* operon (P_{kaiBC}) at a specific site of the chromosome (neutral site I) as the control strain of this study. The *Synechococcus* cells were grown at 30°C in BG-11 liquid medium or solid medium that contained 1.5% (wt/vol) Bacto Agar (Difco) under continuous light (LL) conditions of 46 μmol m^{-2}·s^{-1} from white fluorescent lamps. Plasmids were introduced into *Escherichia coli* DH10B by electroporation. *Synechococcus* cells were transformed with plasmid DNA by natural transformation and selected with 40 μg/ml of spectinomycin sulfate (9).

Site-Directed Mutagenesis of the *kai* Locus. pCkaiABC targeting vector (3) containing the *kaiABC* cluster and a spectinomycin-resistant gene was mutagenized with the overlap extension method by PCR (10). All of the resulting mutations were confirmed by sequencing with a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). The mutant plasmids were introduced into *kaiABC*-deficient P_{kaiBC} reporter cells (NUC38) as described (4).

***In Vitro* Translation of KaiC and Its Derivatives.** Expression vectors for *in vitro* transcription/translation were generated by inserting a wild-type or mutagenized PCR fragment into the *Sal*I–*Bam*HI site of pSP64-poly(A) vector (Promega). The TNT rabbit reticulocyte system (Promega) was used for *in vitro* production and labeling of each KaiC-derived protein as described (4). Briefly, 25 μl of reaction mixture containing 1 μg each of the pSP64-poly(A) derivatives, SP6 RNA polymerase, and 12.5 μl of rabbit reticulocyte was incubated for 2 h at 30°C in the presence of 10 μCi of [^{35}S]methionine (>1,000 Ci/mmol; 1 Ci = 37 GBq). Labeled proteins were confirmed by SDS/10% PAGE and analyzed by autoradiography with a BAS2000 Image Analyzer (Fuji).

Binding of *in Vitro* Translated Proteins to ATP- or GTP-Agarose. An *in vitro* ATP-/GTP-binding assay was performed as described by Iismaa *et al.* (11) with minor modifications. A fraction of the radioactivity (3.0×10^5 cpm) of the *in vitro* translation reaction

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Abbreviations: CI, tandem duplicated domain of KaiC (N-terminal half); CII, tandem duplicated domain of KaiC (C-terminal half); LL, continuous light; GST, glutathione *S*-transferase.

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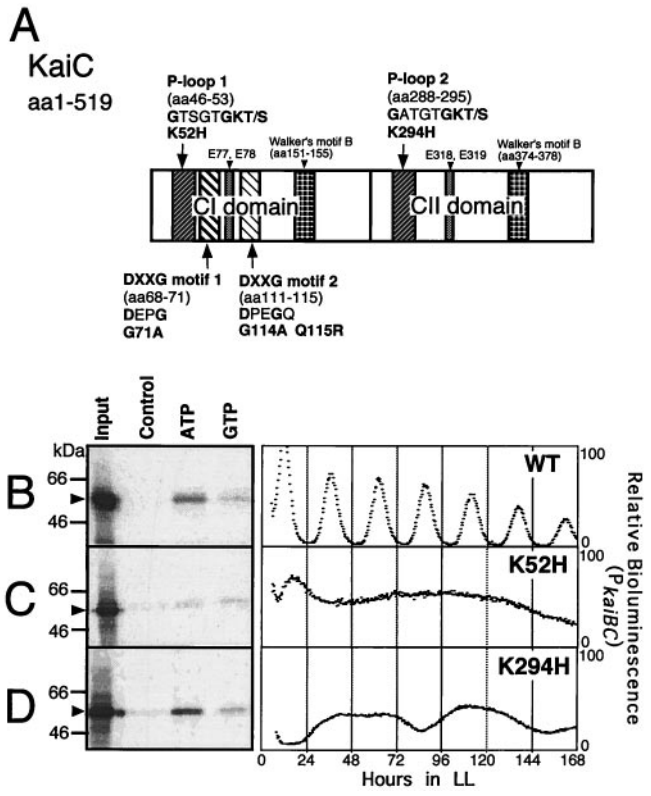

Fig. 1. Effects of point mutations on nucleotide-binding motifs of KaiC and bioluminescence rhythm of P_{kaiBC}. (*A*) Point mutations introduced to nucleotide-binding motifs (P loops and DXXG motifs) of KaiC are illustrated. The amino acid (aa) sequences of these motifs and point mutations are designated by their one-letter codes. Tandem duplicated domains of KaiC, N-terminal and C-terminal halves, are indicated as CI (amino acids 1–250) and CII (amino acids 251–519), respectively. Locations of P loops, DXXG motifs, Walker's motif Bs, and putative catalytic carboxylates are illustrated. Conserved amino acids of each motif are shown in boldface, and X represents any residue. (*B–D*) *In vitro* nucleotide binding assay (*Left*) and bioluminescence profiles for P_{kaiBC} promoter activity (*Right*). ^{35}S-labeled KaiC was incubated with ATP agarose (lane 3), GTP agarose (lane 4), or control agarose (lane 2) resin. Input controls are shown in lane 1. Autoradiograms for ^{35}S-labeled KaiC are shown. Arrowheads indicate the position of the KaiC band (≈57 kDa). For monitoring bioluminescence rhythm, cells were grown to give 30–70 colonies 0.2 mm in diameter under LL conditions. After a 12-h dark treatment, the bioluminescence from each dish was measured with a photomultiplier tube. Bioluminescence intensity per colony was normalized to 100 by the peak value of the first day for wild-type cells (*y* axes). (*B*) Binding to wild-type (WT) KaiC and bioluminescence from wild-type cells. (*C*) Lysine-to-histidine substitution in P loop 1 (K52H). (*D*) Lysine-to-histidine substitution in P loop 2 (K294H).

mixture was diluted to 450 μl with binding buffer [20 mM Tris·HCl, pH 7.0/150 mM NaCl/5 mM $MgCl_2$/0.1% (vol/vol) Triton X-100] and incubated with 50 μl of ATP-agarose or GTP-agarose beads (2.2 μmol/ml; Sigma) at room temperature for 30 min. The beads were pelleted by centrifugation, and the supernatant containing unbound protein was retained. The beads were washed five times with 1 ml of binding buffer to reduce nonspecific protein binding before the subsequent 3-h incubation with the supernatant. Bound protein was eluted by boiling in SDS/PAGE loading buffer and analyzed by SDS/10% PAGE followed by autoradiography.

UV Crosslink Assay. Glutathione *S*-transferase (GST) fused to KaiC, CI, and CII was prepared as described (4). To assess ATP crosslink to KaiC derivative proteins, 1.5 μg each of the fusion proteins was incubated with 0.1 mM [α-^{32}P]ATP (10,000 Ci/mmol) or 0.2 mM [^{35}S]ATPγS (1,000 Ci/mmol) in 20 μl of reaction buffer (100 mM Tris·HCl, pH 7.6/50 mM KCl/5 mM $MgCl_2$/0.4 mM DTT) at 25°C for 30 min. Reactions were terminated by placing the mixture on ice. The mixture was exposed to UV light (Stratalinker 1800, Stratagene) for 40 min at 4°C, and 4 μl of 6× SDS/PAGE loading buffer was added to the mixture. After boiling for 5 min, samples were subjected to SDS/PAGE with 10% gels and autoradiography and then analyzed with the BAS2000 Image Analyzer.

Assay of Bioluminescence Rhythm. *Synechococcus* cells were inoculated onto BG-11 agar plates in a plastic dish (30 mm in diameter) and incubated under LL conditions (46 μmol m^{-2}·s^{-1}) to form 50–100 colonies. Then, cells were subjected to 12 h of darkness to synchronize the circadian clocks, and bioluminescence from the agar plates was automatically monitored under LL in the presence of decanal solution with a photomultiplier-tube-based bioluminescence-monitor system (3).

Protein Phosphorylation Assay. GST and GST fused to KaiA, KaiB, or KaiC were prepared as described (4). An autophosphorylation assay was performed as described by Aiba *et al.* (12) and McCleary and Zusman (13) with some modifications. The protein sample (1.0 μg) was added to 15 μl of a reaction mixture {50 mM Tris·HCl, pH 7.5/100 mM KCl/5 mM $MgCl_2$/0.5 mM EDTA/2 mM DTT/0.1 mM [γ-^{32}P]ATP (10,000 cpm/mmol) or [α-^{32}P]ATP (30,000 cpm/mmol)} at 25°C for 30 min. Reactions were terminated by the addition of 3 μl of 6× SDS/PAGE loading buffer. After heating at 65°C for 5 min, samples were subjected to SDS/PAGE with 10% gels and then blotted onto Immobilon-P membranes (Millipore). The radioactivities of phosphorylated proteins were analyzed with the BAS2000 Image Analyzer.

Chemical Stability Assay of Phospho-Linkage. A chemical stability assay was performed as described by McCleary and Zusman (13) with minor modifications. Equal amounts of GST-KaiC were phosphorylated and blotted onto an Immobilon-P membrane as described above. Individual blots were excised from the membrane and separately incubated in 50 mM Tris·HCl, pH 7.5/3 M NaOH/1 M HCl or 0.8 M hydroxylamine (pH 6.8) at 42°C. After incubation for 1 h, the membranes were washed with distilled water and dried in air, and then the radioactivities were analyzed with the BAS2000 Image Analyzer.

Results

KaiC Binds to ATP and GTP. ^{35}S-labeled KaiC was translated *in vitro* and incubated with ATP-agarose or GTP-agarose. As shown in Fig. 1*B*, radioactive KaiC was detected after incubation with ATP-agarose or GTP-agarose followed by SDS/PAGE (Fig. 1*B*, lanes 3 and 4). The binding of KaiC to ATP was much stronger than that to GTP. No activity was detected after incubation with control agarose resin (Fig. 1*B*, lane 2). In addition, [^{35}S]KaiA associated with neither ATP- nor GTP-agarose (data not shown). These results indicate specific binding of KaiC to ATP and to GTP with lower affinity.

Point Mutations of the P Loops Affect Both ATP-/GTP-Binding of KaiC and Circadian Rhythm. As illustrated in Fig. 1*A*, the first and second P loops (P loop 1 and P loop 2, respectively) were disrupted separately by substitution of the conserved lysine residues with histidine (K52H and K294H, respectively) to examine the effects of these mutations on the ATP-/GTP binding activity of KaiC. Fig. 1*C* shows that the K52H substitution in P loop 1 markedly reduced the ATP-binding activity; however, the P loop 2 mutation (K294H) failed to alter the ATP-binding profile (Fig. 1*D*), and neither mutation altered the GTP-binding activities.

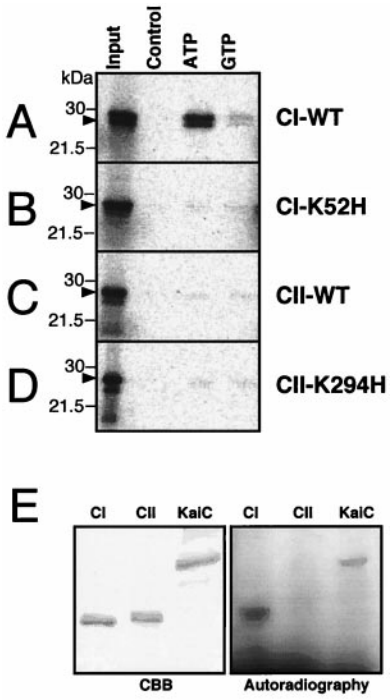

Fig. 2. Nucleotide binding to the CI or CII domain of KaiC. (*A–D*) *In vitro* nucleotide binding to the ^{35}S-labeled CI or CII domain of KaiC was examined. Experimental conditions and the presentation of data were the same as described for Fig. 1 *B–D Left*, except that the ^{35}S-labeled CI or CII domain of KaiC was used instead of full-length KaiC. (*A*) Wild-type (WT) CI domain. (*B*) CI domain containing K52H mutation. (*C*) Wild-type CII domain. (*D*) CII domain containing the K294H mutation. (*E*) UV crosslink assays. Purified GST-KaiC, GST-CI, and GST-CII proteins were visualized by staining with Coomassie brilliant blue (CBB) after SDS/PAGE (*Left*). Each fusion protein was incubated with [α-^{32}P]ATP, crosslinked by UV irradiation, and then analyzed by SDS/PAGE followed by autoradiography (*Right*).

Next, we analyzed the effect of these mutations on the circadian oscillation of *kaiBC* expression. Initially, we constructed a DNA fragment that contained the entire *kai* gene cluster in which the *kaiC* ORF was modified to carry mutations in the nucleotide-binding motifs. Each mutant *kai* cluster was introduced into a *kai*-deficient strain at the original locus. Although the control strain that had regained the normal *kai* cluster had a robust circadian rhythm (Fig. 1*B*), the P loop 1-disrupted transformant (K52H) completely lost the rhythmicity (Fig. 1*C*), suggesting that the lysine residue in P loop 1 was indispensable to the generation of the circadian oscillation. The amino acid substitution at P loop 2 (K294H) resulted in an extremely long-period phenotype ($\approx$70 h; Fig. 1*D*) with lowered amplitude. Neither of these mutations altered the average level of bioluminescence (compare the values of the vertical axes in Fig. 1 *B–D*). Moreover, substitutions of threonine residues with alanine in both P loop 1 and P loop 2 instead of lysine-to-histidine mutations disrupted the rhythm completely (data not shown). These results imply that both P loops are crucial for the circadian clock of cyanobacteria.

ATP Binds Mainly to the CI Domain. Although mutation in either P loop significantly altered the rhythm, binding to ATP was lowered only by the mutation in P loop 1. The contribution of P loop 2 to ATP-binding seemed to be minor. To examine this point, we produced CI and CII domains of KaiC with or without the point mutations by *in vitro* translation. ATP and, to a lesser extent, GTP bound to CI domains (Fig. 2*A*), and the P loop 1 mutation lowered the ATP binding as was the case of full-length

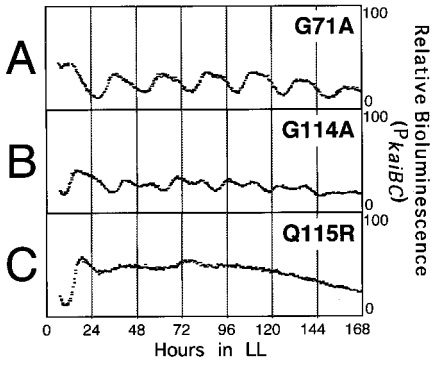

Fig. 3. Effects of point mutations in DXXG motifs on circadian rhythm. Bioluminescence profiles of P_{kaiBC}:*luxAB* reporter strains carrying mutations in each DXXG motif under LL conditions are shown. Measurement of the bioluminescence and representation of data were the same as described for Fig. 1 *B–D Right*. (*A*) Glycine-to-alanine substitution in DXXG motif 1 (G71A). (*B*) Glycine-to-alanine substitution in DXXG motif 2 (G114A). (*C*) Glutamine-to-arginine substitution at position 115.

KaiC (Fig. 2*B*). On the other hand, only a faint signal of ATP-binding was detected for either normal or mutated CII domains (Fig. 2 *C* and *D*). To exclude a possibility that CII is merely inaccessible to agarose-linked ATP, we also performed a UV crosslinking assay to assess the ATP-binding profile of KaiC, CI, and CII fusion proteins. GST fused to KaiC, CI, or CII was incubated with [α-^{32}P]ATP or [^{35}S]ATPγS, and UV crosslinked ATP was analyzed by SDS/PAGE and autoradiography. The results confirmed that ATP bound to KaiC and CI but failed to associate with CII (Fig. 2*E* and data not shown). These results suggest that ATP-binding activity mainly resides on the CI domain and that the P loop 1 is responsible for this activity.

Mutation in DXXG Motifs Affects the Clock. Two DXXG motifs (DXXG1 and DXXG2) in the CI domain were disrupted individually by substitution of the conserved glycine residues with alanine (G71A and G114A). DXXG2 is followed by a glutamine residue (Q115) that is a cognate to the glutamine residue at position 61 in Ras protein. The glutamine of Ras is one of the main sites of oncogenic transformation (14) and is required for GTP hydrolysis (15, 16). Therefore, we also changed the residue Q115 to arginine (Q115R). The G71A mutation in DXXG1 lowered the amplitude and distorted the waveform (Fig. 3*A*). The G114A mutation in DXXG motif 2 extended the period to 27 h and caused a bimodal waveform (Fig. 3*B*). Thus, the circadian oscillation in *kaiBC* expression was modified considerably by these mutations, although periodicity remained in the circadian range. On the other hand, Q115R mutation abolished the circadian rhythm (Fig. 3*C*). None of these mutations affected the average level of *kaiBC* expression. We could not detect any effect of these mutations on nucleotide-binding activities by the method with GTP agarose (data not shown).

Autophosphorylation of KaiC. It is known that some bacterial proteins that contain the P loop and Walker's motif B have autokinase activity (17, 18). To examine the autophosphorylation of KaiC, GST-KaiC (4) was produced in *E. coli*, purified with an affinity resin, and then incubated with [γ-^{32}P]ATP or [α-^{32}P]ATP in the presence of Mg^{2+}. As shown in Fig. 4*A*, GST-KaiC was labeled with ^{32}P after incubation with [γ-^{32}P]ATP but not with [α-^{32}P]ATP. No phosphorylation activity was detected in unfused GST and GST fused to KaiA or KaiB. Therefore, GST-KaiC was specifically autophosphorylated by γ-phosphate.

Based on the stability of the phosphate bond in various ionic

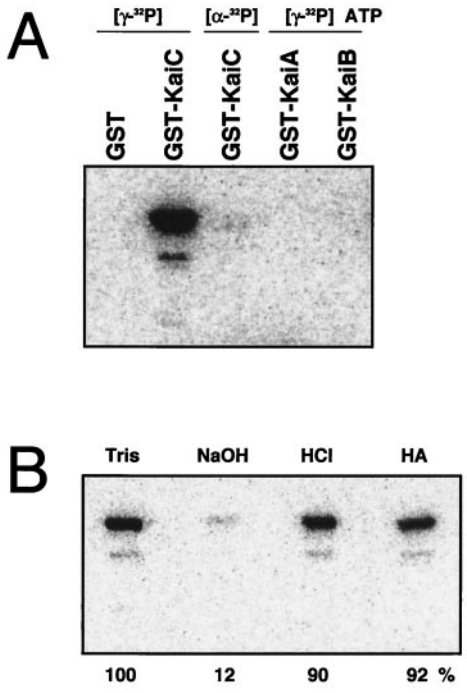

Fig. 4. Autophosphorylation of Kai by ATP and its chemical stability profiles. (*A*) GST fusions of KaiA, KaiB, and KaiC as well as a GST control were incubated with [γ-^{32}P]ATP or [α-^{32}P]ATP in the presence of Mg^{2+}. Proteins were subjected to SDS/PAGE and detected by autoradiography. (*B*) Phosphorylated KaiC separated by electrophoresis was blotted to Immobilon-P membrane. Fractions of the membrane were treated under various ionic conditions and then assayed for binding. Numbers at the bottom are the relative intensity of signal that was normalized with that treated by neutral buffer.

conditions, phosphorylated GST-KaiC was assayed to determine the class of phosphorylated amino acid residue (19). As shown in Fig. 4*B*, 90% or more radioactivity was retained after treatments with HCl and hydroxylamine, whereas base (NaOH) treatment markedly reduced the level to 12% of the control. These results suggest that the phosphate link in phosphorylated GST-KaiC is an *O*-phosphate linkage. Among *O*-phosphate bonds, phosphotyrosine is base stable, whereas phosphoserine and phosphothreonine are base labile (19). Therefore, KaiC was likely to be autophosphorylated at serine or/and threonine residue or residues.

Discussion

Ishiura *et al.* (3) proposed a model of circadian oscillation of cyanobacteria in which negative feedback of the *kaiBC* expression by KaiC and positive regulation by KaiA play key roles. Thus, Kai proteins, especially KaiC, are evidently key components of the circadian oscillator of cyanobacteria (3). In this report, we have presented evidence of the biochemical activity of KaiC. The ATP-binding of KaiC could be essential to generate the circadian oscillation, because a marked decrease in ATP-binding activity caused by the disruption of P loop 1 was strikingly correlated with the complete nullification of circadian rhythms (Fig. 1*B*). Moreover, note that a point mutation of P loop 1 did not alter the average level of bioluminescence from P_{kaiBC} reporter strains. Therefore, a point mutation in P loop 1 likely caused arrhythmicity, not by lowering the average level of *kaiBC* transcription, but by affecting an as-yet unknown biochemical function or functions of KaiC that are driven by bound ATP.

KaiC is composed of two tandem duplicated domains, CI and CII (4). Our results strongly suggest that ATP bound mainly to the CI domain (Fig. 2) and that P loop 1 was a main active site for ATP-binding of KaiC. This observation also suggests distinct functions of CI and CII domains. This possibility is pertinent to our previous findings that the KaiA–KaiB association was enhanced by the CI but not the CII domain (4). However, note also that substitution of the lysine residue of P loop 2 with histidine extremely extended the period of the rhythms ($\approx$70 h) and of conserved threonine with alanine induced arrhythmicity. Thus, it is evident that this region of P loop 2 is also important for normal rhythmicity. Mutation of P loop 2 might affect some unknown function (e.g., maintaining a functional configuration of KaiC) and have a large effect on the circadian oscillator; however, it is still possible that P loop 2 is also a functional ATP-binding motif but that its ATP/GTP-binding affinity is below the detection limit of the current assay.

The GTP-binding activity detected in this study was much weaker than the ATP-binding activity and was not altered by point mutations in the P loops and DXXG motifs (Fig. 1 *B–D Left*; Fig. 2; data not shown). Mutations of invariant glycine to alanine in GXXG motifs 1 and 2 altered the circadian rhythm phenotype; however, rhythmicity still remained obvious, and the period length was in the circadian range (Fig. 3). Moreover, KaiC contained P loops and DXXG motifs but not NXKD and (C/S)AX motifs that are conserved in many GTP-binding proteins (7, 8). Thus, the GTP binding to KaiC seems not to be essential to the timing mechanism of the circadian oscillator. However, because a substitution of Q115, which is cognate to Q61 of Ras (7, 8), with arginine resulted in complete arrhythmia (Fig. 3*C*), it is still possible that GTP hydrolysis by this residue is responsible for the generation of circadian rhythm, as is the case for signal transduction by Ras (15, 16).

Yoshida and Amano (20) revealed a consistency in topologies of ATPases whose crystal structures had already been solved, such as F1-ATPase and RecA. These investigators identified catalytic carboxylate 24 $\pm$ 2 residues from the lysine residue of P loop and Walker's motif B 50–130 residues from the C-terminal side of the P loop (20). In the case of KaiC, the distances between P loops and Walker's motif Bs in CI and CII domains are 87 and 78 residues, respectively, and two continuous glutamate residues are located 25 and 24 residues from the lysine residues of P loops in CI and CII domains, respectively (Fig. 1*A*; ref. 3). Therefore, it is likely that the topology of the CI and CII domains of KaiC are similar to that of the ATPases.

In this study, we have identified *O*-autokinase activity of KaiC at the serine and/or threonine residue or residues in KaiC (Fig. 3). *O*-phosphorylation has been widely found in eukaryotes and prokaryotes including cyanobacteria (21–26). However, to the best of our knowledge, ours is the first report of *O*-autophosphorylation in cyanobacteria. Conserved sequences were found among various *O*-kinases, but such sequences are not found in KaiC (3). It was reported recently that several proteins that contain a P loop have autokinase activities. Point mutations in P loop and Walker's motif B of Ptk protein of *Acinetobacter johnsonii* decreased ATP binding and autophosphorylation activity (17). EpsE protein of *Vibrio cholerae* decreased the autokinase activity by a point mutation in the P loop (18). Therefore, it is possible that KaiC also uses P loops in autophosphorylation.

In various clock models, phosphorylation of clock proteins is considered to be an important part of the circadian timing loop (2). In *Drosophila*, phosphorylation of Period (PER) controls the initial rate of PER accumulation and seems necessary for PER cycling. Double-time (Dbt), a *Drosophila* homolog of casein kinase Iε, was revealed to phosphorylate PER (27, 28). As is the case for PER, autophosphorylation of KaiC, revealed in this study, could alter the stability of KaiC that regulates negative feedback of *kaiBC* transcription. Alternatively, after autophosphorylation, KaiC might change its conformation to regulate as-yet unknown biochemical activities. Our previous analysis

revealed that KaiC interacted with both KaiA and KaiB (4). Autophosphorylated KaiC may alter affinities between the Kai proteins. Conversely, it is possible that the binding of KaiA and/or KaiB regulates the autophosphorylation activity of KaiC.

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