Circadian Autodephosphorylation of Cyanobacterial Clock Protein KaiC Occurs via Formation of ATP as Intermediate*\textsuperscript{[a]}

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Background: The autodephosphorylation mechanism of the KaiC autokinase/autophosphatase is currently unknown.

Results: ATP was transiently formed during autodephosphorylation prior to the formation of Pi.

Conclusion: Autodephosphorylation occurs through the reversal of autophosphorylation, followed by hydrolysis of an ATP intermediate.

Significance: This mechanism is completely different from that of Ser/Thr and Tyr-specific protein phosphatases.

The cyanobacterial circadian oscillator can be reconstituted in vitro; mixing three clock proteins (KaiA, KaiB, and KaiC) with ATP results in an oscillation of KaiC phosphorylation with a periodicity of \( \sim 24 \) h. The hexameric ATPase KaiC hydrolyzes ATP bound at subunit interfaces. KaiC also exhibits autokinase and autophosphatase activities, the latter of which is particularly noteworthy because KaiC is phylogenetically distinct from typical protein phosphatases. To examine this activity, we performed autodephosphorylation assays using \(^{32}\)P-labeled KaiC. The residual radioactive ATP bound to subunit interfaces was removed using a newly established method, which included the dissociation of KaiC hexamers into monomers and the reconstitution of KaiC hexamers with nonradioactive ATP. This approach ensured that only the signals derived from \(^{32}\)P-labeled KaiC were examined. Surprisingly, we detected a transient elevation of [\(^{32}\)P]ATP preceding the formation of \(^{32}\)Pi, suggesting that KaiC dephosphorylates itself through a novel sequential mechanism, \textit{i.e.} KaiC generates ATP as a reaction state of KaiC shows robust circadian rhythms by mixing KaiA, KaiB, KaiC, and ATP in a test tube (1). Eukaryotic circadian clocks have long been understood as transcriptional-translational feedback loops in which clock gene products repress their own transcription (2). Recently, however, protein-based circadian oscillations have been found in human red blood cells (3) and in microalgae (4), suggesting that protein-based circadian clocks exist ubiquitously in living organisms. Among the many experimental systems for studying circadian oscillations, the cyanobacterial \textit{in vitro} system is the simplest one and is expected to be the best model to understand the principle underlying the protein-based oscillations.

KaiC belongs to the P-loop NTPase superfamily, which includes both NTPases and kinases characterized by a strongly conserved nucleotide-binding motif, the P-loop (5). KaiC is a double-domain P-loop ATPase consisting of the N-terminal CI and C-terminal CII domains (6). KaiC forms a double doughnut-like homohexamer with an ATP molecule at each subunit interface in the two rings (7, 8). KaiC exhibits ATPase and autokinase activities (9, 10). KaiA activates both of these activities, whereas KaiB inhibits the effect of KaiA (9, 11, 12). The active sites for ATP hydrolysis reside at the subunit interfaces of CI and CII (9), whereas the autokinase activity is located only in CII (13). The autophosphorylation sites, Ser-431 and Thr-432 (14), face the ATP molecule on the neighboring protomer (8). In addition to these activities, KaiC exhibits autophosphatase activity (15), despite a lack of homology with the Ser/Thr and Tyr-specific protein phosphatases (16, 17). The reaction mechanism for KaiC autodephosphorylation has not yet been elucidated.

To address this issue, we performed an autodephosphorylation assay using KaiC labeled with \(^{32}\)P. Prior to the assay, we removed the residual radioactive ATP bound to subunit interfaces using a newly established method, which included the dissociation of KaiC hexamers into monomers and the reconstitution of KaiC hexamers with nonradioactive ATP. This approach ensured that only the signals derived from \(^{32}\)P-labeled KaiC were examined. Surprisingly, we detected a transient elevation of [\(^{32}\)P]ATP preceding the formation of \(^{32}\)Pi, suggesting that KaiC dephosphorylates itself through a novel sequential mechanism, \textit{i.e.} KaiC generates ATP as a reaction

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\[\text{[Supplemental Figs. S1–S4.]}\]

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intermediate, followed by its hydrolysis. The first step of the reaction is regarded as a reversal of autophosphorylation. On the basis of our observations, we demonstrate that the dephosphorylation of KaiC is catalyzed by the active site of ATPase through a completely different mechanism from those of typical Ser/Thr and Tyr phosphatases.

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains*—We used *Escherichia coli* BL21 cells as the host strain for expressing recombinant Kai proteins.

*Proteins*—Recombinant KaiA was expressed as described previously (6) and purified according to a previously reported method (14). Recombinant KaiB was expressed and purified as described previously (6, 11, 18). Full-length or CII-truncated KaiC hexamers were expressed and purified as described previously (6, 9, 14, 18). Protein concentrations were determined using the Bradford method and bovine serum albumin as a standard.

*Radioactive Chemicals*—[γ-32P]ATP (NEG002Z; 370 MBq/ml, 222 TBq/mmol) and [α-32P]ATP (NEG003H; 370 MBq/ml, 111 TBq/mmol) were purchased from PerkinElmer Life Sciences.

*Reconstitution of Circadian Oscillation in Vitro*—Unless stated otherwise, the phosphorylation rhythm of KaiC was reconstituted and examined as reported previously (1). Briefly, 3.5 μM KaiC was incubated at 30°C in the presence of 1.2 μM KaiA, 3.5 μM KaiB, and 1 mM ATP in 20 mM Tris (pH 8.0), 150 mM NaCl, 5 mM MgCl₂, and 1 mM DTT. Aliquots of the reaction mixture were taken every 4 h and subjected to SDS-PAGE. The calculated concentrations of the molecules that stayed at the thin-layer chromatography origin and of phosphorylated KaiC were plotted against time.

**Preparation of 32P-Labeled KaiC Hexamers**—32P-Labeled KaiC hexamers were obtained using a previously described method with modifications (19). KaiC hexamers (5–10 mg/ml) were incubated on ice for 24 h in buffer A (20 mM Tris (pH 8.0), 150 mM NaCl, 5 mM MgCl₂, and 1 mM DTT) to remove unbound nucleotides. The eluate was subjected to thin-layer chromatography on PEI-cellulose F plates (Merck) using 0.75M KH₂PO₄ as the mobile phase.32P-Derived signals were detected at the position marked with 5% (v/v) 2-mercaptoethanol to release KaiC-bound nucleotides from the proteins. Samples were spotted onto PEI-cellulose plates and subjected to thin-layer chromatography to separate ATP and ADP using KH₂PO₄ as the mobile phase.

**RESULTS**

*Preparation of 32P-Labeled KaiC without KaiA*—To monitor the autodephosphorylation of KaiC, we prepared 32P-labeled KaiC phosphorylated at Ser-431 and Thr-432. At 30°C, KaiC is markedly phosphorylated in the presence of KaiA (11) and dephosphorylated in its absence (15). For autodephosphorylation assays, however, it is desirable to phosphorylate KaiC without KaiA because residual KaiA might interfere with autodephosphorylation. Ito et al. (19) showed that the ratio of phosphorylated KaiC to total KaiC reached ~0.7 after 30 h of incubation at 4°C, suggesting that incubation at low temperature promotes autophosphorylation without KaiA. We incubated KaiC in the presence of [γ-32P]ATP on ice and monitored
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**FIGURE 1. Preparation of $^{32}$P-labeled KaiC monomers for autodephosphorylation assays.** A, KaiC hexamers were phosphorylated by incubating the samples on ice in the presence of 1 mM [γ-$^{32}$P]ATP. At each time point, an aliquot of the reaction mixture was collected and subjected to SDS-PAGE, followed by Coomassie Brilliant Blue (CBB) staining (upper panel) and autoradiography (lower panel). The positions of phosphorylated (P-KaiC) and non-phosphorylated KaiC are indicated on the left. Each lane was loaded with 10 pmol of KaiC subunits. After 24 h of incubation, ~30% of KaiC was newly phosphorylated based on Coomassie Brilliant Blue staining (upper panel). The KaiC-specific radioactive signals increased together with the increasing intensity of phosphorylated KaiC bands (lower panel). Approximately 0.3 mol of phosphate was incorporated into 1 mol of KaiC based on comparisons with serial dilutions of 1 mM [γ-$^{32}$P]ATP with the same specific activity as the radioactive ATP that had been bound to KaiC. Next, we eliminated KaiC-bound [γ-$^{32}$P]ATP to ensure that only signals derived from $^{32}$P-labeled KaiC were examined during the autodephosphorylation assay. We expected that [γ-$^{32}$P]ATP bound to the subunit interface would be released when $^{32}$P-labeled KaiC hexamers were dissociated into monomers. To date, the procedure for the monomerization of *Synechococcus* KaiC hexamers has not been established. Hayashi et al. (23) showed that *Thermosynechococcus* KaiC hexamers dissociated into monomers in the absence of ATP. Therefore, we removed ATP from the reaction mixture and incubated the resulting solution to prepare *Synechococcus* KaiC monomers. Because KaiC formed aggregates in the absence of ATP at temperatures higher than ~10 °C, we incubated the reaction mixture on ice. We added 0.1 mM ADP to stabilize the KaiC monomers during the incubation period. After 24 h of incubation, the KaiC monomers were separated using gel filtration chromatography (Fig. 1C). To verify that the KaiC monomers were functional, we used them to recreate the KaiC phosphorylation rhythm. The KaiC monomers were allowed to re-form hexamers by replacing 0.1 mM ADP with 1 mM ATP, and the resulting KaiC hexamers were incubated at 30 °C in the presence of KaiA and KaiB. It took <5 min for KaiC hexamers to form from monomers (supplemental Fig. S5). As shown in Fig. 1C, this approach did not affect the KaiC phosphorylation rhythm. As expected, the radioactive ATP that had been bound to KaiC was almost completely removed with this procedure (ATP-derived signals at time 0 in Fig. 2A).

**Assay of Autodephosphorylation**—Autodephosphorylation was initiated by adding 1 mM nonradioactive ATP to $^{32}$P-labeled KaiC monomers and incubating the resulting $^{32}$P-labeled KaiC hexamers at 30 °C. At the indicated time points, an aliquot of the reaction mixture was withdrawn and subjected to analysis. Surprisingly, we observed a transient elevation in the ATP concentration prior to an increase in the P$_i$ levels (Fig. 2A). The sum of the concentrations of these molecules was assumed to be constant throughout the reaction (Fig. 2A). These results suggest that KaiC autodephosphorylation occurs through a previously unknown two-step mechanism. First, KaiC forms ATP as an intermediate during autodephosphorylation using ADP as a phosphate acceptor. This reaction can be regarded as the reversal of the autophosphorylation reaction. Subsequently, $^{32}$P-ATP is hydrolyzed by KaiC ATPase to form $^{32}$P$_i$. We normalized the total concentration of these molecules to 1 and plotted the relative concentration of each molecule over time (Fig. 2B). The small standard deviations observed in four independent experiments indicate that the autodephosphorylation process was highly reproducible.

**Characterization and Quantification of KaiC-bound Nucleotides**—Although the reversal of kinase reactions requires ADP as a phosphate acceptor, no reports have previously shown that KaiC binds ADP. To address this issue, we examined the time-dependent changes in the levels of adenine nucleo-
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We reconstituted KaiC hexamers from monomers in the presence of 1 mM $[^{32}P]$ATP and incubated the samples at 30 °C. The number of adenine nucleotides bound to each KaiC protomer remained nearly constant at two (supplemental Fig. S4), indicating that the nucleotide-binding sites of both CI and CII were occupied. The ratio of ADP to the total bound nucleotides increased, reaching −0.7 after 4 h of incubation (Fig. 2C, closed circles). These results suggest that KaiC hexamers contain both ADP and ATP. We also examined the nucleotides bound to CII-truncated KaiC (Fig. 2C, open circles). In contrast to full-length KaiC, >95% of the nucleotides bound to CII-truncated KaiC were ATP. It is possible that the CII domain is responsible for ADP binding to KaiC.

Kinetic Analysis of KaiC Autodephosphorylation—To test our hypothesis of the reaction mechanism, we modeled the autodephosphorylation process (see Equations 1–3 below) and fit our data to the rate equations derived from Equations 1–3. Autodephosphorylation is an intersubunit reaction that occurs at CII subunit interfaces. To simplify the model, we assumed that the hypothetical reaction unit consisted of two subunits facing each other; one contained a nucleotide-binding site, and the other contained a phosphorylation site. We defined KaiCa as the subunit containing the nucleotide-binding site, and KaiCb as the subunit containing the $^{32}P$-labeled phosphorylation site (Fig. 3).

KaiC-bound ADP should be a product of the hydrolysis of bound ATP and should not be incorporated from the reaction mixture because the reaction mixture contained little ADP. We first modeled the hydrolysis of nonradioactive ATP bound to KaiCa, which provides the phosphate acceptor ADP. $k_1$ is the rate constant of ATP hydrolysis, and $k_2$ is the overall rate constant of the opposing reaction, including the reversal of ATP hydrolysis, the release of products, and the incorporation of nonradioactive ATP (Equation 1).

$$KaiC_\alpha\cdot ATP \rightleftharpoons KaiC_\alpha\cdot ADP\cdot Pi \quad (Eq. 1)$$

We modeled the transfer of phosphate from $^{32}P$-labeled KaiCb to ADP on KaiCa. We expected that the amount of both $^{32}P$-labeled KaiCb and KaiCa-bound ADP would affect the rate of this process (Equation 2).

$$^{32}P\text{-KaiCb} + KaiC_\alpha\cdot ADP \rightleftharpoons KaiC_\beta + KaiC_\alpha\cdot [^{32}P]ATP \quad (Eq. 2)$$

$[^{32}P]$ATP is hydrolyzed to form $^{32}P_i$ (Equation 3). The forward and reverse rate constants of ATP hydrolysis are $k_3$ and $k_{-3}$, respectively. The reincorporation of $[^{32}P]$ATP into the KaiC

KaiC-bound nucleotides were detected by autoradiography. The ratio of ADP bound to full-length KaiC (closed circles) or CII-truncated KaiC (open circles) relative to the total bound nucleotides was plotted against time. Data represent means ± S.D. from three or four independent experiments. Although we could not discriminate between CI-bound ADP and CI-bound ATP in this experiment, the CII domain was shown to be responsible for ADP binding.
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FIGURE 3. Schematic model of KaiC autodephosphorylation. KaiC autodephosphorylation occurs at subunit interfaces between CII domains in KaiC hexamers. We assumed that a hypothetical reaction unit consists of two subunits, KaiCa and KaiCb, which contain a nucleotide-binding site and a phosphorylation site, respectively. Because we assayed autodephosphorylation using $^{32}$P-labeled KaiC, the products of autodephosphorylation were detected as radioactive ADP at time 0 was 0.10 by subtracting the amount of ADP bound nucleotides and phosphorylation sites (Equation 2 and 3). The phosphate acceptor ADP, which is required for the reversal of autophosphorylation, is generated by the hydrolysis of nonradioactive ATP prebound to KaiC (Equation 1).

TABLE 1

| Kinetic parameters for KaiC autodephosphorylation | Rate constant | Value | Lower bound | Upper bound |
|---------------------------------------------------|---------------|-------|-------------|-------------|
| $k_1$ and $k_{-1}$                                | $1.19$        | $1.16$ | $1.22$      | $1.22$      |
| $k_{-2}$                                          | $0.793$       | $0.755$ | $0.866$    | $0.866$    |
| $k_3$                                             | $0.00738$     | $0.00959$ | $0.00935$ |

$^a$ Range of parameters is for fits within 20% increase in $\chi^2$ values.

$^b$ Parameters were constrained to the same value during the global fitting process.

$^c$ Only upper limits of these parameters were defined.

We performed analyses using Global Kinetic Explorer (21), a program that allows for the numeric integration of rate equations and the fitting of experimental data based on nonlinear regression analysis. We set the initial values of $^{32}$P-labeled KaiC, and the sum of nonradioactive KaiC-bound nucleotides to 1 and assumed that the initial values of $^{32}$P-ATP and $^{32}$Pi were 0 (Fig. 2B). We estimated that the ratio of KaiCa-bound ADP at time 0 was 0.10 by subtracting the amount of ADP bound to CII-truncated KaiC (Fig. 2C, open circles) from the amount bound to full-length KaiC (Fig. 2C, closed circles). We allowed $k_1$ and $k_2$ to float together, whereas $k_{-1}$ and $k_{-2}$ varied independently. The solid lines in Fig. 2B represent the best fit to the data of Equations 1–3, the $\chi^2$ value of which is 76.4 with 91 degrees of freedom. The rate constants and associated errors for the parameters calculated using FitSpace Explorer (22) are shown in Table 1. The forward rate constants are well constrained by the experimental data. Reverse rate constants of $k_{-1}$ and $k_{-2}$ cannot be accurately resolved on the time scale of the experiment, and only the upper limits are defined.

DISCUSSION

We have shown that KaiC undergoes autodephosphorylation via a previously unknown mechanism that differs from the reactions catalyzed by standard Ser/Thr and Tyr phosphatases (16, 17). This mechanism includes generation of an ATP intermediate, followed by hydrolysis of this molecule (Fig. 3). The former step is regarded as the reversal of autophosphorylation. Previously, we (18) and another group (24) proposed that the phosphorylation cycle of KaiC was understood as the sequential transition among the four phosphorylation states derived from Ser-431 and Thr-432. This model was insufficient to explain the phosphorylation cycle because two independent autokinase and autophosphatase activities were assumed to exist in KaiC. In this study, we have demonstrated that both the phosphorylation and dephosphorylation of KaiC are mediated by a single phosphotransfer reaction catalyzed by the active site of ATP hydrolysis in CII. On the basis of our findings, we propose a revision of the previous model: the KaiC phosphorylation rhythm is sustained by a periodical shift in the equilibrium of the intersubunit phosphate transfer reaction between KaiC-bound nucleotides and phosphorylation sites (Equation 2 and Fig. 3).

It is possible that the direction in which the intersubunit phosphate transfer reaction proceeds is determined by KaiA, which functions as an autokinase activator. The reaction favors ATP formation in the absence of KaiA at 30 °C. In the presence of KaiA, the reaction proceeds to phosphorylated KaiC formation (11). The local structure of the active site may be affected by the presence or absence of KaiA, which determines the direction of phosphate transfer. In agreement with this idea, Kim et al. (25) suggested that KaiA regulated the orientation of ATP bound at the active site through a structural change in a looped segment near the C terminus. KaiB inactivates KaiA by forming a ternary complex with KaiA and Ser-431-phosphorylated KaiC (18), which should result in a circadian change in the direction of phosphate transfer.

We previously proposed that the phosphorylation cycle was coupled with the ATPase activity of KaiC (9). The precise relationship was not elucidated because the autodephosphorylation mechanism was not understood correctly. Now, then, is the KaiC phosphorylation cycle regulated by the ATPase? The ATPase activity of CII has two critical roles: one is the degradation of the ATP intermediate of autophosphorylation and the other is providing ADP, a substrate for the reversal of the autokinase reaction. We believe that ADP bound to the active site was not incorporated from the reaction mixture but was a product of the hydrolysis of bound ATP because the reaction mixture contained little ADP, if any. In agreement with this idea, Rust et al. (26) reported that increasing the ADP/ATP ratio in the reaction mixture did not affect autodephosphorylation. It is possible that ADP is not released immediately after ATP hydrolysis, which suggests that ADP release is the rate-
limiting step in the CII ATPase reaction. The ATPase activity of CII should regulate the phosphorylation cycle as a partial reaction of autodephosphorylation, whereas the ATPase activity of CI may function as a pacemaker of circadian oscillation (9).

We anticipate that other as-yet unknown proteins that catalyze both forward and reverse kinase reactions exist to regulate reversible or cyclic physiological processes. One candidate is *E. coli* AceK, which exhibits kinase, phosphatase, and ATPase activities (27). Although direct evidence has not yet been reported, the phosphatase activity of AceK has been postulated to result from a reversal of the kinase reaction (27). AceK phosphorylates and dephosphorylates isocitrate dehydrogenase, reversibly controlling the flux of metabolites at branching points in metabolic pathways (27).

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