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Effect of pH on the cyanobacterial circadian oscillator in vitro

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The cyanobacterial clock protein KaiC exhibits robust 24-h oscillation of phosphorylation when incubated with KaiA, KaiB, and ATP in vitro. This study shows that the period of the in vitro phosphorylation rhythm of KaiC was correlated with solution pH, varying from 15 h at pH 6.5–36 h at pH 8.5, without affecting the period's temperature compensation. The solution pH altered the autophosphorylation and autodephosphorylation of KaiC and the effect of KaiB on KaiC but had little effect on its ATPase activity. It also modified the surface charge of the interface between two ATPase domains in KaiC, thereby affecting the autophosphorylation and autodephosphorylation activity of this protein via interdomain communication. These findings not only reveal key biochemical properties of the Kai oscillator but also provide insight into its evolutionary adaptation to environmental changes in cyanobacteria.

The circadian clock is an intracellular timing mechanism that coordinates the lives of living organisms with daily environmental changes[1]. To function as autonomous time-keeping devices, circadian clocks have three prominent characteristics: a free-running rhythm with a period of approximately 24 h, temperature compensation of the period, and entrainment to environmental cycles. The circadian clock of cyanobacteria, particularly in the *Synechococcus elongatus* PCC 7942 strain (hereafter referred to as *Synechococcus*), has been extensively studied as it serves as a simple and useful model for understanding the fundamental principles of circadian clocks. This model can be reconstituted in vitro[2] by incubating the core protein KaiC with KaiA, KaiB, and adenosine triphosphate (ATP), resulting in a 24-h rhythm. KaiC exhibits circadian rhythms in ATPase activity, phosphorylation, and interactions with KaiA and KaiB[2–4].

KaiC, a duplicate P-loop ATPase belonging to the RecA superfamily, consists of two domains, N-terminal CI and C-terminal CII, and forms an ATP-dependent hexametric ring[5–7]. The ATPase activity of KaiC is extremely low (10–15 ATPs per day per KaiC molecule at 30 °C), and even in the absence of KaiA and KaiB it exhibits temperature compensation, and correlation with a frequency (reciprocal of the period) of the rhythm[3,8,9]. Therefore, it is thought that KaiC functions as a circadian pacemaker that determines period and temperature compensation. In addition to ATPase activity, KaiC exhibits autophosphorylation and autodephosphorylation activities[10,11]. The two autophosphorylation sites, serine 431 and threonine 432, are located at the subunit interface of CII[12,13], which harbors both ATPase and autophosphorylation activities, while the CI domain exhibits only ATPase activity[3,8]. KaiA and KaiB control the phosphorylation state of KaiC. Specifically, KaiA enhances the autophosphorylation of KaiC[14], whereas KaiB inhibits the effect of KaiA, allowing KaiC to dephosphorylate itself[15,16]. Therefore, in the presence of KaiA and KaiB, the phosphorylation state of KaiC can generate a robust and stable circadian oscillation.

Solution pH alters the surface charge of proteins, thereby affecting several of their properties, such as biochemical activity and stability. Although it has been shown that solution pH also modifies the properties of many P-loop ATPases[17–20], its effect on KaiC and the Kai-protein-based circadian oscillator have not been elucidated yet. In this study, we showed that buffer pH altered the periods of the in vitro phosphorylation rhythm of KaiC without affecting the period's temperature compensation. Specifically, pH modified the autophosphorylation and autodephosphorylation of KaiC and the effect of KaiB on this protein, while it did not affect its ATPase activity. We suggest that solution pH alters the surface charge of the interface between the two ATPase domains (CI and CII) in KaiC, thereby affecting its autophosphorylation and autodephosphorylation activities.

Results

Solution pH alters the period of the in vitro phosphorylation rhythm of KaiC

Circadian oscillators with Kai proteins have been characterized at pH 8.0 or 7.8 using Tris-HCl buffer as a standard condition (Fig. 1A)[2,9]. To study the effect of buffer pH on them, we investigated the in vitro phosphorylation rhythm of KaiC at pH values ranging from 6.0 to 9.0 using four different buffer solutions, i.e., MES-NaOH, PIPES-NaOH, HEPPS-NaOH, and TAPS-NaOH (Supplementary Table S1, Fig. 1B). At pH 6.0, the

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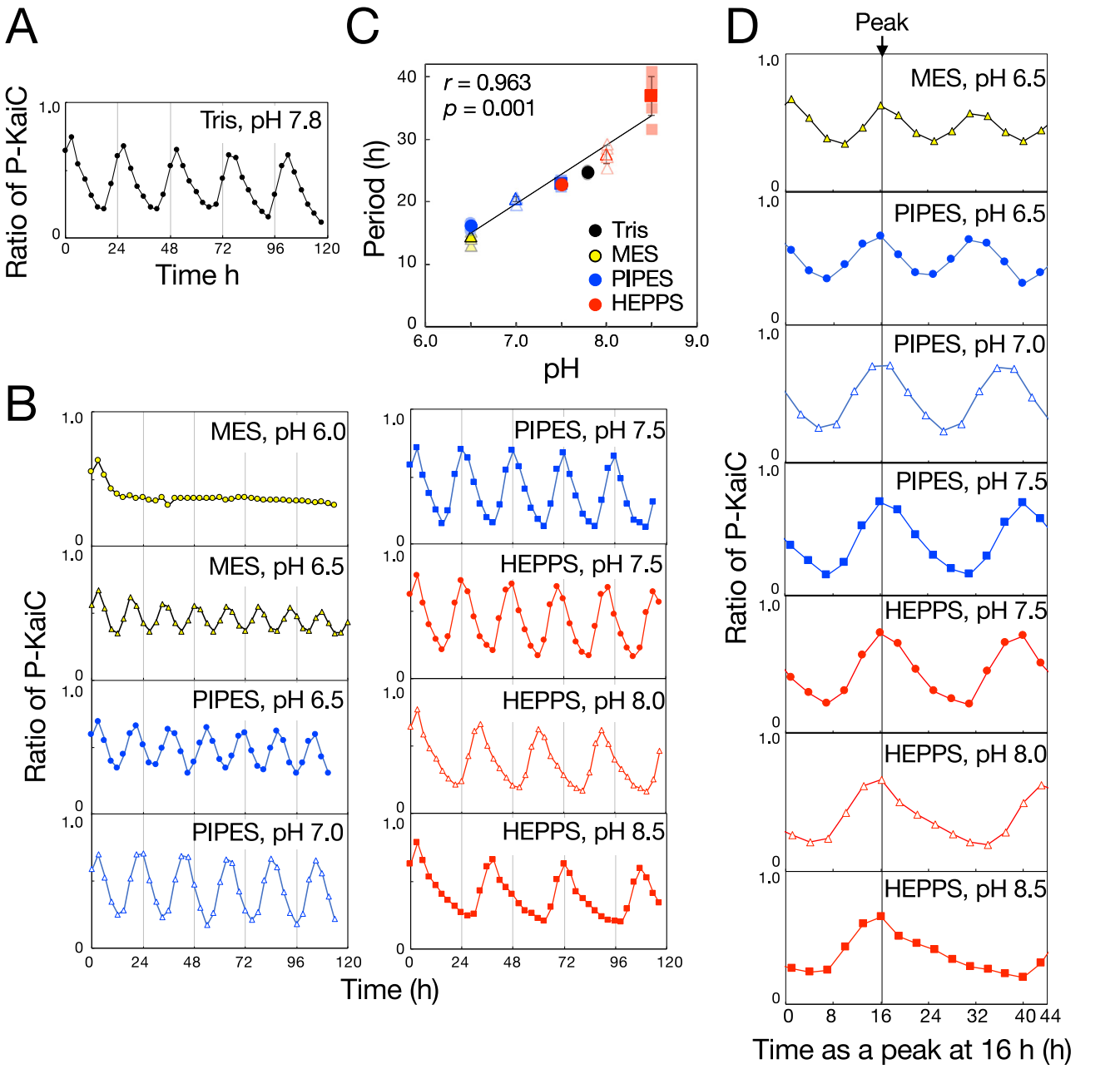

Fig. 1 | Effect of pH on the in vitro phosphorylation rhythm of KaiC. Representative KaiC phosphorylation rhythm under standard conditions (Tris-HCl buffer, pH 7.8) (**A**) or at pH values ranging from 6.0 to 8.5 at 30 °C (**B**). The following reaction buffers were used for the assay: 20 mM Tris-HCl (pH 7.8), 20 mM MES-NaOH (pH 6.0, or 6.5), 20 mM PIPES-NaOH (pH 6.5, 7.0 or 7.5) or 20 mM HEPPS-NaOH (pH 7.5, 8.0 or 8.5), in addition to 150 mM NaCl, 5 mM $MgCl_2$, and 1 mM ATP. The in vitro phosphorylation rhythm of KaiC at pH 9.0 obtained using TAPS-NaOH buffer is shown in Supplementary Fig. S2. **C** Period of the in vitro phosphorylation rhythm of KaiC, plotted against buffer solution pH. *r and p* indicate the correlation coefficient and *P* value, respectively. Values are represented as means ± SD. The sample sizes (*n*) are shown in Supplementary Table. S1. **D** The in vitro phosphorylation rhythm of KaiC with the second peak aligned at 16 h.

phosphorylation ratio of KaiC increased during the first 4 h of incubation, after which it decreased and remained at approximately 0.5. Between pH 6.5 and 8.5, the phosphorylation state of KaiC exhibited rhythms with periods ranging from 15 h (pH 6.5) to 36 h (pH 8.5) (Fig. 1B). With1.0-increases in pH, these periods were extended by 9.5 h, which indicated a correlation between their length and buffer pH (Fig. 1C). It was observed that as the buffer pH was increased, the dephosphorylation phase of the KaiC phosphorylation rhythm became longer (Fig. 1D). In different buffer solutions with the same pH values (MES or PIPES buffer at pH 6.5, and PIPES or HEPPS buffer at pH 7.5), the periods and amplitudes of the KaiC phosphorylation rhythm were the same, indicating that it was not the composition of the buffer but its pH that affected rhythm behavior. In addition, the KaiC phosphorylation rhythm stably persisted for 5 days at pH values ranging from 6.5 to 8.5, indicating that buffer pH did not affect the stability of KaiA, KaiB, and KaiC, but it did affect the amplitude as well as the period length of the KaiC phosphorylation rhythm (Supplementary Fig. S1). At pH values ranging from 6.5 to 7.5, the higher the pH, the higher the amplitude. Above pH 7.5, the amplitudes remained constant, but the periods became longer, which was indicative of the multifaceted effects of buffer pH on Kai-protein-based oscillators. At pH 9.0, only one of seven different Kai protein preparations exhibited an oscillation with a period of ~44 h, while the remaining six were unstable in oscillation (Supplementary Fig. S2).

Temperature compensation is a key property of circadian clocks[21], with the ratio of the period at a 10 °C difference (Q_{10}) being 0.8–1.2 much closer

Table 1 | Period of the in vitro phosphorylation rhythm of KaiC at 25 °C, 30 °C, 35 °C and 40 °C

Buffer	pH	25 °C hours ± SD (*n*)[a]	30 °C hours ± SD (*n*)	35 °C hours ± SD (*n*)	40 °C hours ± SD (*n*)	Q_{10}
PIPES	6.5	AR (3)	16.1 ± 0.6 (6)	18.6 ± 0.1 (5)	19.0 ± 0.1 (3)	0.85
PIPES	7.5	22.7 ± 0.2 (3)	22.9 ± 0.4 (6)	22.6 ± 0.2 (5)	22.5 ± 0.3 (4)	1.01
HEPPS	8.5	38.8 ± 0.7 (3)	36.9 ± 3.1 (6)	42.4 ± 3.7 (5)	AR (3)	0.93

[a]Number of experiments.

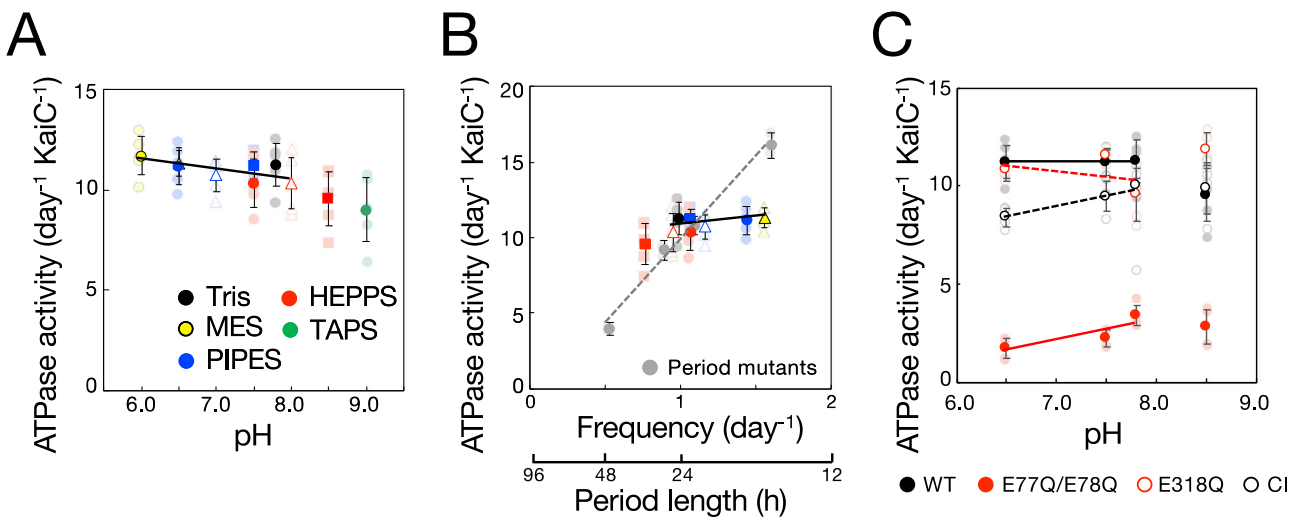

Fig. 2 | Effect of pH on the ATPase activities of KaiC at 30 °C. ATPase activities of KaiC plotted against buffer solution pH (**A**) and frequencies of the in vitro phosphorylation rhythm of KaiC under the pH conditions used for the assay (**B**). The ATPase activities and frequencies of short-period (F470Y and S157P) and long-period (A251V and T42S) mutants of KaiC under standard conditions (Tris buffer, pH 7.8) are also shown in (**B**) (gray circle). **C** ATPase activities of KaiC-WT, KaiC-E77QE78Q, KaiC-E318Q, and KaiC-CI at pH 6.5 (PIPES), pH 7.5 (PIPES), pH 7.8 (Tris-NaOH), and pH 8.5 (HEPPS). In the absence of KaiA and KaiB, KaiC was incubated at 30 °C for 72 h (KaiC-WT) or 48 h (mutant KaiC), and data after 24 h were used for analysis ($n = 5$ for KaiC-WT; $n = 3$ for F470Y, S157P, A251V and T42S; $n = 4$ for KaiC-E77QE78Q, KaiC-E318Q and KaiC-CI). Values are presented as means ± SD.

to 1 than what would be expected from the temperature dependence of normal biochemical reactions[1]. We investigated the effect of buffer pH on temperature compensation for the KaiC phosphorylation rhythm. The obtained Q_{10} values were 0.85, 1.01, and 0.93 at pH 6.5, pH 7.5, and pH 8.5, respectively, indicating that the period of the KaiC phosphorylation rhythm was temperature-compensated regardless of pH (Table 1, Supplementary Fig. S3). The oscillation of KaiC phosphorylation was not detected at pH 6.5 and 25 °C and was unstable at pH 8.5 and 40 °C, suggesting that buffer pH affects the temperature range within which KaiC phosphorylation oscillates.

The ATPase activity of KaiC is stable against buffer pH

It has been shown that the frequency (reciprocal of the period) of circadian oscillations reconstituted with Kai proteins correlates with the ATPase activity of KaiC[3,9]. The period of the in vitro phosphorylation rhythm of KaiC was correlated with solution pH (Fig. 1C). In this study, we investigated whether KaiC ATPase activity varied with buffer pH. The results showed that KaiC ATPase activity tended to decrease with increasing buffer pH, and it was 15% lower at pH 8.5 than at pH 6.5 (Fig. 2A). A correlation was observed between the frequency of oscillation and the ATPase activity of KaiC due to solution pH variation (Fig. 2B). However, the effect of pH on ATPase activity had a greater impact on the period than the effects of mutations in KaiC.

KaiC consists of two ATPase domains, N-terminal CI and C-terminal CII, and the ATPase activity of CI mainly contributes to the total ATPase activity of KaiC. To determine whether buffer pH specifically affected the ATPase activity of the CI or CII domains, we investigated the ATPase activity of a truncated version of KaiC (KaiC-CI), which consists solely of the N-terminal domain, and KaiC mutants with mutations in the putative catalytic glutamate residues of ATP hydrolysis in either the CI or CII domain (E77QE78Q or E318Q) at pH 6.5, 7.5, and 8.5. At pH 7.5, the ATPase activity of E318Q was close to that of WT, while the activities of KaiC-CI and E77QE78Q were approximately 80% and 20% of WT, respectively (Fig. 2C). At pH values ranging from 6.5 to 8.5, the ATPase activities of E77QE78Q, E318Q, and KaiC-CI increased slightly with buffer pH, indicating that the ATPase activity of these KaiC mutants was not significantly affected by pH changes. These results showed that the ATPase activities of CI, CII, and full-length KaiC are relatively stable across different solution pH values, suggesting that other factors influence the period of the in vitro phosphorylation rhythm based on buffer pH.

Buffer pH modifies the autophosphorylation and autodephosphorylation of KaiC

We investigated the reasons for the variation in the period of the KaiC phosphorylation rhythm depending on buffer pH. We found that the dephosphorylation phase of the KaiC phosphorylation rhythm became longer as buffer pH increased (Fig. 1D). Two phosphorylation sites, serine 431 and threonine 432, located in the CII domain and ATPase activity of this domain, are crucial for the autokinase and autophosphatase activities of KaiC. In the presence of KaiA and KaiB, these activities switch alternately, resulting in the circadian oscillation of the phosphorylation status of KaiC[22]. Therefore, because buffer pH alters the period of the KaiC phosphorylation rhythm, it is possible that the autokinase and autophosphatase activities of KaiC, and/or the interaction of KaiC with KaiA and KaiB, are affected by pH.

First, to investigate the effect of buffer pH on the balance between the autophosphatase and autokinase activities, we prepared highly phosphorylated and dephosphorylated KaiC via incubation at 4 °C and 30 °C, respectively[10]. Highly phosphorylated KaiC was incubated at 30 °C in

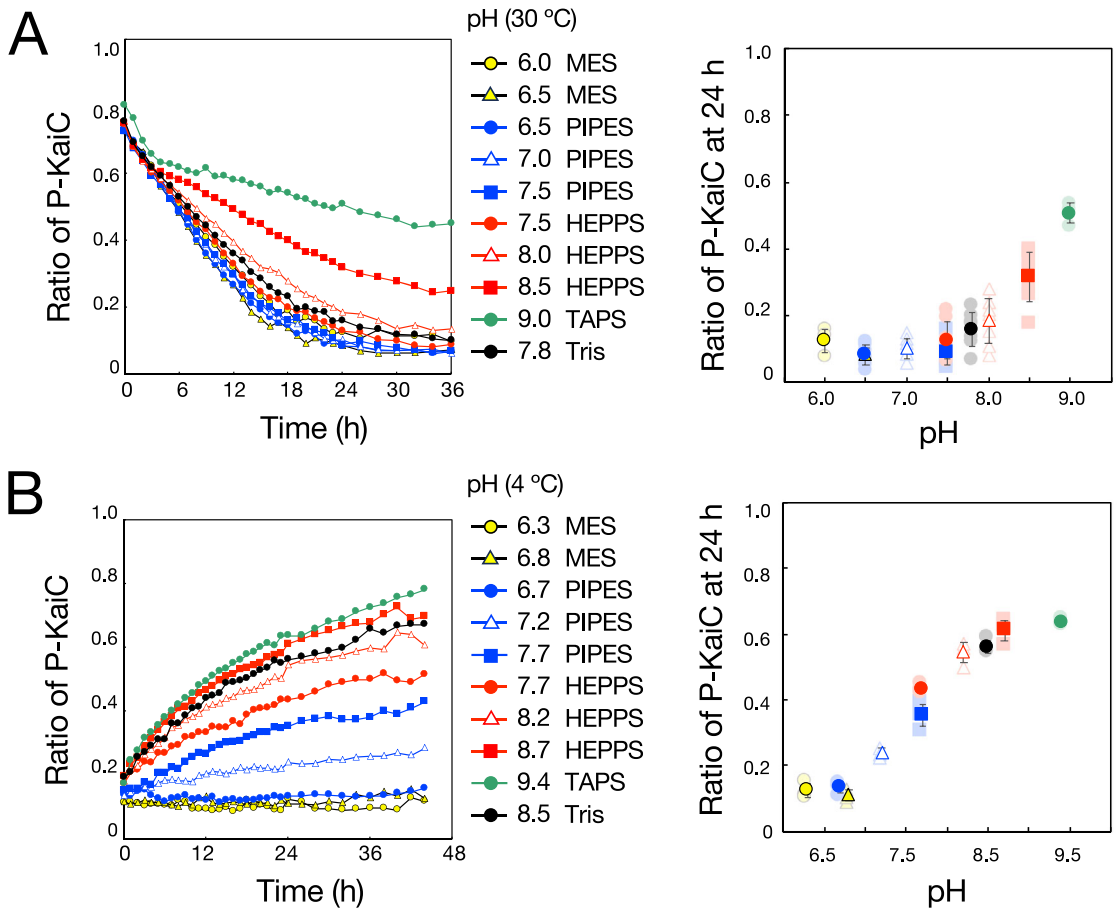

Fig. 3 | Effect of pH on the dephosphorylation and phosphorylation of KaiC without KaiA and KaiB. A Dephosphorylation of KaiC under pH values ranging from 6.0 to 9.0 (left) and the phosphorylation ratio of KaiC at 24 h shown in the left panel (right). Highly phosphorylated KaiC was dephosphorylated by incubation at 30 °C. **B** Phosphorylation of KaiC under pH values ranging from 6.3 to 9.4 (left) and the phosphorylation ratio of KaiC at 24 h shown in the left panel (right). Highly dephosphorylated KaiC was phosphorylated by incubation at 4 °C. The buffer solution pH was adjusted at 30 °C, and pH values were determined at 4 °C. The values for the phosphorylation status of KaiC are presented as means in this figure and as means ± SD in Supplementary Fig. S4. In (**A**), the sample sizes are as follows : $n = 3$ for pH 6.0 (MES) and pH 6.5 (MES); $n = 7$ for pH 6.5 (PIPES), pH 7.0 (PIPES), pH 7.5 (PIPES), pH 7.5 (HEPPS), pH 8.0 (HEPPS), pH 8.5 (HEPPS), and pH 7.8 (Tris); and $n = 4$ for pH 9.0 (TAPS). In (**B**), $n = 3$.

buffers with pH ranging from 6.5 to 9.0 to observe dephosphorylation. At pH 6.0–7.0, dephosphorylation was almost the same, whereas at pH 7.5 and above, the dephosphorylation reaction slowed down as the buffer pH increased (Fig. 3A, Supplementary Figs. S4A and S5A). At 4 °C, dephosphorylated KaiC was phosphorylated across buffer pH values ranging from 6.7 to 9.4, and phosphorylation occurred more rapidly as the buffer pH increased. This phosphorylation process did not occur at pH <6.5 (Fig. 3B, Supplementary Figs. S4B and S5B). These results indicated that buffer pH affected the balance between the autophosphatase and autokinase activities of KaiC.

Buffer pH modifies the KaiB–KaiC interaction

Next, we investigated the effect of buffer pH on the interaction of KaiC with KaiA or KaiB. KaiA interacts with the CII domain of KaiC via a segment known as the A-loop, which is located near the enzymatic active site of the CII domain, and promotes autophosphorylation by facilitating nucleotide exchange in the ATPase active site of the CII domain[11,23]. To determine whether buffer pH affected the KaiA–KaiC interaction, we examined the phosphorylation of KaiC with the addition of KaiA at pH 6.5, 7.5, or 8.5 (Fig. 4A). Adding KaiA to dephosphorylated KaiC promoted the autophosphorylation of this protein in a similar manner at any of the tested buffer pH conditions, indicating that pH had little effect on the function of KaiA on KaiC.

KaiB binds to phosphorylated KaiC at the B-loops of its CI domain[24]. To examine the effect of KaiB on KaiC at different buffer pH values, we added KaiB to phosphorylated KaiC at pH 6.5, 7.5, 8.5, and 9.0 then examined the dephosphorylation of KaiC (Fig. 4B, C). The addition of KaiB had little effect on KaiC dephosphorylation at pH 6.5 and 7.5. However, at pH 8.5, it enhanced the process after 12 h, with the maximum enhancement being observed at pH 9.0. It has previously been shown that the addition of KaiB does not affect the phosphorylation status of KaiC[15] but affects the dephosphorylation of this protein by inhibiting the effect of KaiA. Notably, a direct effect of KaiB on the phosphorylation of KaiC was not previously known but was detected here under high buffer pH conditions.

Based on the results shown in Fig. 1, higher buffer pH values resulted in a longer dephosphorylation phase. The pS/T (S431-phosphorylated) and pS/pT (S431- and T432-phosphorylated) forms of KaiC have been reported to increase during the dephosphorylation phase in the KaiC phosphorylation rhythm, with KaiB predominantly binding to either of them[24]. Here, we investigated the ratios of one nonphosphorylated (S/T) and three phosphorylated (pS/T, pS/pT, and S/pT) forms of KaiC during dephosphorylation at pH 6.5, 7.5, and 8.5 (Supplementary Fig. S6A) and found that the ratios of the pS/T and pS/pT forms increased with pH (Supplementary Fig. S6B). Therefore, at higher pH values, the binding of KaiB to KaiC would be stronger and the dephosphorylation phase in the KaiC phosphorylation rhythm would be longer.

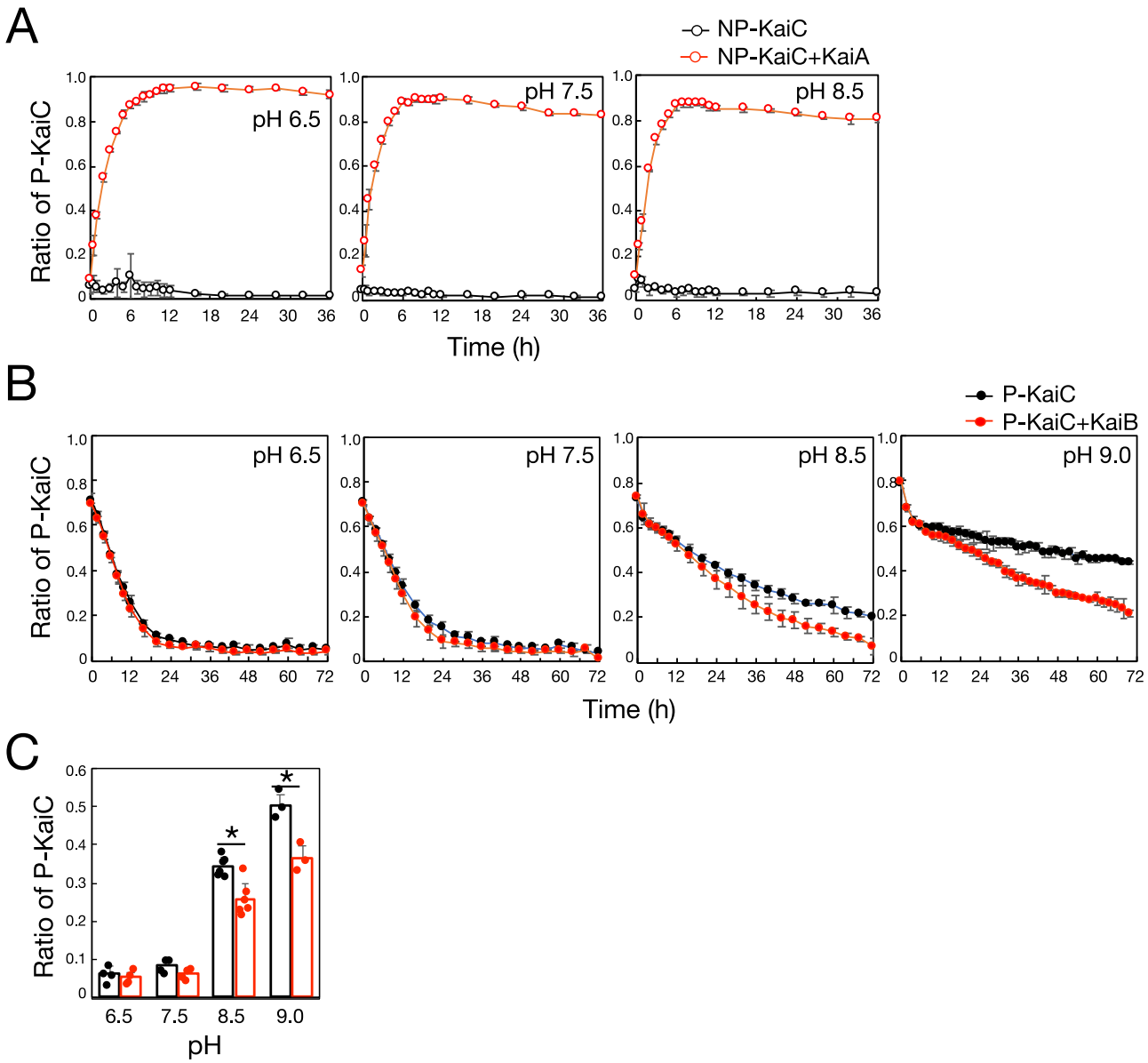

Fig. 4 | Effect of pH on the function of KaiA or KaiB in the phosphorylation of KaiC. A KaiC phosphorylation by KaiA at pH 6.5 (PIPES), pH 7.5 (PIPES), or pH 8.5 (HEPPS). **B** Effect of KaiB on KaiC dephosphorylation at pH 6.5 (PIPES), pH 7.5 (PIPES), pH 8.5 (HEPPS), and pH 9.0 (TAPS). **C** Phosphorylation ratio of KaiC at 36 h in (**B**), showing KaiC (black) or KaiC with KaiB (red). Values are presented as means ± SD ($n > 4$). *$p < 0.01$. In (**A**), the sample sizes are as follows : $n = 3$. In (**B**), $n = 4$ for pH 6.5 and pH 7.5; $n = 6$ for pH 8.5; $n = 3$ for pH 9.0).

Surface electrostatic potential

To understand the structural basis of the responses to pH described above, we calculated the electrostatic potentials of Kai proteins over a wide pH range. In particular, we focused on the interface of CI-CII domains in KaiC and that of the KaiC–KaiB complex (Fig. 5 and Supplementary Fig. S7). On the interface of KaiC–KaiB, KaiC was negatively charged and KaiB was positively charged, suggesting that electrostatic interactions contributed to the interaction of these proteins. Around the neutral pH range (pH 6–9), the charged states of the interface did not change (Fig. 5B), indicating that the response of KaiC activities to pH did not depend on the KaiB–KaiC interaction.

Conversely, the interface of the CI-CII domains in KaiC showed a more complicated distribution of charges. Importantly, on the surface of CI, positively charged patches were detected at pH 6 (indicated by an arrow in pH 7.5 panel, Fig. 5A), their intensities gradually decreased as pH increased, and they finally disappeared at pH 9. This behavior implied that the interaction between domains in KaiC influenced protein function. In general, amino acid residues with a pKa value close to neutral pH are typically either Cys or His. However, no such residues were present in the interface of CI and CII in KaiC. In the analysis, the pKa value of the side chain of lysine at residue 232 of KaiC (K232) was assigned at a neutral pH, implying that the residue was responsible for the response to pH (Supplementary Fig. S8).

To determine the relationship between buffer pH and lysine charge at residue 232 of KaiC, we investigated the KaiC phosphorylation rhythm of K232N and K232F, period mutants of KaiC with K232 replaced by asparagine or phenylalanine. We found that the effect of pH on the period of K232N and K232F was stronger and weaker compared with that on the period of WT, respectively (Supplementary Fig. S9). The periods of K232N, K232F, and WT at pH 7.5 were 1.61, 1.11, and 1.18 times longer than those at pH 6.5. These results suggested that the lysine charge at residue 232 of KaiC is involved in the pH effect on KaiC activities. It should be noted that in this experiment we compared the KaiC phosphorylation rhythm in these two mutants and WT at 40 °C because it was unstable for K232N and K232F

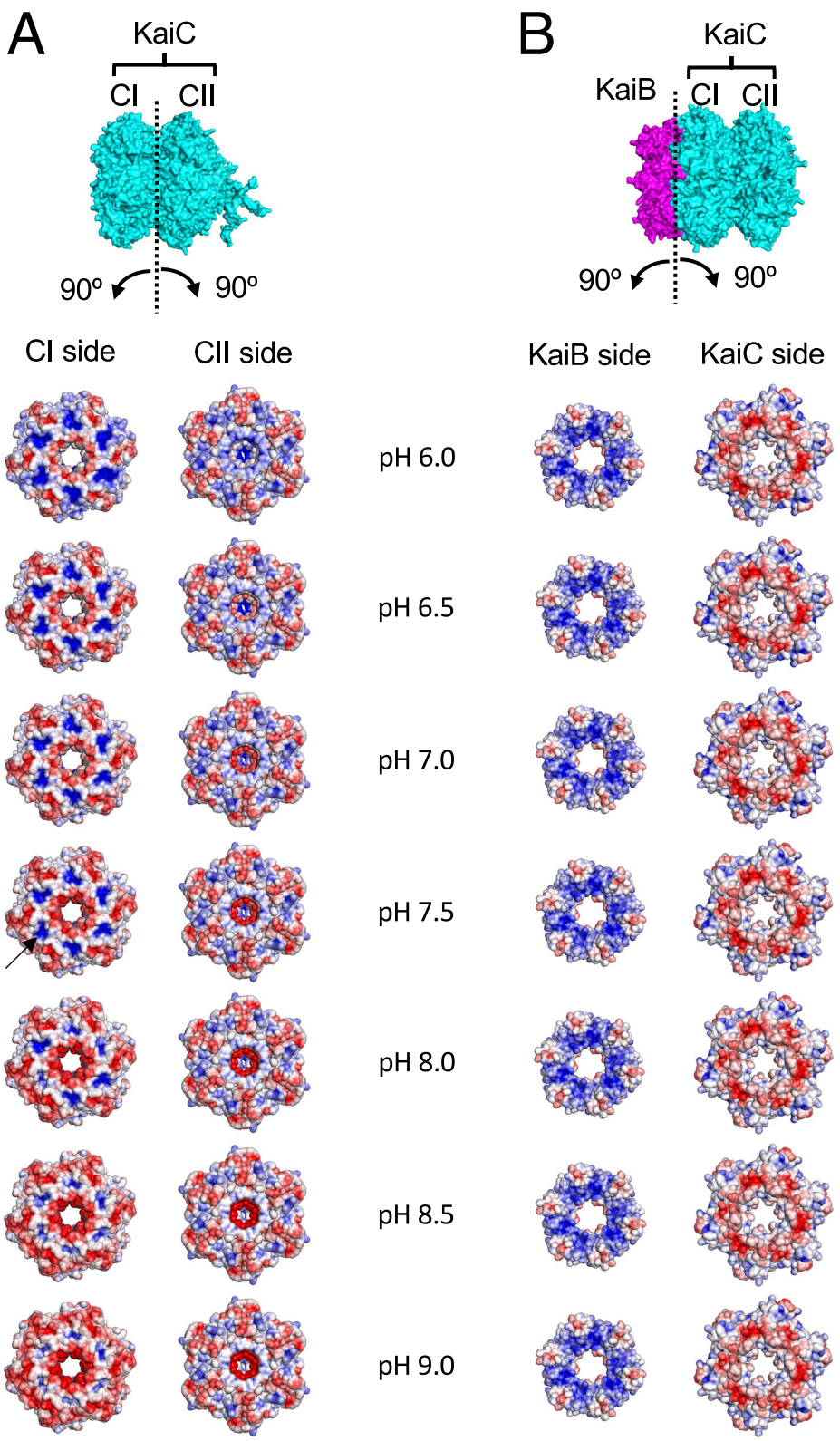

Fig. 5 | Surface electrostatic potential of Kai proteins. Surface electrostatic potential of the interface between the CI and CII domains in KaiC (**A**) and of the KaiC–KaiB complex (**B**) at pH values ranging from 6 to 9. Positively charged (4 kT/e) and negatively charged (−4 kT/e) residues are colored in blue and red, respectively. k is the Boltzmann constant, T is the absolute temperature (T = 310 [K]), and e is the elementary charge. An arrow on the surface of the CI side at pH 7.5 in (**A**) is pointed to one of six symmetric positive patches whose potential gradually decreases as pH increase.

at 30 °C. As observed for WT, the KaiC phosphorylation rhythm of the mutants was not detected at pH 8.5 and 40 °C (Supplementary Fig. S9).

Discussion

In this study, we showed that solution pH altered the periods of the in vitro phosphorylation rhythm of KaiC, which was longer and shorter at high and low pH values, respectively, ranging from 15 h (at pH 6.5) to 36 h (at pH 8.5), regardless of buffer composition (Fig. 1). The results are consistent with a recent report on the relationship between the period of the phosphorylation rhythm of KaiC and pH[25]. Buffer pH had little effect on the ATPase activity of KaiC (Fig. 2) but altered the autophosphorylation or autodephosphorylation of this protein (Fig. 3) and the KaiB–KaiC interaction (Fig. 4). Our findings reveal a new mechanism controlling the period of Kai-protein-based circadian oscillators.

In the absence of KaiA and KaiB, KaiC tended to be phosphorylated and dephosphorylated at high and low pH values, respectively (Fig. 3), which indicated that high pH slows the dephosphorylation of KaiC and prolongs the circadian period. The autophosphorylation and autodephosphorylation of KaiC are mediated by a reversible phosphotransfer reaction between KaiC-bound nucleotides and the hydroxyl group of S431/T432 that is catalyzed by the active site of ATP hydrolysis located in the subunit boundaries of CII[10,11,26]. Therefore, buffer pH may affect the balance of the phosphotransfer reaction in terms of its direction at the two phosphorylation sites in CII. In the presence of KaiA, KaiC autophosphorylation is promoted[14,27]. Our results show that the effect of KaiA was not affected by pH (Fig. 4). Even in the absence of KaiA, the phosphorylation state of KaiC increased at higher pH (Fig. 3B), which was observed at 4 °C under conditions where KaiC autophosphorylation activity exceeded its dephosphorylation activity. The effect of pH on KaiC phosphorylation will require further studies measuring autophosphorylation and dephosphorylation separately. In the presence of KaiA and KaiB, the dephosphorylation phase of KaiC lengthened at higher pH, resulting in a longer period (Fig.1). This can be explained by the experimental data showing that KaiC autodephosphorylation was slower at higher pH (Fig. 3A). The length of the phosphorylation phase was little affected by pH, possibly because the effect of KaiA did not change with pH (Fig. 4).

High pH inhibited the dephosphorylation of KaiC and strengthened the effect of KaiB on KaiC (Fig. 4). This indicated that, in the presence of KaiA and KaiB, high pH slowed down the switching from KaiC dephosphorylation to phosphorylation in the reconstituted system and prolonged the protein's circadian period. Although it is possible that buffer pH alters the functional state of KaiB, the electrostatic potential of Kai proteins calculated in this study suggested that the response of KaiC activity to pH was independent of the KaiB–KaiC interaction (Fig. 5B). The variations in the phosphorylation status of KaiC caused by buffer pH could affect the capacity of KaiB or KaiA to bind to KaiC. KaiB has been shown to bind to the CI domain of KaiC when serine 431 at the CII domain is phosphorylated, and the resulting KaiB–KaiC complex sequesters KaiA on CI[22]. In this study, the pS/T form of KaiC was maintained at high pH (Supplementary Fig. S6). Therefore, high pH may delay the switching to the phosphorylation phase by promoting the binding of KaiB to the CI domain of KaiC, thus inhibiting the function of KaiA that stimulates the autophosphorylation of KaiC. At low pH values, the period and amplitude of the KaiC rhythm are shortened, and the trough of the KaiC phosphorylation rhythm remains high (Fig. 1 and Supplementary Fig. S1). In the KaiABC reconstitution system, many short-period mutants of KaiC have been reported to exhibit low-amplitude rhythms[2,9]. The results of the short-period rhythm induced by low pH also showed low amplitude. This may be due to the weaker binding of KaiB to KaiC at low pH, which facilitates the separation of KaiB from KaiC. KaiABC interactions should be involved in these phenotypes, but further studies are needed to provide a reasonable explanation.

The calculated electrostatic potential of the Kai proteins showed that pH altered the interfacial behavior of the CI-CII domain of KaiC, highlighting the importance of K232 at the interface between these two domains (Fig. 5). If the pH dependence of the interaction between CI and CII is controlled by the charge distribution state of a specific amino acid, such as K232, then it should be possible for parameters such as period and activity to exhibit a sigmoidal pH dependence. Most of the data we presented here showed a linear dependence over a wide pH range. It can be assumed that the pH-dependent contributions from many amino acid residues, including K232, are averaged out, resulting in the linear dependence observed. Since K232 is located at the ATP binding site (Supplementary Fig. S8), this residue could be an indicator of the nucleotide state of CI. Additionally, it would be interesting to analyze the pH dependence, focusing on the residues around the active site of ATP hydrolysis. In the analysis of the electric potential, ATP was removed during calculations because, in general, the electric properties of a ligand are difficult to predict and it is impossible to calculate the pKa values. Therefore, the results represent the electric potential of the transient nucleotide-free state. Nevertheless, in the crystal structure of the KaiC and KaiC-CI domains[8,13], anions are located on the top of the side chain of K232 (Supplementary Fig. S8), which is strong evidence of the presence of a positive charge on K232 and that the interaction between CI and CII is mediated by these anions. Solution pH was shown to alter the surface charge of the interface in KaiC, thereby affecting the autophosphorylation and autodephosphorylation activity of KaiC through interdomain communication.

Compared to the significant variations in the period of the KaiC phosphorylation rhythm caused by buffer pH, the ATPase activities of both the CI and CII domains of KaiC and the full-length WT KaiC were stable against buffer pH (Fig. 2). As pH generally affects the activity and stability of numerous enzymes, including ATPase, it is noteworthy that KaiC maintained a stable ATPase activity for several days at pH values ranging from 6.0 to 9.0. Our results suggested that KaiC is intrinsically stable against pH, in addition to temperature. Given that the CI and CII ATPases communicate with each other through the interface between the CI and CII domains in KaiC[28–31], the variation in surface charge at this interface due to pH changes may affect this interdomain communication. There may be an unknown mechanism that allows KaiC ATPase activity to remain stable despite pH fluctuations.

This study explored the implications of pH-dependent variations in the periodicity of the circadian oscillator of Kai proteins in cyanobacterial cells. The three Kai proteins studied were derived from the cyanobacterium *Synechococcus*. We considered whether the difference in periodicity due to solution pH adjusts the period by compensating for the difference in the KaiA/KaiC ratio in cyanobacterial cells under light and dark conditions. In the dark, the de novo transcription and translation of clock genes in *Synechococcus* cells are abolished, the transcription-translation feedback loop in *kai* genes does not function, and only the Kai-protein-based circadian oscillator functions as the circadian clock[32]. It has been reported that the KaiA/KaiC ratio decreases in cells from light-to-dark conditions[15,33]. In addition, the period of the KaiC rhythm is lengthened by lowering the KaiA concentration[34]. The intercellular pH of *Synechococcus* cells has been reported to be 7.4 and 7.0 in light and dark conditions, respectively[35]. Based on our results and these intracellular pH values, it is likely that the circadian clock period is shorter in the dark than under light conditions. Therefore, it is possible that the shortening of the period induced by low pH compensates for its prolongation caused by the decrease in the KaiA/KaiC ratio in the dark. It has been recently shown that cytosolic pH in the cyanobacterium *Synechocystis* sp. PCC 6803 transiently varies when exposed to light-to-dark and dark-to-light conditions[36]. Such variations in intracellular pH between light and dark conditions may be involved in the circadian clock entrainment in cyanobacterial cells.

In *Synechococcus*, intracellular pH remains between 7.2 and 7.6 under light conditions, even when the external pH range is 5–10[37]. This stability suggests that the period of the circadian rhythm driven by KaiC in these cells remains largely unchanged, as inferred from our results. Consequently, KaiC may not need to evolve mechanisms to strictly maintain constant lengths of the period of the circadian rhythm in response to variations in external pH. While pH affects the period of the Kai protein oscillator, other factors that fluctuate within the cell, such as temperature, magnesium ion concentrations, Kai protein accumulation, and ADP/ATP ratios, do not play significant roles[2,4,34,38–40]. The protein-based circadian clock may have evolved mechanisms to compensate for period length changes due to fluctuations in the intracellular environment. Elucidating the relationship between the circadian period and solution pH contributes to revealing the intrinsic biochemical properties of Kai-protein-based circadian oscillators and how these adapt to variations in the intercellular environment.

Methods

Preparation of Kai proteins

Recombinant KaiA, KaiB, KaiC, and KaiC mutants proteins were expressed in *Escherichia coli* and purified as described previously[9,15,41], with details as follows. Previously constructed pGEX-6P-1 plasmids[14,15], each carrying an individually cloned open reading frame (ORF) of *kaiA*, *kaiB*, or *kaiC* from

Synechococcus elongatus PCC 7942, were used. Each plasmid was separately transformed into *Escherichia coli* BL21 cells to express the corresponding GST-fusion protein. Cells expressing GST-fusion protein were harvested and lysed by sonication in extraction buffer specific to each protein: 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.5 mM EDTA for KaiA; 20 mM Tris-HCl (pH 8.0), 10 mM NaCl, and 0.5 mM EDTA for KaiB; and 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1 mM ATP, and 5 mM $MgCl_2$ for KaiC. After lysis, samples were centrifuged at 24,000 × *g*, and the resulting supernatant was subjected to affinity purification using a glutathione Sepharose 4B column (GE Healthcare). For KaiA and KaiC, the bound GST-fusion proteins were digested with PreScission protease (GE Healthcare) directly on the column after affinity purification. In the case of KaiB, the GST–KaiB fusion protein was eluted with a buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM NaCl, and 20 mM reduced glutathione. The eluate was then desalted using a HiPrep Desalting 26/10 column (Bio-Rad) to remove glutathione, followed by digestion with PreScission protease.

Further purification of KaiA was carried out by sequential Resource Q anion exchange chromatography (90–450 mM NaCl gradient), hydroxyapatite chromatography using a Bio-Scale CHT2-I column (Bio-Rad), and size-exclusion chromatography using a Superdex 200 column (GE Healthcare). Further purification of KaiB was conducted by Resource Q anion exchange chromatography (0–300 mM NaCl gradient), followed by hydroxyapatite chromatography using a Bio-Scale CHT2-I column (Bio-Rad), and size-exclusion chromatography using a Superdex 75 column (GE Healthcare).Further purification of KaiC, the eluate was applied to a HiPrep Sephacryl S300 column (GE Healthcare) followed by Resource Q anion exchange chromatography using a 90–450 mM NaCl gradient.

KaiC assay conditions across various pH levels

The following reaction buffers were used to assess the pH dependence of KaiC activity: 20 mM Tris-HCl (pH 7.8), 20 mM MES-NaOH (pH 6.0, or 6.5), 20 mM PIPES-NaOH (pH 6.5, 7.0 or 7.5) or 20 mM HEPPS-NaOH (pH 7.5, 8.0 or 8.5), 20 mM TAPS-NaOH (pH 9.0). All buffers contained 150 mM NaCl, 5 mM $MgCl_2$, and 1 mM ATP. The pH was adjusted at 30 °C. Before the reaction, the recombinant KaiC protein was passed through Micro BioSpin P-30 columns (Bio-Rad) or a PD MiniTrap G-25 column (cytiva) equilibrated with the reaction buffer. After estimating the KaiC concentration using the Quick Start Bradford Protein Assay (Bio-Rad), the reaction mixture was prepared accordingly.

Reconstitution of in vtro KaiC phosphorylation rhythm

The KaiC phosphorylation rhythm was reconstituted in vitro as previously described[2] and as in detail below. The assay was performed using the reaction buffer described above. The KaiA, KaiB, and KaiC concentrations were 1.2, 3.5, and 3.5 μM, respectively. The samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to separate the phosphorylated forms of KaiC. To determine period, KaiC phosphorylation ratio after 12 h incubation was fitted to a cosine function of time[34].

ATPase assay using ultraperformance liquid chromatography (UPLC)

The ATPase activity of KaiC was measured using an ACQUITY UPLC system (Waters) as in detail below[9]. ADP was separated from ATP on a BEH C18 column (2.1 ID × 50 mm, 1.7 μm) (Waters) at a flow rate of 0.8 mL min^{-1} with a mobile phase of 14 mM ammonium phosphate, 7 mM tetrabutylammonium hydrogen sulfate (pH 8.5), and 15% (vol/vol) acetonitrile. ADP concentrations were calculated from the corresponding peak areas.

Autodephosphorylation and autophosphorylation of KaiC without KaiA and KaiB

To investigate the autodephosphorylation or autophosphorylation of KaiC, two preparations of this protein, i.e., highly phosphorylated or highly dephosphorylated, were obtained via incubation on ice or at 30 °C for 3 days. The preparations were passed through Micro BioSpin P-30 columns (Bio-Rad) equilibrated with the reaction buffer [20 mM PIPES-NaOH (pH 6.5 or 7.5) or 20 mM HEPPS-NaOH (pH 8.5) in addition to 150 mM NaCl, 5 mM $MgCl_2$, and 1 mM ATP]. After estimating the KaiC concentrations, a reaction mixture with 3.5 μM KaiC was prepared. To investigate the autodephosphorylation or autophosphorylation activity of KaiC without KaiA and KaiB, the highly phosphorylated or highly dephosphorylated KaiC preparations were subjected to dephosphorylation or phosphorylation via incubation at 30 °C or 4 °C, respectively. To separate the phosphorylated forms of KaiC, the samples were subjected to SDS-PAGE. One nonphosphorylated and three phosphorylated KaiC forms were separated by either by conventional SDS-PAGE[41] or via Phos-tag SDS-PAGE. For the former method, samples were subjected to SDS-PAGE on a gel of 11% T with 0.67% C using an EPL156DA (Advantec) or a NA-1040 (Nihon Eido) electrophoresis apparatus. For the latter method, a separation gel of 7% (w/v) 149:1 acrylamide: N,N -methylene-bis-acrylamide containing Tris-HCl, pH 8.8, 0.1% SDS, supplemented with 20 μM of Phos-tag™ acrylamide reagent and 40 μM Mn^{2+}, was prepared using acrylamide containing Mn^{2+} Phos-tag (FUJIFILM Wako Pure Chemical).

Effects of KaiA and KaiB on the autophosphorylation and autodephosphorylation of KaiC

To investigate the effect of KaiA on the autophosphorylation of KaiC, highly dephosphorylated KaiC was prepared via incubation at 30 °C for 3 days. KaiC was passed through Micro BioSpin P-30 columns (Bio-Rad) equilibrated with the reaction buffer [20 mM PIPES-NaOH (pH 6.5 or 7.5) or 20 mM HEPPS-NaOH (pH 8.5) in addition to 150 mM NaCl, 5 mM $MgCl_2$, and 1 mM ATP]. After estimating the KaiC concentrations, a reaction mixture with KaiC and KaiA at 3.5 and 1.2 μM, respectively, was prepared and incubated at 30°C.

To investigate the effect of KaiB on the autodephosphorylation of KaiC, highly phosphorylated KaiC was prepared via incubation on ice for 3 days. KaiC was passed through Micro BioSpin P-30 columns (Bio-Rad) equilibrated with the same reaction buffer described above. After estimating the KaiC concentration, a reaction mixture with KaiC and KaiB at 3.5 and 3.5 μM, respectively, was prepared and incubated at 30 °C.

Calculation of the surface electrostatic potentials

The structure of full-length KaiC from *Synechococcus* PDB code 2GBL[42] was used as a template for the analysis of the interface between the CI and CII domains. Amino acid residues 1–247 and 252–519 were defined as belonging to CI and CII, respectively. A SWISS-MODEL[43] based on the KaiC–KaiB complex structure from *Thermosynechococcus elongatus* (PDB code, 5JWQ)[22] was used for the analysis of the interface of the same complex in this study. Electric charges were assigned to the above-mentioned structures using PDB2PQR[44]. The pH was set from 4 to 11 as shown in Fig. 5 and Supplementary Fig. S7, and all the other parameters were set to the default settings. The N-terminus and C-terminus of KaiC and KaiB were set as neutral. Surface potential maps were created using the APBS plugin within PyMOL[45] with the following default parameters: 2.0 for protein dielectric constant, 78.0 for solvent dielectric constant, 310 K for temperature, and 0.15 M for ionic concentration.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials published with the paper or in external repositories. Source data are available at Figshare :https://doi.org/10.6084/m9.figshare.29097302.v1[46].

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References

1. Johnson, C. H. in *Chronobiology biological timekeeping* (eds. Loros, J.J., Dunlap, J.C., DeCoursey, P. J.) 67–105 (Sinauer Associates, 2004).
2. Nakajima, M. et al. Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. *Science* **308**, 414–415 (2005).
3. Terauchi, K. et al. ATPase activity of KaiC determines the basic timing for circadian clock of cyanobacteria. *Proc. Natl Acad. Sci. USA* **104**, 16377–16381 (2007).
4. Kageyama, H. et al. Cyanobacterial circadian pacemaker: Kai protein complex dynamics in the KaiC phosphorylation cycle in vitro. *Mol. Cell* **23**, 161–171 (2006).
5. Ishiura, M. et al. Expression of a gene cluster kaiABC as a circadian feedback process in cyanobacteria. *Science* **281**, 1519–1523 (1998).
6. Iwasaki, H., Taniguchi, Y., Ishiura, M. & Kondo, T. Physical interactions among circadian clock proteins KaiA, KaiB and KaiC in cyanobacteria. *EMBO J.* **18**, 1137–1145 (1999).
7. Mori, T. et al. Circadian clock protein KaiC forms ATP-dependent hexameric rings and binds DNA. *Proc. Natl Acad. Sci. USA* **99**, 17203–17208 (2002).
8. Abe, J. et al. Circadian rhythms. Atomic-scale origins of slowness in the cyanobacterial circadian clock. *Science* **349**, 312–316 (2015).
9. Ito-Miwa, K., Furuike, Y., Akiyama, S. & Kondo, T. Tuning the circadian period of cyanobacteria up to 6.6 days by the single amino acid substitutions in KaiC. *Proc. Natl Acad. Sci. USA* **117**, 20926–20931 (2020).
10. Nishiwaki, T. & Kondo, T. Circadian autodephosphorylation of cyanobacterial clock protein KaiC occurs via formation of ATP as intermediate. *J. Biol. Chem.* **287**, 18030–18035 (2012).
11. Nishiwaki-Ohkawa, T., Kitayama, Y., Ochiai, E. & Kondo, T. Exchange of ADP with ATP in the CII ATPase domain promotes autophosphorylation of cyanobacterial clock protein KaiC. *Proc. Natl Acad. Sci. USA* **111**, 4455–4460 (2014).
12. Nishiwaki, T. et al. Role of KaiC phosphorylation in the circadian clock system of Synechococcus elongatus PCC 7942. *Proc. Natl Acad. Sci. USA* **101**, 13927–13932 (2004).
13. Pattanayek, R. et al. Visualizing a circadian clock protein: crystal structure of KaiC and functional insights. *Mol. Cell* **15**, 375–388 (2004).
14. Iwasaki, H., Nishiwaki, T., Kitayama, Y., Nakajima, M. & Kondo, T. KaiA-stimulated KaiC phosphorylation in circadian timing loops in cyanobacteria. *Proc. Natl Acad. Sci. USA* **99**, 15788–15793 (2002).
15. Kitayama, Y., Iwasaki, H., Nishiwaki, T. & Kondo, T. KaiB functions as an attenuator of KaiC phosphorylation in the cyanobacterial circadian clock system. *EMBO J.* **22**, 2127–2134 (2003).
16. Xu, Y., Mori, T. & Johnson, C. H. Cyanobacterial circadian clockwork: roles of KaiA, KaiB and the kaiBC promoter in regulating KaiC. *EMBO J.* **22**, 2117–2126 (2003).
17. Weinstock, G. M., McEntee, K. & Lehman, I. R. Hydrolysis of nucleoside triphosphates catalyzed by the recA protein of Escherichia coli. Hydrolysis of UTP. *J. Biol. Chem.* **256**, 8856–8858 (1981).
18. Metrick, M. A., Temple, J. E. & MacDonald, G. The effects of buffers and pH on the thermal stability, unfolding and substrate binding of RecA. *Biophys. Chem.* **184**, 29–36 (2013).
19. Salonikidis, P. S., Kirichenko, S. N., Tatjanenko, L. V., Schwarz, W. & Vasilets, L. A. Extracellular pH modulates kinetics of the Na(+),K(+)-ATPase. *Biochim. Biophys. Acta* **1509**, 496–504 (2000).
20. Camberg, J. L. & Sandkvist, M. Molecular analysis of the Vibrio cholerae type II secretion ATPase EpsE. *J. Bacteriol.* **187**, 249–256 (2005).
21. Hastings, J. W. & Sweeney, B. M. On the mechanism of temperature independence in a biological clock. *Proc. Natl Acad. Sci. USA* **43**, 804–811 (1957).
22. Tseng, R. et al. Structural basis of the day-night transition in a bacterial circadian clock. *Science* **355**, 1174–1180 (2017).
23. Kitayama, Y., Nishiwaki-Ohkawa, T., Sugisawa, Y. & Kondo, T. KaiC intersubunit communication facilitates robustness of circadian rhythms in cyanobacteria. *Nat. Commun.* **4**, 2897 (2013).
24. Chang, Y. G. et al. Circadian rhythms. A protein fold switch joins the circadian oscillator to clock output in cyanobacteria. *Science* **349**, 324–328 (2015).
25. Mukaiyama, A. et al. Evolutionary origins of self-sustained Kai protein circadian oscillators in cyanobacteria. *Nat. Commun.* **16**, 4541 (2025).
26. Egli, M. et al. Dephosphorylation of the core clock protein KaiC in the cyanobacterial KaiABC circadian oscillator proceeds via an ATP synthase mechanism. *Biochemistry* **51**, 1547–1558 (2012).
27. Williams, S. B., Vakonakis, I., Golden, S. S. & LiWang, A. C. Structure and function from the circadian clock protein KaiA of Synechococcus elongatus: a potential clock input mechanism. *Proc. Natl Acad. Sci. USA* **99**, 15357–15362 (2002).
28. Chang, Y. G., Kuo, N. W., Tseng, R. & LiWang, A. Flexibility of the C-terminal, or CII, ring of KaiC governs the rhythm of the circadian clock of cyanobacteria. *Proc. Natl Acad. Sci. USA* **108**, 14431–14436 (2011).
29. Chang, Y. G., Tseng, R., Kuo, N. W. & LiWang, A. Rhythmic ring-ring stacking drives the circadian oscillator clockwise. *Proc. Natl Acad. Sci. USA* **109**, 16847–16851 (2012).
30. Furuike, Y. et al. Regulation mechanisms of the dual ATPase in KaiC. *Proc. Natl Acad. Sci. USA* **119**, e2119627119 (2022).
31. Furuike, Y. et al. Elucidation of master allostery essential for circadian clock oscillation in cyanobacteria. *Sci. Adv.* **8**, eabm8990 (2022).
32. Tomita, J., Nakajima, M., Kondo, T. & Iwasaki, H. No transcription-translation feedback in circadian rhythm of KaiC phosphorylation. *Science* **307**, 251–254 (2005).
33. Hosokawa, N., Kushige, H. & Iwasaki, H. Attenuation of the posttranslational oscillator via transcription-translation feedback enhances circadian-phase shifts in Synechococcus. *Proc. Natl Acad. Sci. USA* **110**, 14486–14491 (2013).
34. Nakajima, M., Ito, H. & Kondo, T. In vitro regulation of circadian phosphorylation rhythm of cyanobacterial clock protein KaiC by KaiA and KaiB. *FEBS Lett.* **584**, 898–902 (2010).
35. Ritchie, R. J., Trautman, D. A. & Larkum, A. W. D. Phosphate uptake in the cyanobacterium synechococcus R-2 PCC 7942. *Plant Cell Physiol.* **38**, 1232–1241 (1997).
36. Nakamura, S. et al. A luminescent Nanoluc-GFP fusion protein enables readout of cellular pH in photosynthetic organisms. *J. Biol. Chem.* **296**, 100134 (2021).
37. Ritchie, R. J. Membrane potential and pH control in the cyanobacterium synechococcus R-2 (anacystis nidulans) PCC 7942. *J. Plant Physiol.* **137**, 409–418 (1991).
38. Kitahara, R. et al. Pressure accelerates the circadian clock of cyanobacteria. *Sci. Rep.* **9**, 12395 (2019).
39. Jeong, Y. M. et al. Magnesium regulates the circadian oscillator in cyanobacteria. *J. Biol. Rhythms* **34**, 380–390 (2019).
40. Rust, M. J., Golden, S. S. & O'Shea, E. K. Light-driven changes in energy metabolism directly entrain the cyanobacterial circadian oscillator. *Science* **331**, 220–223 (2011).
41. Nishiwaki, T. et al. A sequential program of dual phosphorylation of KaiC as a basis for circadian rhythm in cyanobacteria. *EMBO J.* **26**, 4029–4037 (2007).
42. Pattanayek, R. et al. Analysis of KaiA-KaiC protein interactions in the cyano-bacterial circadian clock using hybrid structural methods. *EMBO J.* **25**, 2017–2028 (2006).
43. Waterhouse, A. et al. SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res.* **46**, W296–W303 (2018).

44. Dolinsky, T. J., Nielsen, J. E., McCammon, J. A. & Baker, N. A. PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. *Nucleic Acids Res.* **32**, W665–W667 (2004).
45. Baker, N. A., Sept, D., Joseph, S., Holst, M. J. & McCammon, J. A. Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc. Natl Acad. Sci. USA* **98**, 10037–10041 (2001).
46. Ito-Miwa, K., Onoue, Y., Kondo, T. & Terauchi, K. Raw data for:Effect of pH on the cyanobacterial circadian oscillator in vitro. *Figshare* https://doi.org/10.6084/m9.figshare.29097302.v1 (2025).

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Author contributions

K.M. and K.T. conceived and designed the experiments. K.M., Y.O. and K.T. performed and analyzed the experiments. K.M., Y.O., T.K., and K.T. interpreted and discussed the data. K.M., Y.O. and K.T. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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