Mutation of alanine-422 in KaiC leads to a low amplitude of rhythm in the reconstituted cyanobacterial circadian clock

(Received August 27, 2019; Accepted January 19, 2020; J-STAGE Advance publication date: March 30, 2020)

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The cyanobacterial circadian oscillator can be reconstituted by mixing the purified clock proteins KaiA, KaiB, and KaiC with ATP in vitro, leading to a 24-h oscillation of KaiC phosphorylation. The cyanobacterial mutant prl carrying valine instead of alanine at position 422 of KaiC (KaiC-A422V) lost the ability to shift the phase of the circadian rhythm. In this study, we analyzed KaiC-A422V to investigate the effect of this single-residue substitution on the in vitro reconstitution of KaiC oscillation. KaiC-A422V exhibited low amplitude oscillations of phosphorylation with a smaller amount of Kai complex than wild-type KaiC (KaiC-WT). Although KaiA can stimulate KaiC phosphorylation, the phosphorylation level of KaiC-A422V is much lower than that of KaiC-WT even at higher KaiA concentrations. It has been suggested that monomer shuffling of KaiC is involved in entraining the in vitro rhythm. To examine whether KaiC-A422V has the capacity for monomer shuffling, we used the difference in the amplitude of the phosphorylation rhythms between KaiC-WT and KaiC-A422V as the indicator of monomer shuffling. When KaiC-A422V and KaiC-WT were mixed, the amplitude of the phosphorylation rhythm changed according to the mixing ratio. This suggests that KaiC-A422V has a reduced ability to shuffle monomers in hexameric KaiC. In addition, the A422V mutation resulted in a change of the stability of the KaiC protein.

Key Words: ATPase; circadian clock; cyanobacteria; KaiC; phosphorylation; Synechococcus elongatus PCC 7942

Abbreviations: BN-PAGE, blue native polyacrylamide gel electrophoresis; cs-KaiC, competent-state KaiC; gs-KaiC, ground-state KaiC

Introduction

The circadian clock is an endogenous timing mechanism in many organisms that coordinates various biological activities in alternating day/night environments (Pittendrigh, 1993). It shares three characteristics: first, they generate self-sustained oscillations under constant conditions with an approximately 24-h (circadian) period; second, the circadian period is stable against changes in temperature, unlike typical biochemical processes, facilitating circadian timing under different external conditions; and third, circadian clocks can be easily entrained to external time cues, such as light and temperature, derived from day-night alternation (Golden et al., 1997).

Cyanobacteria are the simplest organisms known to possess a circadian clock (Golden et al., 1997). In the cyanobacterium Synechococcus elongatus PCC 7942, a prokaryotic model organism for circadian clock research, three clock genes, namely kaiA, kaiB, and kaiC, are essential for the circadian clock (Ishiura et al., 1998). KaiC displays auto-phosphorylation and auto-dephosphorylation activities, and its phosphorylation level exhibits circadian oscillation in cells (Iwasaki et al., 2002). The KaiC protein coordinates genome-wide gene expression (Ito et al., 2009), including its own (Nakahira et al., 2004), and moreover, KaiC-based transcription/translation oscillation persists in cells to generate robust circadian rhythms via coupling to biochemical KaiC phosphorylation oscillation (Kitayama et al., 2008).
In a previous study, the cyanobacterial mutant strain prl was found to not undergo a circadian phase shift following a 5-h dark pulse (Kiyohara et al., 2005). This mutant carried a base substitution in the kaiC coding region, resulting in a codon change from alanine to valine at position 422 of KaiC (KaiC-A422V), and the phase shift phenotype was rescued via introduction of the wild-type (WT) kai gene cluster. The prl mutant failed to respond to a dark pulse, but it exhibited circadian rhythms with a period and amplitude similar to those of WT cells. In the prl mutant, the mutation of Ala to Val has rather drastic consequences that include abnormal phase-resetting responses to dark pulses, abolished rhythms of KaiC accumulation, and a reduced amplitude of the KaiC phosphorylation rhythm (Kiyohara et al., 2005). An X-ray structural study of KaiC-A422V reported that Ala422 is located in a loop that is involved in KaiA-stimulated KaiC phosphorylation (Egli et al., 2013).

The circadian clock of S. elongatus PCC 7942 is the sole example that can be reconstituted in vitro. Circadian oscillation of KaiC phosphorylation can be reconstituted by mixing KaiA, KaiB, and KaiC with ATP in vitro (Nakajima et al., 2005). The simplicity of the cyanobacterial in vitro system makes it ideal for understanding the principles underlying circadian clocks. The biochemical oscillator possesses the three hallmark features of circadian clock: (1) autonomous oscillations of approximately 24 h, (2) temperature compensation, and (3) a phase shift of the KaiC rhythm in response to external temperature stimulation (Nakajima et al., 2005; Terauchi et al., 2007; Yoshida et al., 2009). The physiological characteristics of the circadian system should be based on the molecular features of the Kai protein oscillator.

KaiC consists of two homologous domains, namely N-terminal CI and C-terminal CII, and both domains possess P-loop ATPase motifs. KaiC forms a homohexameric structure that is essential for circadian oscillation (Pattanayek et al., 2004). KaiC has auto-phosphorylation and auto-dephosphorylation activities in addition to ATPase activity, and KaiA stimulates KaiC auto-phosphorylation and ATPase activities. Conversely, KaiB inhibits the effects of KaiA (Iwasaki et al., 2002; Kitayama et al., 2003; Terauchi et al., 2007; Xu et al., 2003). KaiA binds to the CII domain of KaiC to promote KaiC phosphorylation at Ser431/Thr432 (Pattanayek and Egli, 2015), and then KaiA re-binds to KaiB bound to the CI domain of KaiC (Snijder et al., 2017; Tseng et al., 2017).

It was assumed that an insight into the mechanism regarding the ability to phase shift, an important property of cyanobacterial clocks, would be obtained by analyzing the KaiC-A422V variant. In this study, we examined the effect of the substitution of A422V mutation of KaiC on in vitro KaiC phosphorylation oscillation.

Materials and Methods

Plasmid construction for KaiC-A422V. The expression plasmid for KaiC (Oyama et al., 2016) was mutagenized to obtain KaiC-A422V according to the Quick-Change mutagenesis protocol (Stratagene). The oligo-nucleotide primers used in the present study were as follows: kaiCA422V-f, 5'-TTTATGGGAGTGCCATTGGAT-3'; kaiCA422V-r, 5'-AATCGAATGCACTCCCATAAA-3' (the mutated codon is underlined).

Purification of recombinant Kai proteins. S. elongatus PCC 7942 KaiA, KaiB, and KaiC proteins were expressed in Escherichia coli DH5α and purified as previously described (Oyama et al., 2016). KaiC-A422V was purified in the same manner as KaiC-WT. Recombinant Strep-tagged fusion proteins were produced in E. coli, followed by purification using a Strep-tactin Sepharose column (IBA). Proteins were further purified using a RESOURCE Q column (GE Healthcare) and gel filtration chromatography as previously described (Oyama et al., 2016).

In vitro reconstitution of the KaiC phosphorylation cycle. The KaiC phosphorylation cycle in vitro was reconstituted as previously described (Nakajima et al., 2005; Oyama et al., 2016). KaiC was incubated with KaiA and KaiB in a buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM ATP, and 5 mM MgCl₂ at 30°C. The final concentrations of KaiA, KaiB, and KaiC were 1.2, 3.5, and 3.5 µM, respectively. The phosphorylated forms of KaiC were separated via SDS-polyacrylamide gel electrophoresis (PAGE), and the SDS-PAGE gels were stained with Quick-CBB-plus (Wako). The relative amount of phosphorylated KaiC in each sample was determined by densitometric analysis using ImageJ software (Schneider et al., 2012).

Blue native (BN)-PAGE. BN-PAGE was performed using a NativePAGE™ Novex Bis-Tris Gel System (Invitrogen) according to the manufacturer’s protocols with some modifications as previously described (Oyama et al., 2016). The concentration of each Kai protein was 2-fold higher than that in the reconstitution experiment to enhance protein signals.

Preparation of KaiC monomers and reconstitution of the hexamer. KaiC monomers were prepared from hexameric KaiC as previously described with some modifications (Nishiwaki and Kondo, 2012). Briefly, solutions of purified hexameric KaiC with 1 mM ATP were exchanged for a buffer supplemented with 0.1 mM ADP using Spin-X UF (Corning). The resulting KaiC solutions were incubated on ice for 24 h. KaiC monomer fractions were obtained via gel filtration chromatography using a HiPrep 16/60 Sephacryl S-300 HR column (GE Healthcare). Hexameric KaiC was reconstituted from monomers in a buffer containing 1 mM ATP.

Results

Reconstitution of circadian phosphorylation with KaiC-A422V. We purified the KaiC-A422V variant as a Strep-tagged fusion protein. Initially, we confirmed the phosphorylation oscillation of KaiC-A422V in vitro by mixing KaiA, KaiB, and ATP. The migration pattern of KaiC-A422V on the SDS-PAGE gel was slightly different from that of WT KaiC (KaiC-WT). KaiC has two phosphorylation sites, namely Ser431 and Thr432. Thus, during oscillation, three phosphorylated and one non-phosphorylated form of KaiC
can appear periodically, and they can be separated into four bands on a specific SDS-PAGE gel (Nishiwaki et al., 2007). In the gel used for this experiment, three phosphorylated KaiC forms were indistinguishable and detected as a single band. The lower band corresponded to non-phosphorylated KaiC. In contrast to KaiC-WT, the three phosphorylated KaiC-A422V forms were detected as a doublet upper band, and non-phosphorylated KaiC-A422V was detected as a lower band (Fig. 1A).

The KaiC-A422V phosphorylation level oscillated with a period of approximately 21 h, which was shorter than that of KaiC-WT (Fig. 1B). The amplitude of the KaiC-A422V rhythm was remarkably reduced compared with that of KaiC-WT. This in vitro behavior is consistent with in vivo reports of pr1 cyanobacterial mutants carrying KaiC-A422V (Kiyohara et al., 2005).

KaiC forms a hetero-complex with KaiA and KaiB, and the formation pattern changes with circadian oscillation (Kageyama et al., 2006; Snijder et al., 2017; Tseng et al., 2017). We have reported the complex formation of the three Kai proteins over time using BN-PAGE, in which native proteins are charged with the anionic dye Coomassie brilliant blue (CBB), and demonstrated that BN-PAGE analysis is a powerful and effective technique for detecting conformational differences in KaiC hexamers and Kai protein complexes (Oyama et al., 2016). In BN-PAGE of KaiC-WT, a band with an apparent molecular mass of approximately 50 kDa that was observed at 6–18 h corresponded to cs-KaiC (cs-KaiC in Fig. S1). cs-KaiC was observed every 24 h using an in vitro reconstitution system as previously reported (Oyama et al., 2016) (Fig. 2A). In contrast to KaiC-WT, a much smaller amount of cs-KaiC was detected in KaiC-A422V, and the profile of the relative amount of cs-KaiC did not show robust circadian oscillation (Fig. 2B).
Effect of KaiA on KaiC-A422V

Ala422 is in the proximity of the phosphorylation sites in the CII domain, and it is also located in a loop that is involved in KaiA-stimulated KaiC phosphorylation (Egli et al., 2013). The effect of KaiA on the phosphorylation of KaiC-A422V was examined. When 3.5 µM KaiC was incubated with 1.2 µM KaiA at 30°C, the ratio of phosphorylated KaiC-WT was increased until 6 h, remaining constant thereafter, whereas the phosphorylated states of KaiC-A422V more gradually increased and reached a slightly lower level than was observed for the WT after 24 h (Fig. 3A), indicating that the enhancing effect of KaiA on KaiC phosphorylation was significantly reduced in the KaiC-A422V variant. In addition, the effects of higher concentrations of KaiA, specifically 3.0 and 6.0 µM, on KaiC-A422V phosphorylation were examined. Higher concentrations of KaiA were not effective (Fig. 3B); that is, the low level of phosphorylation of KaiC-A422V could not be compensated by higher concentrations of KaiA.

Effects of temperature on KaiC-A422V stability

The pr1 mutant carrying the A422V mutation in KaiC exhibited an impaired phase shift response to a dark pulse in vivo (Kiyohara et al., 2005). In the in vitro reconstitution system, a temperature shift was applied to entrain the KaiC phosphorylation rhythm because Kai proteins do not sense light/dark signals (Yoshida et al., 2009). Thus, we applied temperature shifts from 30 to 45°C and analyzed the effects on the KaiC-A422V phosphorylation rhythm. Regarding the KaiC-WT phosphorylation rhythm, the temperature shift during the dephosphorylation phase led to the subsequent peaks and troughs approximately 3 h earlier than the reference rhythm (Supplementary Fig. S2A). This result is consistent with a previous report (Yoshida et al., 2009). We applied the same temperature shift to KaiC-A422V (Fig. S2B) and found that the KaiC-A422V phosphorylation rhythm disappeared after the shift to 45°C.

Similarly, we analyzed phase shifting after a reverse temperature shift from 45 to 30°C (Fig. S2C). The phase of KaiC-WT phosphorylation was delayed by approximately 3 h after the temperature shift. As shown in Fig. S2D, we unexpectedly found that KaiC-A422V did not exhibit an oscillation of phosphorylation at 45°C. Moreover, a temperature shift from 45 to 30°C did not recover the rhythm. These results suggest that the A422V substitution resulted in a decreased stability of KaiC.

Amplitude of the phosphorylation rhythm after mixing KaiC-WT and KaiC-A422V hexamers

Previous reports suggested that monomer shuffling of KaiC is involved in entraining the in vitro rhythm (Ito et al., 2007; Kageyama et al., 2006). Because the pr1 mutant could not entrain the rhythm (Kiyohara et al., 2005), monomer shuffling may not occur in KaiC-A422V. To examine whether KaiC-A422V hexamers can shuffle monomers, we used the difference in the amplitude of the phosphorylation rhythms between KaiC-WT and KaiC-A422V as the indicator of monomer shuffling. KaiC-WT displayed a high amplitude rhythm, whereas KaiC-A422V displayed a low amplitude rhythm (Fig. 1). If monomer shuffling occurs between KaiC-WT and KaiC-A422V hexamers, the amplitude of the rhythm wave should change depending on the mixing ratio of KaiC-WT and KaiC-A422V in the reconstitution solution.

The phosphorylation rhythm at hexameric KaiC-WT/ hexameric KaiC-A422V ratios of 3:7, 5:5, and 7:3 mixtures were examined in the presence of KaiA and KaiB (Fig. 4A). The three different mixtures exhibited KaiC phosphorylation rhythms with individual amplitude according to their mixture ratios. The amplitude of the rhythm was highest in the 7:3 mixture. As the contents of KaiC-A422V increased, the amplitude decreased. As expected, the observed rhythm amplitude was proportional to the mixture ratios of KaiC-WT and KaiC-A422V (Fig. 4B), which was in good agreement with the theoretical amplitude assuming that KaiC-WT and KaiC-A422V...
hexamers operate independently. This result suggests that monomer shuffling did not occur between KaiC-WT and KaiC-A422V in the reconstitution system and that the KaiC-A422V variant is incapable of monomer shuffling.

To examine the phosphorylation rhythm of the case in which monomer shuffling did occur between KaiC-WT and KaiC-A422V, we prepared hetero-hexamers containing both KaiC-A422V and KaiC-WT protomers (Fig. 4C). Monomerization and homo-hexamer reconstitution had no effect on KaiC activity (Supplementary Fig. S3). We mixed the monomers of KaiC-WT and KaiC-A422V with molar ratios of 3:7, 5:5, and 7:3 and then reconstituted hexamers, assuming that the hetero-hexamer composition changed according to the mixture ratios. The reconstituted hexamers were incubated in the presence of KaiA and KaiB, and we monitored the phosphorylation rhythm (Fig. 4D). The three mixtures of reconstituted hexamers displayed similar phosphorylation rhythm patterns regardless of the mixing ratios. The amplitude was almost identical to the average value of the KaiC-WT and KaiC-A422V phosphorylation rhythm (Figs. 4B and D). The monomer compositions in KaiC hexamers containing both WT and A422V variants thus did not contribute to the phosphorylation rhythm. This result for the hetero-hexamers was apparently different from that for the simple mixtures of KaiC-WT and KaiC-A422V hexamers (Fig. 4B).

**Discussion**

KaiC-A422V exhibited a phosphorylation rhythm with a lower amplitude in the circadian reconstitution system (Fig. 1). Our data are consistent with the rhythm of KaiC phosphorylation in the cyanobacterial prf mutant, which exhibits a lower amplitude than the WT (Kiyohara et al., 2005). Although KaiA can stimulate KaiC phosphorylation (Iwasaki et al., 2002), the phosphorylation level of KaiC-A422V was significantly lower than that of KaiC-WT, even at higher KaiA concentrations (Fig. 3). The KaiA dimer binds to the CII domain of KaiC to induce a conformation change in the A loop region near the C-terminus (Kim et al., 2008). A structural study revealed that Ala422 is located at a loop near the phosphorylation sites, and the loop is important for the activation of KaiC phosphorylation by KaiA (Egli et al., 2013). This implies that the Ala to Val substitution of KaiC weakens the interaction between KaiA and KaiC, resulting in the low amplitude rhythm.

BN-PAGE demonstrated that complex formation of KaiC-A422V with KaiA and KaiB oscillated in a circadian manner, whereas the complex tends to dissociate faster than that of KaiC-WT, which would be caused by the weak KaiA stimulation (Fig. 2). KaiB binds to KaiC depending on the phosphorylation of Ser431 after ATP
Mutation in circadian oscillator KaiC

It has been proposed that KaiA stimulates the KaiC auto-phosphorylation via a concerted allosteric mechanism (Egli et al., 2013; van Zon et al., 2007). Recent studies provided insights into the phosphorylation mechanism, in which inter-subunit communication regulates KaiC activity (Kitayama et al., 2013; Lin et al., 2014). It is probable that through those mechanisms, KaiA may stimulate kinase activity in the reconstituted hetero-hexamers consisting of both KaiC-WT and KaiC-A422V.

Acknowledgments

The authors thank Kazuma Umehara and Yoshitaro Sanbayashi for their technical support. This work was financially supported by JSPS KAKENHI Grant Number 16H00784, 17K19247, 19K05833 to K.T.

Supplementary Materials

Supplementary figures are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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hydrolysis in the CI domain (Oyama et al., 2016; Phong et al., 2013). The cycle of the complex formation on KaiC-A422V suggests that the same phosphorylation sequence as KaiC-WT occurs in this variant despite a finding in X-ray crystallography that Ser431 is not phosphorylated in the KaiC-A422V crystal structure (Egli et al., 2013). In addition, BN-PAGE analysis revealed that a much smaller amount of KaiC-A422V is converted to cs-state KaiC than KaiC-WT (Fig. 2). Conversion from gs-KaiC to cs-KaiC is induced by ATP hydrolysis in the CI domain following phosphorylation of Ser431 in the CI domain (Oyama et al., 2016). Because the CI domain of KaiC-A422V is no different from that of KaiC-WT, the significant decrease of the amount of cs-state KaiC-A422V is attributable to the poor phosphorylation of Ser431.

We analyzed the phase shift ability of KaiC-A422V through the effects of temperature shifts on the KaiC-A422V phosphorylation rhythm (Fig. S2). A study of protein denaturation with circular dichroism reported that KaiC-WT and this variant have an identical melting temperature of approximately 85°C, suggesting that thermal stability is similar between KaiC-A422V and KaiC-WT (Egli et al., 2013). Contrary to the reported stability of KaiC-A422V, no phosphorylation rhythm of KaiC-A422V was observed at 45°C, and the phosphorylation rhythm was not restored when the temperature was lowered from 45 to 30°C (Fig. S2). This behavior suggested that this variant underwent irreversible denaturation functionally following exposure to high temperatures. Due to the loss of phosphorylation oscillation at 45°C, we failed to examine the phase shift of KaiC-A422V in response to temperature changes. It has been reported that the oscillation phase could be reset via a transient change of the ratio of ATP to ADP in vitro, which may mimic metabolic changes caused by dark pulses in vivo (Rust et al., 2011). By examining whether a transient change in the ATP/ADP ratio causes a phase shift in the phosphorylation rhythm of KaiC-A422V, it would be possible to analyze the phase shift ability of KaiC-A422V.

Monomer shuffling of KaiC has been revealed to be involved in entraining the in vitro rhythm (Ito et al., 2007; Kageyama et al., 2006). The phosphorylation rhythms in the mixtures of KaiC-WT and KaiC-A422V in monomeric or hexameric states suggest that KaiC-A422V is incapable of monomer shuffling (Fig. 4). This result can rationalize that the cyanobacterial mutant strain *prl* carrying the KaiC-A422V variant lost the ability to entrain the rhythm (Kiyohara et al., 2005). Although the detailed mechanism of monomer shuffling is unknown, Ala422 of KaiC is an important residue for elucidating the mechanism.

The hetero-hexamers consisting of KaiC-WT and KaiC-A422V prepared through monomerization treatment exhibited a stable phosphorylation rhythm with a constant amplitude regardless of the mixture ratios (Fig. 4D). A ratio of one KaiA dimer to one KaiC hexamer is reportedly sufficient to drive the phosphorylation oscillation in KaiC (Hayashi et al., 2004). It is likely that a single KaiA dimer stimulates one KaiC-WT protomer in the reconstituted hexamer to initiate phosphorylation followed by sequential phosphorylation from one protomer to the next.
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