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ABSTRACT

MOLECULAR MECHANISM OF CYANOBACTERIAL CIRCADIAN CLOCK OSCILLATOR AND EFFECT OF COFACTORS ON ITS OSCILLATION

by
Manpreet Kaur

The circadian rhythms arise as an adaptation to the environmental 24-hour day and night cycle due to Earth's rotation. These rhythms prepare organisms to align their internal biological activities and day to day behavior or events with the environmental change of the 24-hour day and night cycle. Circadian rhythms are found widely in all living kingdoms of life on Earth. Cyanobacteria are photosynthetic prokaryotes which first used to study these circadian rhythms. Among cyanobacterial species, Synechococcus elongatus PCC 7942 (henceforth, *S. Elongatus*) is the simplest organism with a durable and sturdy circadian clock and is study as a model organism. The *S.Elongatus* central pacemaker is a post-translational oscillator(PTO) consisting of three proteins KaiA, KaiB, and KaiC, and these proteins can replicate in a test tube with ATP and Magnesium. The circadian oscillator mechanism revolves around KaiC phosphorylation at residues S431 and T432 in the CII domain throughout ~24-hours. KaiA initiates KaiC phosphorylation by binding to the KaiC region known as A-loop. KaiB then commences the process of dephosphorylation by sequestering KaiA from A-loop. This ordered phosphorylation with A loop confirmation change does not provide a detailed explanation about the molecular mechanism acting downstream of A loop. This oscillator mechanism is not well studied, yet the shift in protein concentrations, the addition of cofactors in an oscillator, and protein mutations in the circadian period are still unknown.

This dissertation addresses how important it is to investigate each clock component, their protein mutations, and interactions to solve the oscillator mechanism and the whole developmental biological chronometer's hidden complexity. It discusses the Aloop downstream mechanism for KaiC phosphorylation and how cofactors and mutations influence its oscillation in this report. And also propose the current self-sustained circadian oscillator possibly evolved from the KaiC-alone hourglass controlled by magnesium.

MOLECULAR MECHANISM OF CYANOBACTERIAL CIRCADIAN CLOCK OSCILLATOR AND EFFECT OF COFACTORS ON ITS OSCILLATION

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A Dissertation
Submitted to the Faculty of
New Jersey Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Chemistry

Department of Chemistry and Environmental Science

December 2020

APPROVAL PAGE

MOLECULAR MECHANISM OF CYANOBACTERIA CIRCADIAN CLOCK OSCILLATOR AND EFFECT OF COFACTORS ON ITS OSCILLATION

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I would like to dedicate this dissertation to my family for their love and support. Without them being with me, none of this would have been possible.

***My parents**: Satinder Kaur and Joginder Singh,*
***My husband**: Manvinder Singh,*
***My Twins:** Jason Singh Bhatti and Guran Singh Bhatti,*
***My sisters and brother**: Maninder Kaur, Harvinder Kaur and Ranjeet Singh*

Thank You!

ACKNOWLEDGMENT

I am very grateful and profoundly thankful to my Advisor, Dr. Yong Ick Kim. For giving me this wonderful opportunity to join his lab as a graduate student, Prof. Kim shares his valuable expertise, experience, and work on this fascinating project. His mentorship continuously improves my skills, encourages me to believe in my dreams, and giving independence throughout my Ph.D. enabled me to achieve my aspirations. His commitment, time, and conviction to perfection for his graduate students leave me with a deep sense of gratitude.

I would like to express my heartfelt thanks to my committee members: Dr. Kevin Belfield, Dr. Casey Diekman, Dr. Tamara Gund, and Dr. Yuanwei Zhang, for their consistent encouragement, helpful discussions, and scholarly advice. I am also grateful to Dr. Diekman for designing mathematical models, simulations and Dr. Dias for analyzing the Crystal structure for my research projects. I want to thank Dr. Gund for her guidance and support as chair of the department during my admission. I thank our research associate, Young M. Jeong, for being a good friend, all the help and advice she has given me over the years.

I would also like to thank all the Department of Chemistry and Environmental Sciences members for their complete support during my Ph.D. I would also like to acknowledge the many outstanding undergraduates and graduates— Amy Ng, Neha Thatti, Helene Brochon, Pyonghwa Kim, for their contributions to my dissertation chapters. I want to thank my fellow graduate student Sweety Singh who has been very close to me and accompanied me on this Ph.D. journey. My fellow graduate students, graduate professors,

and undergraduate mentees: these past four years of work would not have been as much fun or informative without you all. Finally, I would like to thank Manvinder Singh, my beloved husband, who inspired me to pursue a Ph.D. and my newborn twins, for their cooperation and love to complete it. Thank you very much!

TABLE OF CONTENTS

TABLE OF CONTENTS
(Continued)

Chapter **Page**

TABLE OF CONTENTS
(Continued)

LIST OF FIGURES

LIST OF FIGURES
(Continued)

CHAPTER 1

INTRODUCTION OF CYANOBACTERIA AND CIRCADIAN CLOCK

This chapter aims to introduce cyanobacteria and the basics of circadian rhythms. It intended to describe the concepts, characteristics, and properties used to study circadian biology. This dissertation will also talk about the cyanobacterial clock model to elucidate the mechanisms for its functioning oscillator.

1.1 Introduction

The changing 24-hour environmental conditions such as the light/dark cycle and temperature fluctuations result from the Earth's spinning on its axis around the sun. These periodic changes influence many of the living body's internal and external activities, which impose stress on all the organisms to build a timing mechanism called the "circadian clock." This natural clock operates within an organism to calibrate its cognitive, physiochemical, and metabolic processes with the 24-hour daily sunrise and sunset cycles. The resetting process of internal circadian rhythms with external day/night 24-hour cycles is called "entrainment" (Edmunds, 1983; Koukkari & Sothern, 2006; Pittendrigh, 1981). Maintaining correct circadian timing is complex and challenging to understand and explain biochemically at the cellular and structural level for different organisms. However, it is a crucial phenomenon for the development and longevity of an organism. For instance, humans can develop significant health problems such as cancer, neurological disorders, and metabolic diseases due to this clock's disturbance or dysfunction (Arble, Ramsey, Bass, & Turek, 2010; Richards & Gumz, 2013; Zelinski, Deibel, & McDonald, 2014). To overcome the dysfunction or achieve the synchronization between the biological clock and

the environmental clock, we need to study and understand the circadian clock's molecular mechanism.

Internal clock mechanism varies across a broad range of organisms, but they all serve the same essential clock functions:

Timekeeping: the ability of an organism's clock to independently generate a circadian period of ~24 hours, regardless of environmental feedback, indicating that the circadian clock is not environmentally driven but is endogenous and biologically programmed (Dunlap, Loros, & DeCoursey, 2004).

Entrainment: sensing environmental signals (light/dark, temperature variation, food) to align the clock phase, known as the phase setting phenomenon.

Temperature compensation: It can sustain nearly 24 hours across an organism's intrinsic temperature fluctuations, despite the high sensitivity of standard biochemical response rates to temperature.

In all forms of life, the circadian mechanism considers being consists of three components: the oscillator, an input pathway, and an output pathway. The clock's input component involves organs (invertebrate or non-vertebrate) or proteins (in unicellular bacteria and fungi) that sense external day/night signals. It conveys these signals to the central oscillator, thus leading to entrainment. However, the input pathway is not only restricted to photoreception, as dietary constraints and temperature can also serve as external stimuli. A central oscillator synchronizes and generates a 24-h rhythm in response to external time cues received through the input pathways to adapt to the sun's 24-h day/night cycle. Based on changes in the circadian clock's central oscillator, the output

component regulates an organism's gene expression. The detailed biochemical mechanisms of how circadian clocks generate these oscillations are unknown.

The single-celled *S. elongatus* PCC 7942 strain of cyanobacteria is considered an ideal model organism for studying the circadian rhythm's biochemical mechanism. Fossil records suggest that this cyanobacterium first surfaced about 2.5 billion years ago. It undergoes photosynthesis to acquire energy from sunlight to produce oxygen as a byproduct. It was the first known prokaryote containing oxygen in the Earth's atmosphere, and it also fixes nitrogen (Rasmussen, Fletcher, Brocks, & Kilburn, 2008; Schopf & Packer, 1987). However, this raises how an organism can fix nitrogen and produce oxygen, as oxygen irreversibly inhibits the nitrogenase enzyme. Researchers were able to find that unicellular cyanobacteria can temporally separate photosynthesis and nitrogen fixation (Mitsui et al., 1986). This separation requires control over circadian rhythms that a circadian clock can facilitate.

1.2 *S. Elongatus* PCC 7942

S. elongatus PCC 7942 cyanobacteria clock is considered an ideal circadian model organism to study the circadian rhythm because it is most stable and grows over a wide range of temperatures, i.e., 25–35-degrees C, in the presence of light and nutrients. It can sense continuous changes in the environment and respond to them quickly, allowing for adaptation. This species, amid sunshine and dark cycle, also can separate photosynthesis and nitrogen fixation processes by temporal differentiation, and the circadian clock can regulate them (Grobbelaar et al., 1986; Mitsui et al., 1986; Schneegurt, Sherman, Nayar, & Sherman, 1994). It is the simplest clock composed of three Kai proteins, KaiA, KaiB,

and KaiC, which can be purified and replicated in the test tube, and in the presence of ATP and other constant conditions, the clock can operate for an indefinite time (Mihalcescu Hsing & Leibler, 2004). The phosphorylation/dephosphorylation cycles of KaiC occur over a 24-hour circadian duration (M. Ishiura et al., 1998; Nakajima et al., 2005). Additionally, the clock proteins can be changed regularly by physical and biochemical properties, and protein-protein interactions can control such changes. Genetic manipulation is simple to execute, and molecular tools are available for this species, providing maximum flexibility (Golden, 1988).

1.3 Cyanobacterial Circadian Clock

In *S. elongatus,* the circadian period can be tracked via the luciferase gene's insertion as a luminescence reporter for gene expression. While monitoring this luminescence from *S. elongatus* genetically mutated colonies, and several rhythmic genes were isolated. The KaiABC three genes were essential for generating a circadian oscillation (M. Ishiura et al., 1998). There is a variety of data in most species to support the circadian operating mechanism as a transcription-translation feedback loop (TTFL) based on negative clock gene feedback regulation, i.e., when a gene product inhibits its level of expression (M. Ishiura et al., 1998). But the clock of *S. elongatus* is a post-translational oscillator (PTO), consisting of three proteins KaiA, KaiB, and KaiC, which can replicate in the test tube by mixing them with ATP and magnesium (Nakajima et al., 2005). The invention of an in vitro method made cyanobacterial circadian clock research is straightforward and manageable. However, the clock is still not precise to understand biochemically and structurally. The effect of mutations in other clock proteins suggests that the watch is an

interacting network. All components contribute to timekeeping, phase resetting, and output signaling functions to sustain the circadian period.

The circadian clock is composed of three components, which are described in detail as follows:

A. Oscillator

The S. Elongatus model approach can be driven by biochemical, protein-protein interactions, or structural studies of cyanobacterial circadian oscillator consisting of KaiABC. KaiC is a crucial player in the central oscillator of cyanobacteria. It is a multifunctional protein that shows autophosphorylation, auto dephosphorylation, and ATPase activity. *S. elongatus* KaiC's crystal structure is a twin doughnut consisting of ATP activity at the N-terminal CI domain and KaiC active residue site at the C-terminal CII domain. These two domains are stacked with 15 amino acid chain to set the oscillation time close to 24h and control multiple output processes, including gene regulation and metabolism through bond formations and binding site changes in protein structure (Nakajima et al., 2005; Tomita, Nakajima, Kondo, & Iwasaki, 2005).

KaiC's phosphorylation/dephosphorylation 24-hour cycles are modulated by KaiA and KaiB proteins with a ten residue segment (residues 488–497) referred to as the A-loop (Kim, Dong, Carruthers, Golden, & LiWang, 2008). KaiA binds to the exposed A-loop of KaiC and initiates the phosphorylation of KaiC at two adjacent residues (S431 and T432). It first phosphorylates the singly phosphorylated threonine form (T-KaiC). Then serine is phosphorylated to create the fully phosphorylated KaiC (ST-KaiC). KaiB then commences dephosphorylation by taking KaiA away from the A-loop and resulting in the A-loop being in the buried conformation. T432 becomes dephosphorylated first (S-KaiC), followed by

Ser431 to form dephosphorylated KaiC (U-KaiC). This rhythmic KaiC cycle repeats 24 hours (Figure 1) (Nishiwaki et al., 2007; Rust, Markson, Lane, Fisher, & O'Shea, 2007; Snijder et al., 2017; Tseng et al., 2017).

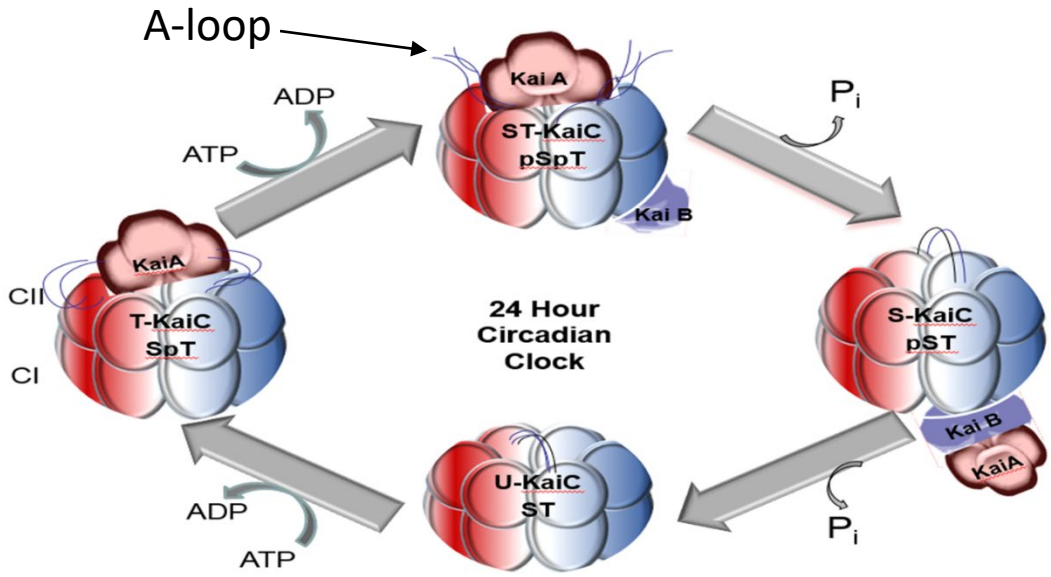


Figure 1.1 The circadian oscillator is comprised of KaiC, KaiA, and KaiB. During the first 12 hours, the A-loop gets exposed by KaiA, and non-phosphorylated KaiC (ST, U-KaiC) first phosphorylates T432 (SpT, T-KaiC), then S431 (pSpT, ST-KaiC). In the next 12 hours, the A-loop gets buried as KaiB sequesters KaiA and dephosphorylates T432 (pST, S-KaiC), then S431 (back to ST, U-KaiC).

The circadian clock 24-hour oscillations were readily detected, both in vivo and in vitro, via SDS-PAGE. A phosphorylated KaiC is shown as an upper triple band, and a dephosphorylated KaiC can be viewed as a lower single band on the gel (Figure 1.2). The ratio of phosphorylated KaiC to total KaiC is determined by densitometry of each band, and the percentage is plotted as a function of the incubation time. The graph represents the oscillations in a circadian manner.

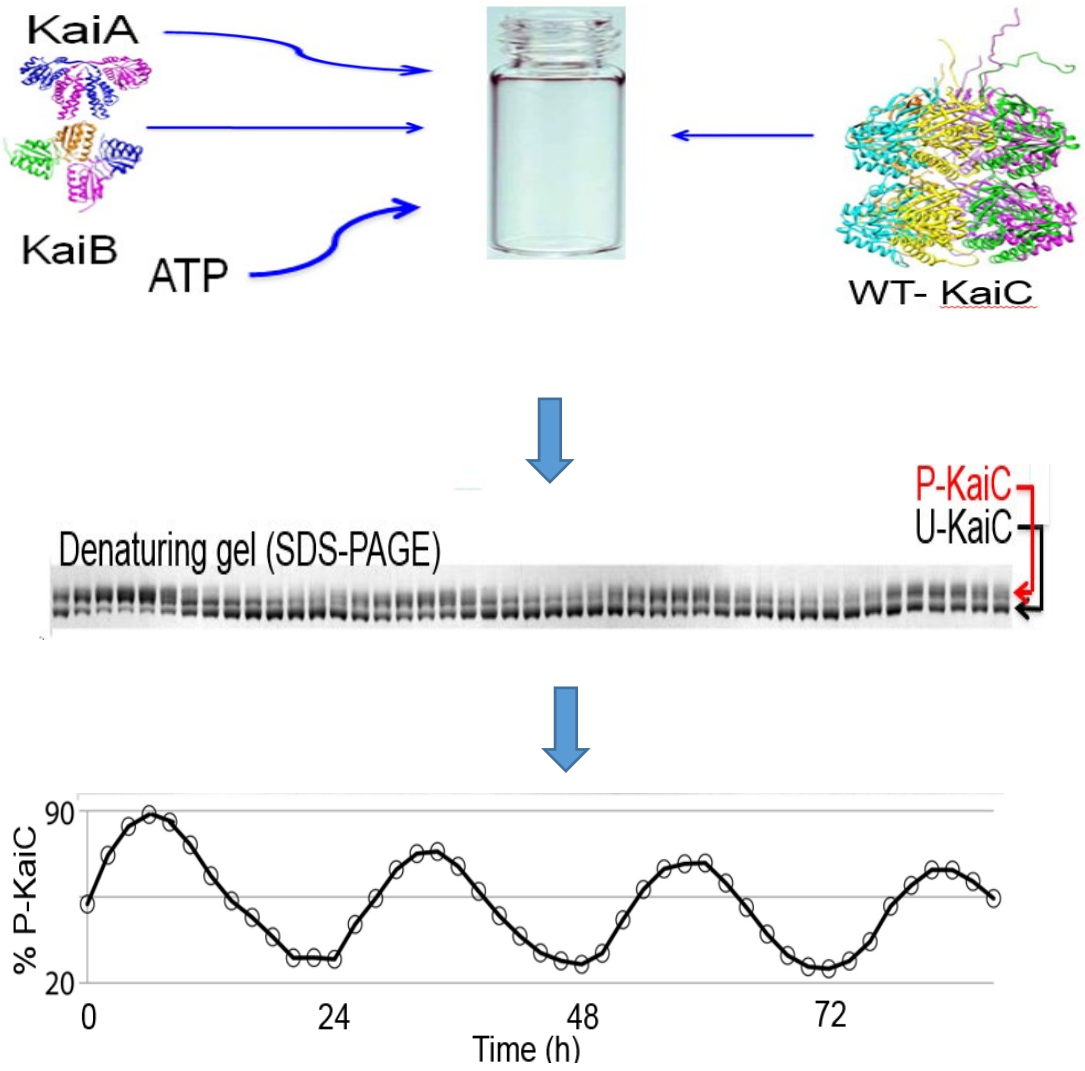


Figure 1.2 In vitro cyanobacterial clock.

The discovery of ordered phosphorylation and KaiC A-loop confirmation, which switches between the KaiC phosphorylation and dephosphorylation state, leads to the following questions: who plays the role of timekeeper in the oscillator, and how do these proteins interact with each other, to the ATP binding site, to the active residues (S431 and T432) or to the A-loop to generate stable oscillations? It is believed that KaiA is the primary regulator in KaiC phosphorylation. KaiA gets activated by breaking a hydrogen bond network that holds the A-loop in the buried state, which in turn keeps the KaiC

phosphorylation active site two residues (S431 and T432) away from the γ-phosphate of the ATP substrate (Kim et al., 2008). Besides, KaiA can also activate KaiC autophosphorylation activity by sharing and exchanging base pairs at the site of ATP (Nishiwaki-Ohkawa, Kitayama, Ochiai, & Kondo, 2014). Though KaiA can perform various functions, the mechanism of its interaction is not clear. It's known that the addition of KaiA is the only way for KaiC phosphorylation to be activated. Still, our lab can show that magnesium can act as a master regulator of KaiC phosphorylation downstream of the A-loop confirmation.

B. Input

A majority of studies focus on the in vitro circadian clock oscillator mechanism and overlook the contributions from the other components of clock whose mutation resulted in the disruption of circadian rhythms in vivo (Boyd, Bordowitz, Bree, & Golden, 2013; Ivleva, Gao, LiWang, & Golden, 2006). In most organisms, the circadian clock's period of oscillation is not precisely 24 hours. Organisms need to adjust their clock daily to maintain their internal physiological and behavioral activities in synchrony with the external 24-hour day/night clock. This synchronization of the phase between an organism's circadian oscillator and the sun's day/night cycles is becoming an essential factor in understanding and solving the oscillator's molecular mechanism. The transition from day to night in an organism's biological system can be seen through the use of photoreceptors, which detect light (Liu, 2003; Millar, 2003). However, it is interesting that in cyanobacteria, no direct light detection mechanism is found yet. Instead, it uses intrinsic means for sensing the light-dark (LD) cycle by a few genes and their subsequent proteins, the ratio of ATP/ADP, and the quinone pool's oxidation state. Research has shown that resetting the KaiC

phosphorylation circadian phase can be done by adjusting the ATP/ADP ratio in vitro to mimic changes in vivo during phase-resetting of darkness (Rust, Golden, & O'Shea, 2011). There are three known redox sensing proteins: LdpA, CikA, and KaiA (Figure 1.3) (Schmitz et al. 2000; Katayama et al. 2003; Ivleva et al. 2005). CikA senses the plastoquinone pool's redox state, which, varies in photosynthetic organisms, depending on the light. In addition, CikA interacts with the KaiA proteins of the circadian oscillator and affects the phosphorylation state of KaiC during the resetting of the circadian phase by a dark pulse. The input pathway's role is two-fold: to recognize environmental zeitgebers and transmit the information to the oscillator.

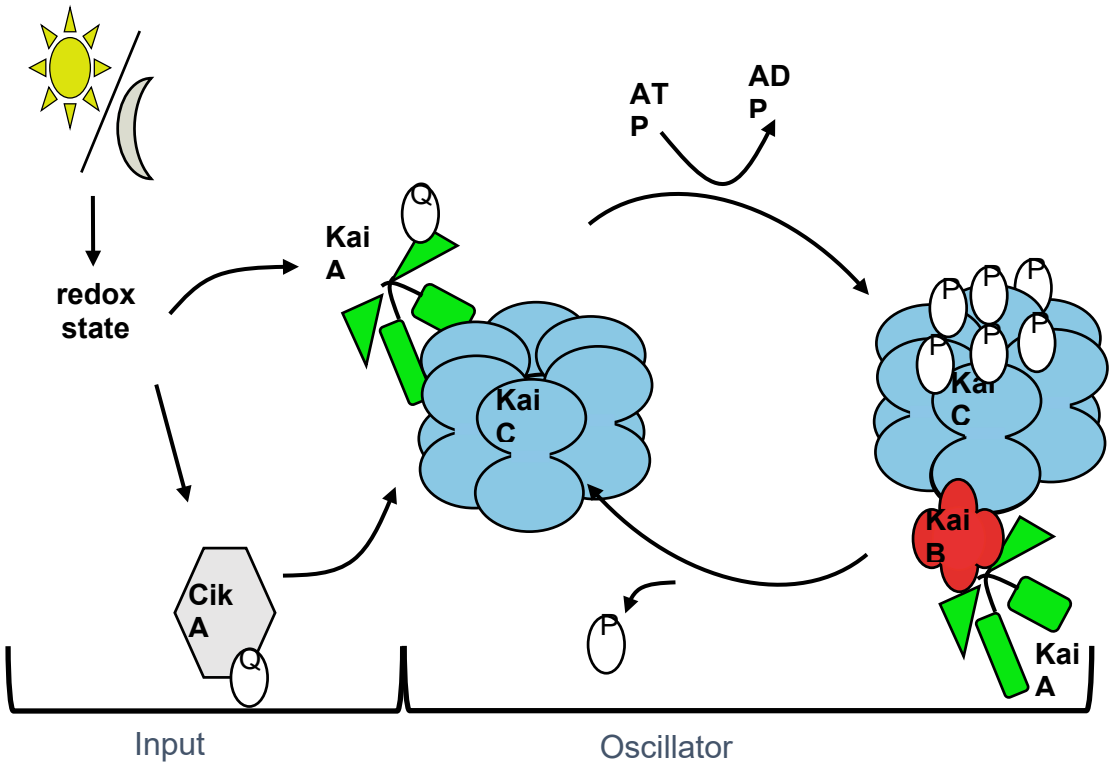


Figure 1.3 The input component interaction with the oscillator component.

C. Output Component

The circadian output components CikA, SasA, and RpaA, connect to the Kai oscillator to regulate gene expression. They relay the circadian oscillator's timing information to output pathways that control rhythmic biological phenomena within a single-celled cyanobacterium (Takai N et al. 2006, Taniguchi et al., 2010; Markson et al., 2013).

The circadian clock output component interaction with the oscillator component takes place (Figure 1.4) when SasA gets bound to phosphorylated KaiC and becomes phosphorylated on its own. The phosphorylated SasA transfers its phosphate group to RpaA, and, in turn, RpaA becomes phosphorylated. RpaA-P then suppresses the expression of class 2 genes, which are associated with phosphorylation, and activates class 1 genes, resulting in dephosphorylation. As light diminishes, KaiC enters a state where KaiB can bind it. KaiB is structurally similar to the SasA domain and competes with SasA for binding. As the night progresses, KaiB sequesters KaiA and attracts CikA. CikA gets dephosphorylated on binding with KaiB-C complex, resulting in the dephosphorylation of RpaA. This interaction between SasA, CikA, and the Kai oscillator creates a rhythm of RpaA phosphorylation that peaks at night (Markson, Piechura, Puszynska, & O'Shea, 2013).

Moreover, RpaA is a transition factor, and its phosphorylated form binds to activate gene transcription. The non-phosphorylated form cannot bind to the promoter of the gene. Thus, no transcription occurs when RpaA is not phosphorylated. The oscillation of phosphorylated RpaA is reduced at dawn and increases at dusk, creating a rhythmic expression.

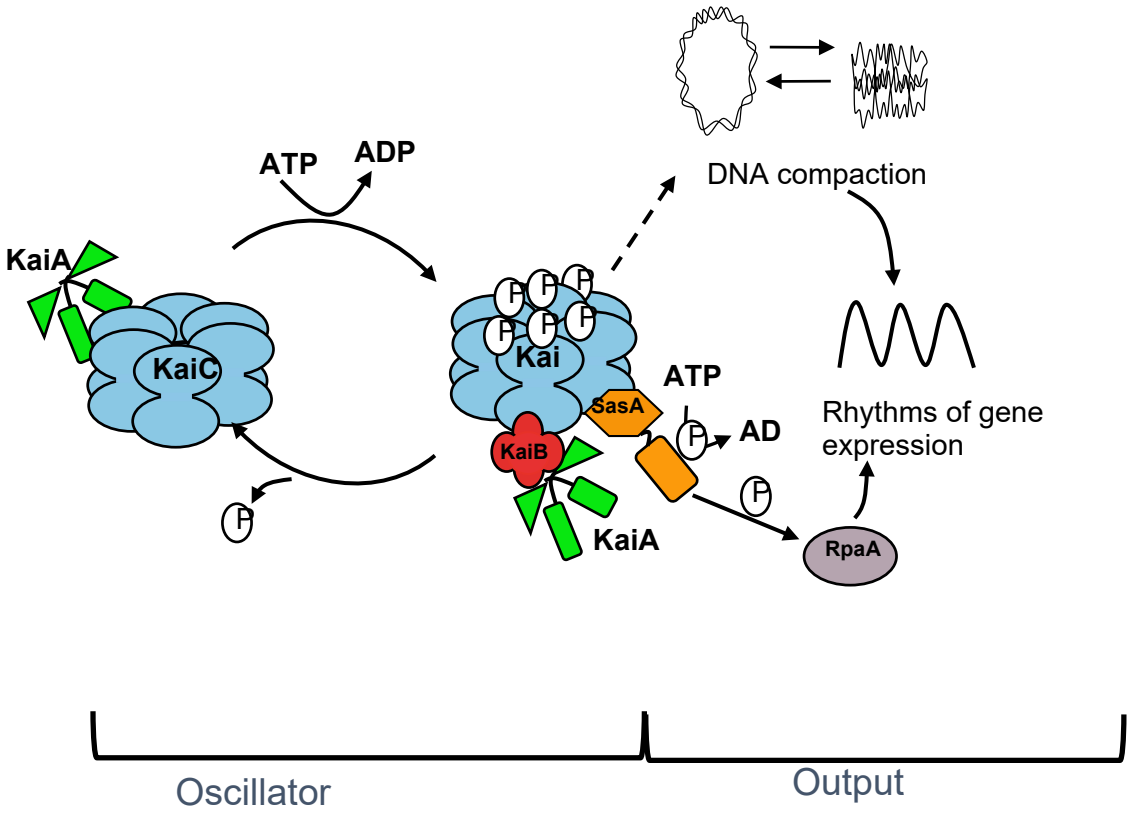


Figure 1.4 The output component interaction with the oscillator component.

The circadian clock is an endogenous timekeeping mechanism that is advantageous for life in a rhythmically changing environment. The gene expression, metabolism, physiology, and behavior of almost all light-perceiving organisms living on Earth are governed by the circadian (~24-hour) clock, which anticipates a daily rhythm in sunlight and ambient temperature.

1.4 Cyanobacteria Circadian Clock Entrainment and Biochemical Activity

Photoautotrophic cyanobacteria are microorganisms that entirely depend on photosynthesis to extract energy from the environment. The cyanobacterial circadian clock KaiABC generates ~24-h oscillations. Still, the clock's ability to reset its phase in response to

external cues is critical for proper synchronization with the Earth's rotation. The dark/light cycles of KaiABC proteins are not the only key factors that control the clock oscillations. Together with input and output pathways, it makes a complete clock system that coordinates the Kai oscillation with the environmental day-night cycle, which controls cellular processes at the time of day that is most beneficial to the organism. The metabolic rhythms produced during the day due to photosynthesis help cyanobacterial growth while anticipating night; it allows the cell to prepare for the absence of photosynthetic energy by storing its energy in the form of glycogen granules. This biochemical finding implies that cyanobacteria circadian clock is derived from the photosynthesis mechanism, independent of light or dark.

Biochemical studies also revealed that the cyanobacteria oscillations regulate by the light/dark cycle and magnesium concentration, ATP/ADP ratio, and redox metabolites (Wood et al., 2010) can also regulate it. Researchers had shown that the KaiC phosphorylation phase resets by altering the ATP/ADP ratio. During the dark phase, the ATP/ADP ratio drops gradually rather than acutely and reaches a threshold ratio, which induces a phase shift after ~two h of darkness. Pulses of oxidized quinone affect the ability of KaiA to stimulate KaiC autophosphorylation, due to which the cyanobacterial circadian clock phase gets reset. My recent publication on how magnesium regulates the circadian clock used structural analysis to reveal that magnesium regulates the phosphorylation and dephosphorylation of KaiC by association or dissociation on catalytic Glu residues that activate phosphorylation (Young M. Jeong et al., 2019). In the absence of KaiA, high magnesium concentration can dephosphorylate KaiC, whereas low magnesium concentration phosphorylates KaiC. The circadian input kinase protein (CikA; Schmitz et

al. 2000) appears to help synchronize the circadian clock photosynthetic activity: When KaiC is fully phosphorylated (Kim et al., 2008), KaiB binds to the B-loop of KaiC and sequesters KaiA from the A-loop of KaiC (Chang et al., 2015). If CikA is present at this point, it competes with KaiA for the binding site on KaiB. The displaced KaiA can bind to the A-loop to activate the kinase activity of KaiC. As a result, CikA indirectly alters the phosphorylation state of KaiC. These biochemical findings move the focus of cyanobacterial circadian clock research from studying the molecular mechanism of core proteins to exploring how they sense metabolic changes that are driven by photosynthesis, perhaps without the need for direct perception of light or dark cycles

CHAPTER 2

CIKA MODULATES THE EFFECT OF KAIA ON THE PERIOD OF THE CIRCADIAN OSCILLATION IN KAIC PHOSPHORYLATION

2.1 Abstract

Cyanobacteria contain a circadian oscillator that can be reconstituted in vitro. In the reconstituted circadian oscillator, the phosphorylation state of KaiC oscillates with a circadian period, spending about 12 h in the phosphorylation phase and another 12 h in the dephosphorylation phase. Although some entrainment studies have been performed using the reconstituted oscillator, they were insufficient to fully explain entrainment mechanisms of the cyanobacterial circadian clock due to the lack of input pathway components in the in vitro oscillator reaction mixture. Here, we investigate how an input pathway component, CikA, affects the phosphorylation state of KaiC in vitro. In general, CikA affects the amplitude and period of the circadian oscillation of KaiC phosphorylation by competing with KaiA for the same binding site on KaiB. In the presence of CikA, KaiC switches from its dephosphorylation phase to its phosphorylation phase prematurely, due to an early release of KaiA from KaiB as a result of competitive binding between CikA and KaiA. This causes hyperphosphorylation of KaiC and lowers the amplitude of the circadian oscillation. The period of the KaiC phosphorylation oscillation is shortened by adding increased amounts of CikA. A constant period can be maintained as CikA is increased by proportionally decreasing the amount of KaiA. Our findings give insight into how to reconstitute the cyanobacterial circadian clock in vitro by the addition of an input pathway component, and explain how this affects circadian oscillations by directly interacting with the oscillator components.

Keywords CikA, cyanobacteria, Circadian clock, input pathway, in vitro

2.2 Introduction

A cyanobacterium is the simplest organism to have a circadian clock (Iwasaki and Kondo, 2004). Circadian clocks consist of three major components: a central oscillator, an input pathway, and an output pathway (Takahashi, 2004). The central oscillator in cyanobacteria is known as the minimal self-sustained circadian oscillator and is composed of three proteins, KaiA, KaiB, and KaiC. The oscillator can be reconstituted in vitro by mixing the Kai proteins with adenosine triphosphate (ATP) and magnesium (Kim et al., 2015; Nakajima et al., 2010). The phosphorylation/ dephosphorylation state of KaiC exhibits a circadian rhythm regulated by alternating autokinase and autophosphatase activity (Kitayama et al., 2008). KaiC alone undergoes dephosphorylation in vitro due to its innate phosphatase activity (Kim et al., 2008). KaiA turns on the kinase activity of KaiC by binding to the A-loop, whereas KaiB turns the kinase activity off by sequestering KaiA from the A-loop (Chang et al., 2015; Kim et al., 2008).

Although the Kai proteins alone produce a robust circadian rhythm in vitro, there are several other proteins whose mutation results in abnormal rhythms in vivo (Boyd et al., 2013; Ivleva et al., 2006). In this study, we focus on the properties of CikA, a histidine kinase that has multiple effects on the in vivo clock (Schmitz et al., 2000). CikA-null mutants exhibit smaller magnitude phase shifts in response to dark pulses, and oscillate with a shortened period and reduced amplitude (Schmitz et al., 2000; Zhang et al., 2006). Based on these observations, CikA was originally classified as an input pathway component (circadian input kinase A). However, recent biochemical evidence that CikA acts to dephosphorylate the output pathway transcription factor RpaA suggests this may be a misnomer, and that the CikA-null phenotype arises from a disrupted output signal (Gutu

and O'Shea, 2013). To distinguish between these possibilities, the physical properties of the oscillator must be monitored instead of the output. For this purpose, examining the reconstituted oscillator is advantageous because monitoring KaiC phosphorylation is easier in vitro than in vivo. Here we used the reconstituted in vitro oscillator to study the effect of CikA on KaiC phosphorylation.

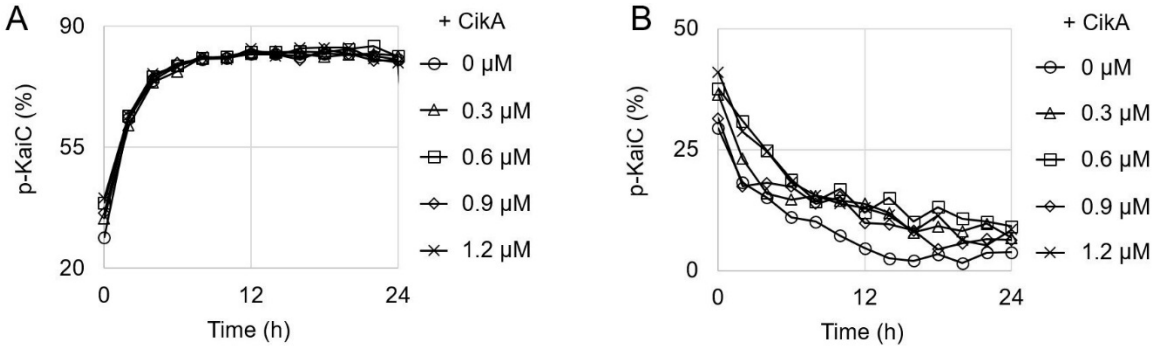


Figure 2.1 Direct effect of CikA on KaiC phosphorylation. (A) Phosphorylation traces of KaiC. KaiA (1.2 μM), KaiC (3.4 μM), and CikA (see the legend for concentrations) were mixed with adenosine triphosphate (ATP). The p-KaiC represents the percentage of total KaiC that is phosphorylated. The markers represent the experimental data points for KaiC phosphorylation (every 2 h). The graph is the best representative of 3 replicates. (B) Dephosphorylation traces of KaiC. KaiB (3.4 μM) was added instead of KaiA in the same reaction mixtures as in (A). Everything else is the same as in (A).

As mentioned above, it has been suggested that CikA also functions as an output pathway component by acting as a phosphatase against RpaA, a transcription factor regulated by the circadian clock (Gutu and O'Shea, 2013; Markson et al., 2013). CikA's phosphatase activity against RpaA is activated when CikA binds to KaiB (Chang et al., 2015; Tseng et al., 2017). Analysis of the three-dimensional structure of the KaiBC protein complex has shown that CikA binds to KaiB at the same binding site that is used to sequester KaiA (Tseng et al., 2017). In this study, we explore the consequences of this competitive interaction between CikA and KaiA on the ability of KaiA to stimulate KaiC

phosphorylation. To examine whether CikA directly affects the phosphorylation and dephosphorylation of KaiC, the phosphorylation state of KaiC was monitored with CikA added to the reaction mixtures (3.4 μM KaiC, 150 mM NaCl, 20 mM Tris-HCl, 5 mM MgCl2, 0.5 mM EDTA, 1 mM ATP; pH 8.0). All assays were performed as described previously (Kim et al., 2015) without any modification. CikA was purified using the same method as used for KaiC purification. Various concentrations of CikA were added to the reaction mixtures at time zero, with KaiA (1.2 μM) or KaiB (3.4 μM) for phosphorylation (Figure 2.1A) or dephosphorylation (Figure 2.1B), respectively. The phosphorylation state of KaiC was monitored for 24 h by collecting 20 μL of the reaction mixture every 2 h to run sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Because the addition of CikA did not change the phosphorylation state of KaiC appreciably (Figure 2.1A and 2.1B), we concluded that CikA does not directly affect the phosphorylation and dephosphorylation of KaiC. It has been suggested that CikA competes with KaiA for the binding site on KaiB to transfer the circadian signal generated by the circadian oscillator to the transcription factor (Chang et al., 2015). Structural analysis reveals that both CikA and KaiA bind competitively to the same site on KaiB bound to fully phosphorylated KaiC (Tseng et al., 2017). To observe the effect of this competitive binding on KaiC phosphorylation, we added various concentrations of CikA to the circadian oscillation reaction mixtures composed of KaiA, KaiB, and KaiC (Figure 2.2A). The amplitudes of the oscillations decreased with each increase in CikA concentration (Figure 2.2B). Compared with the oscillation in the absence of CikA, the amplitudes were decreased by 17% at 0.3 μM, 32% at 0.6 μM, 37% at 0.9 μM, and 54% at 1.2 μM of CikA.

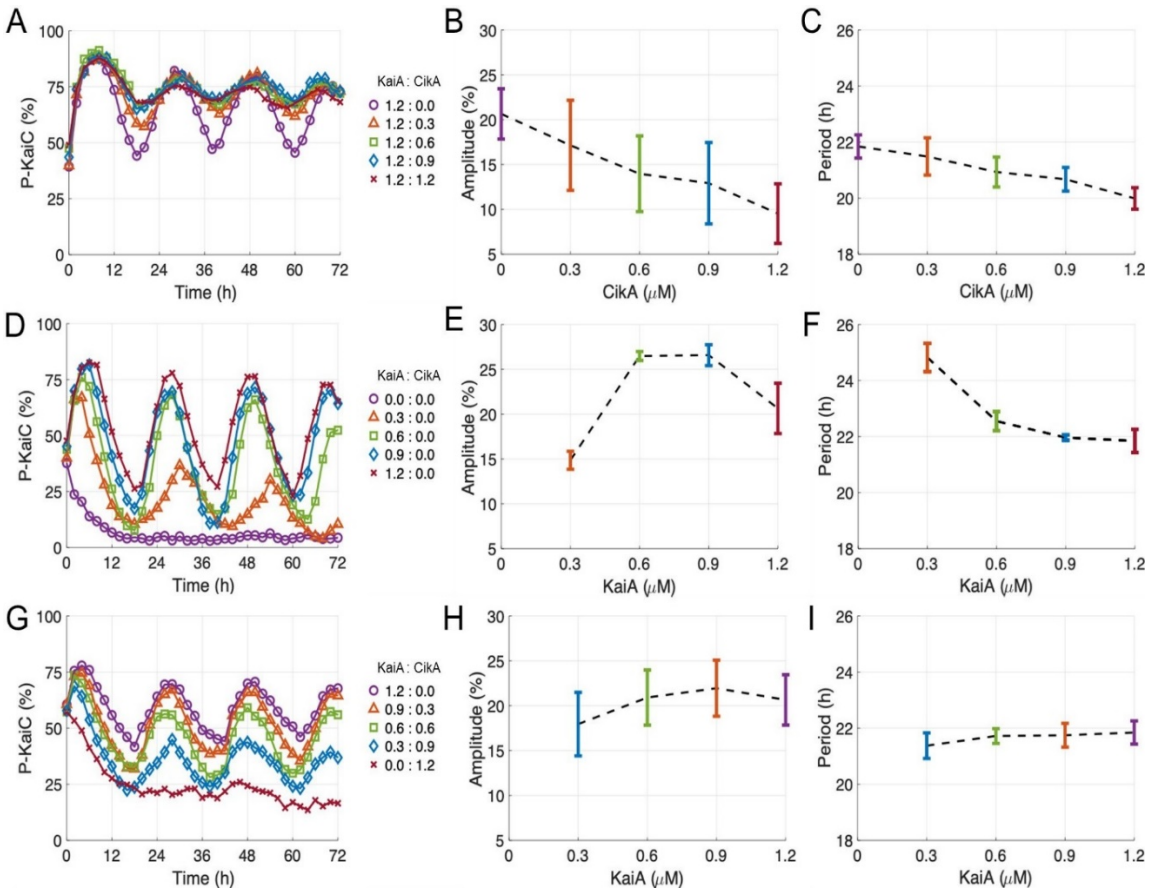


Figure 2.2 Effect of CikA on KaiC phosphorylation. Oscillation amplitude and period for various concentrations of KaiA and CikA (μM, listed as KaiA: CikA in legends) were calculated using FFT-NLLS. Error bars show standard error of the mean (SEM) from triplicates (except for the KaiA: CikA = 1.2:0.0 case, which had 6 replicates). (A) Circadian oscillation of KaiC phosphorylation with various amounts of CikA added to the reaction mixture of KaiA (1.2 μM), KaiB (3.4 μM), and KaiC (3.4 μM). Everything else is the same as in (Figure 2.1A). (B) Amplitude of the KaiC phosphorylation oscillation for the CikA concentrations in (A). (C) Period of the KaiC phosphorylation oscillation with the CikA concentrations in (A). (D) Circadian oscillation of KaiC phosphorylation with various amounts of KaiA added to the reaction mixture of KaiB (3.4 μM) and KaiC (3.4 μM). Everything else is the same as in (Figure 2.1A). (E) Amplitude of the KaiC phosphorylation oscillation with the KaiA concentrations in (D). (F) Period of the KaiC phosphorylation oscillation with the KaiA concentrations in (D). (G) Circadian oscillation of KaiC phosphorylation with various amount of KaiA and CikA added to the reaction mixture of KaiB (3.4 μM) and KaiC (3.4 μM). Everything else is the same as in (Figure 1A). (H) Amplitude of the KaiC phosphorylation oscillation with the KaiA and CikA concentrations in (G). (I) Period of the KaiC phosphorylation oscillation with the KaiA and CikA concentrations in (G).

Phosphorylation levels at the peaks of the oscillation were not affected much by increasing the CikA concentration in the reaction mixtures (4% decreased with 1.2 μM of

CikA); however, levels at the troughs were increased by 22% with 1.2 μM of CikA (Figure 2.2A). Thus, overall, KaiC maintains a hyperphosphorylated state in the presence of CikA. CikA affected not only the amplitude but also the period of the circadian oscillation of KaiC phosphorylation. We computed period estimates from our data using two different methods: the Biodare2 (https://biodare2.ed.ac.uk/) implementation of the fast Fourier trans form-nonlinear least squares (FFT-NLLS) procedure with linear detrending (Zielinski et al., 2014), and Lomb-Scargle periodograms with the MATLAB function plomb (Mathworks Inc., Natick, MA). These two approaches yielded very similar period estimates. We found that periods were shortened by increasing the CikA concentration in the reaction mixture (Figure 2.2C). This result is compatible with a previous report performed with a controlled expression of CikA in cyanobacteria, in which increasing CikA expression results in shorter period (Zhang et al., 2006). This hyperphosphorylation and the shortened period closely resemble the result of excess KaiA in the circadian oscillation reaction mixture (Kageyama et al., 2006; Nakajima et al., 2010). Because CikA competes with KaiA for binding to KaiB (Tseng et al., 2017), the addition of CikA in the circadian oscillation mixture leads to an increase in the concentration of free KaiA, which can bind to the A-loop of KaiC to activate the autokinase activity of KaiC (Kim et al., 2008). Since the addition of CikA produces a similar effect as the presence of excess KaiA in the reaction mixture (Kageyama et al., 2006; Nakajima et al., 2010; Rust et al., 2007), we decided to explore how varying CikA and KaiA concentrations in tandem affects the oscillation. Earlier, it was reported that KaiA concentration in the reaction mixture is an important variable for the circadian period of KaiC phosphorylation. Previous reports (Kageyama et al., 2006; Nakajima et al., 2010) and our data demonstrate that decreasing

KaiA concentration lengthens the period in the absence of CikA (Figure 2.2D-F). We hypothesized that, in the presence of CikA, decreasing KaiA would not lead to lengthened periods, because more free KaiA would be released during the dephosphorylation phase of KaiC due to the competition with CikA for the binding site of KaiB. To test our hypothesis, we decreased the concentration of KaiA in the reaction mixture while simultaneously increasing the CikA concentration. The sum of the KaiA and CikA concentrations was maintained at 1.2 μM, which is the KaiA concentration specified in the original cyanobacterial circadian oscillation reaction mixture (Nakajima et al., 2005). The phosphorylation state of KaiC was monitored for 3 days to analyze the oscillatory properties of the reaction mixture. Differing from Figure 2.2D, here the phosphorylation state of KaiC maintained robust circadian rhythms with a stable circadian period as the CikA concentration was increased (Figure 2.2G-I). In particular, the rhythms with small amplitudes at high concentrations of CikA in Figure 2.2B (up to a CikA concentration of 0.9 μM) can be recovered (at least 86%) into a robust amplitude circadian rhythm by decreasing the KaiA concentration (Figure 2.2H). All reaction mixtures showed a similar circadian period (within an hour difference of each other), even though the KaiA and CikA concentration ratios were different in each reaction mixture (Figure 2.2I). Thus, in the presence of CikA, a stable circadian oscillation can be generated with decreased KaiA concentrations despite KaiA concentration being an important variable for the period of KaiC phosphorylation (Kageyama et al., 2006; Nakajima et al., 2010). To explain this observation, we propose that CikA enhances the ability of KaiA to stimulate KaiC phosphorylation by competing KaiA out of its sequestered state. Specifically, because CikA competes with KaiA for the binding site on KaiB, it increases free KaiA, which

stimulates the phosphorylation of KaiC by stabilizing the exposed conformation of the A-loop. Therefore, more CikA in the reaction mixture can release more free KaiA by occupying its binding site on KaiB, keeping the kinase activity of KaiC turned on with comparatively small amounts of total KaiA available.

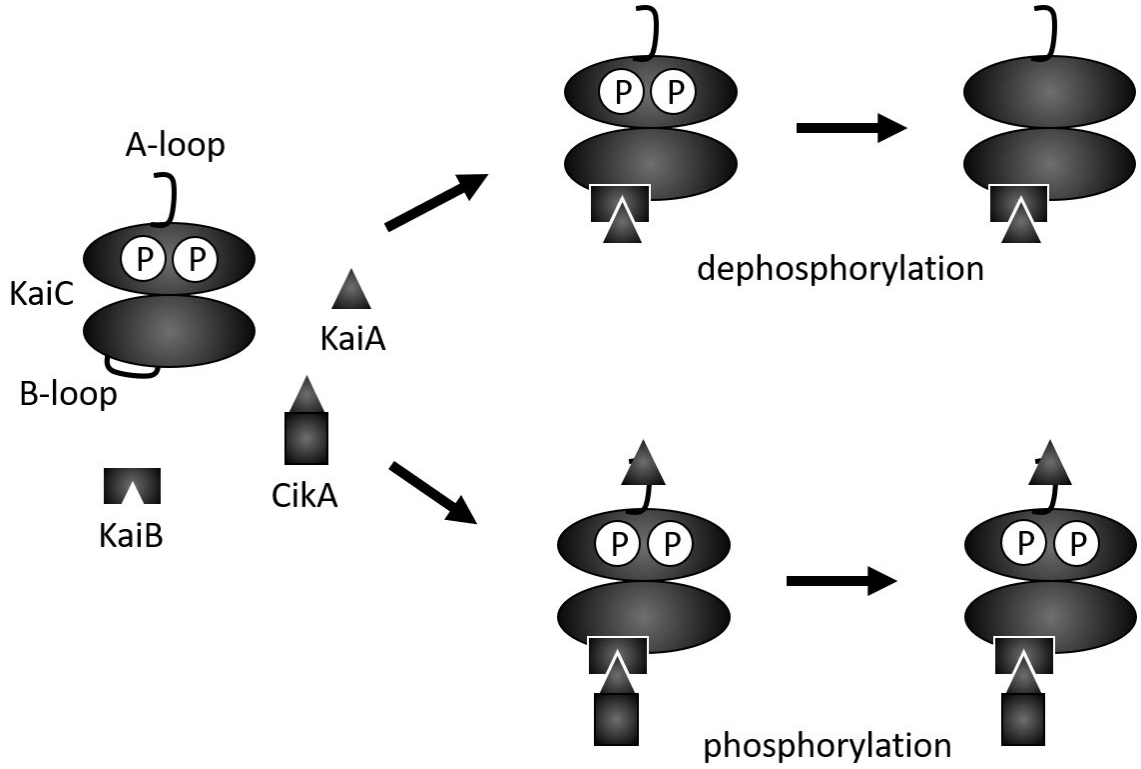


Figure 2.3 Model for the effect of CikA on KaiC phosphorylation. When KaiC is fully phosphorylated during the day, KaiB binds to the B-loop of KaiC and sequesters KaiA. CikA competes with KaiA for the binding site on KaiB. If CikA occupies the binding site, the KaiA released from KaiB binds to the A-loop of KaiC and keeps KaiC hyperphosphorylated.

In conclusion, we found that CikA affects the physical properties of the circadian oscillator, which implies input functionality. Although CikA does not have a large effect on the self-sustained period, it is still possible that day/night alternations in CikA

abundance in cyanobacteria (Ivleva et al., 2005) play a role in entraining the KaiABC oscillator, since small changes in the self-sustained period can have a large effect on the entrained phase (Granada et al., 2013). We also propose a model for the mechanism of how CikA influences KaiC phosphorylation (Figure 2.3): When KaiC is fully phosphorylated (Kim et al., 2008), KaiB binds to the B-loop of KaiC and sequesters KaiA from the A-loop of KaiC (Chang et al., 2015). If CikA is present at this point, it competes with KaiA for the binding site on KaiB. The displaced KaiA can bind to the A-loop to activate the kinase activity of KaiC (Figure 2.3). As a result, CikA indirectly alters the phosphorylation state of KaiC.

Many current mathematical models of the cyanobacterial circadian oscillator have been made only considering interactions between the Kai proteins (Das et al., 2017; Leypunskiy et al., 2017; Miyoshi et al., 2007). By introducing CikA into the cyanobacterial circadian oscillator as an input component, building a more accurate model of the cyanobacterial circadian clock is possible. Furthermore, the functionality of CikA as an input component has not been examined extensively in vitro. Although a few in vitro entrainment studies have been performed CikA, their results were partially inconsistent with in vivo experiments (Kim et al., 2012; Rust et al., 2011). Here, we have successfully generated a stable circadian period in the presence of CikA by varying the ratio between KaiA and CikA. This finding leads us one step closer to the circadian clock's in vitro reconstitution, not just the circadian oscillator, by adding the input and the output components to the reaction mixture. This will enable further studies on the entrainment of cyanobacterial circadian rhythms using phase response curves (Johnson, 1999) and entrainment maps (Diekman and Bose, 2016).

CHAPTER 3

MAGNESIUM REGULATES THE CIRCADIAN OSCILLATOR IN CYANOBACTERIA

3.1 Abstract

The circadian clock controls 24-h biological rhythms in our body, influencing many time-related activities such as sleep and wake. The simplest circadian clock is found in cyanobacteria, with the proteins KaiA, KaiB, and KaiC generating a self-sustained circadian oscillation of KaiC phosphorylation and dephosphorylation. KaiA activates KaiC phosphorylation by binding the A-loop of KaiC, while KaiB attenuates the phosphorylation by sequestering KaiA from the A-loop. Structural analysis revealed that magnesium regulates the phosphorylation and dephosphorylation of KaiC by dissociating from and associating with catalytic Glu residues that activate phosphorylation and dephosphorylation, respectively. High magnesium causes KaiC to dephosphorylate, whereas low magnesium causes KaiC to phosphorylate. KaiC alone behaves as an hourglass timekeeper when the magnesium concentration is alternated between low and high levels in vitro. We suggest that a magnesium-based hourglass timekeeping system may have been used by ancient cyanobacteria before magnesium homeostasis was established.

Keywords circadian clock, KaiC, phosphorylation, hourglass, autokinase, PhoQ

3.2 Introduction

Almost all organisms on earth have adapted to environmental cycles by developing their own timekeeping system, a circadian clock, to predict daily changes. The simplest circadian model system is found in cyanobacteria, a single-celled organism. The central oscillator of the cyanobacterial circadian clock is composed of only 3 proteins, KaiA, KaiB, and KaiC (Golden and Canales, 2003). Because oscillations continue without transcriptional-translational feedback loops (Tomita et al., 2005), the cyanobacterial circadian clock can be reconstituted in vitro by mixing those three proteins and adenosine 5′-triphosphate (ATP) with magnesium ions (Nakajima et al., 2005). KaiC undergoes rhythmic autophosphorylation and autodephosphorylation with a 24-h period in the presence of both KaiA and KaiB (Iwasaki and Kondo, 2004). These autokinase and autophosphatase activities in KaiC are regulated by the conformation of the A-loop, which is known as a day/night switch located in the C-terminus of KaiC (residues 487-519; Kim et al., 2008). Without KaiA and KaiB, KaiC alone undergoes dephosphorylation in the in vitro reaction because the default conformation of the A-loop is the "buried" state (Kim et al., 2008). The A-loop changes its conformation from the buried to the "exposed" state by binding KaiA (Kim et al., 2008; Vakonakis and LiWang, 2004). When the A-loop is in the exposed conformation, kinase activity is predominant and KaiC is phosphorylated. KaiA keeps the A-loop in the exposed conformation by binding the A-loop with differential affinity, while KaiB returns the A-loop to a buried conformation by sequestering KaiA far from the A-loop (Snijder et al., 2017; Tseng et al., 2017). At the active site of KaiC, S431 and T432 residues are phosphorylated and dephosphorylated sequentially over the daily cycle (Nishiwaki et al., 2007; Rust et al., 2007). Because the active site is distal from the

A-loop, the kinase and phosphatase activity of KaiC may be regulated by an allosteric effect that changes the conformation of the active site. Currently, the mechanism acting downstream of the A-loop is unknown. In this study, we found that magnesium is a key element regulating the kinase and phosphatase activity of KaiC as a downstream regulatory element of the A-loop. Based on our findings, we suggest a possible direction of the evolution of the cyanobacterial circadian oscillator in cyanobacteria.

3.3 Materials And Methods

3.3.1 Cloning, Protein Purification, and Expression

Cloning and purifications were performed essentially as described previously (Kim et al., 2015; Kim et al., 2012). The KaiC E318D protein expression plasmid was generated using the site-directed mutagenesis method.

3.3.2 Structure Analysis of KaiC Hexamer

Every structure analysis was performed with UCSF Chimera (Pettersen et al., 2004). Distance measurements between OGlu and OThr were performed with the "Structure Analysis" function in UCSF Chimera.

3.3.3 In Vitro Assay of KaiC Phosphorylation

All assays were performed essentially as described previously (Kim et al., 2015) with the following modification: ethylenediaminetetraacetic acid (EDTA) was completely removed for all reactions that were performed without KaiA. The magnesium concentration was modified as indicated in the main text. Disodium ATP (initial concentration: 1 mM), rather

than MgATP, was used for all in vitro reactions to avoid the addition of magnesium ions from another source.

3.3.4. Molecular Dynamics Simulation of KaiC Hexamer

All-atom molecular dynamics simulations were performed using GROMACS version 5.1. The Charmm36 force field was used to account for the different interactions between the atoms of the system that comprised the KaiC protein in the hexameric state solvated with 60,000 to 70,000 TIP3P water molecules. For the initial conformation of the KaiC hexamer, we used its experimentally resolved crystal structure (i.e., PDB ID: 1U9I), wherein positions of each atom of residues 14-497 of KaiC were given. We also studied a variant of this experimental structure in which the A-loop (residue 489-497) was deleted from the PDB entry. The latter mimics the exposed conformation of the A-loop, in which KaiC becomes phosphorylated. Moreover, to study the effects of magnesium, two sets of simulations were performed for these two initial conformations: one with magnesium ions and the other without them. The initial location of the magnesium atoms was indicated in the PDB structure. The net charge of the system was neutralized by adding Na and Cl ions to the solvent. The four systems examined in this study underwent energy minimization as well as equilibration in the NVT ensemble for 10 ns at 300 K, with heavy atoms of the protein restrained to their initial positions via 1000 kcal mol−1Å−2 springs. The leap-frog algorithm with a time step of 2 fs was used to integrate the equations of motion, and the neighbor list was updated every 10 steps. A Lennard-Jones cutoff of 1.0 nm was used. Electrostatics were treated using the smooth particle mesh Ewald method with a grid spacing of 0.13 nm and a 1.0 nm real-space cutoff. The production run was performed in the NPT ensemble (1 atm and 300 K), in which the temperature was controlled using the

v-rescale thermostat (τT = 0.1 ps) and the pressure was controlled using the Parrinello-Rahman barostat (τP = 1 ps). The total simulation time of the production runs was 50 ns.

3.3.5. Hourglass Timer In Vitro

The KaiC (3.4 μM, 2 mL)–alone reaction mixture was prepared with the modified in vitro reaction buffer (150 mM NaCl, 20 mM Tris-HCl, 5 mM MgCl2, pH = 8.0). The reaction mixture was incubated at 30 °C for 16 h to completely dephosphorylate KaiC. The incubated reaction mixture was passed through a spin desalting column (Zeba spin desalting column, Fisher Scientific, Waltham, MA) at 100 × g for 3 min. The protein concentration was measured using the Bradford protein assay kit and adjusted by concentrating it with a spin concentrator (Vivaspin 2, Millipore, Burlington, MA). The reaction mixture was incubated at 30 °C for 12 h for phosphorylation. During the incubation, 20 μL of the reaction mixture was mixed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading dye every 2 h for 12 h. Then, 5 μL of highly concentrated magnesium solution in the modified in vitro reaction buffer was added for dephosphorylation and continuously incubated at 30 °C for 12 h. Sampling was performed in the same manner. The removal and addition of magnesium were performed repeatedly for 2 days to simulate light/dark alterations in magnesium concentration. After collecting all the reaction samples, SDS-PAGE was performed with the previously reported method to analyze the phosphorylation state of KaiC (Kim et al., 2015). The KaiB and KaiC reaction mixture for testing the hourglass timer was performed in the same manner as the KaiC-alone reaction mixture.

3.3.6 Mathematical Modeling of Hourglass Timer

Our mathematical model of magnesium-dependent phosphorylation and dephosphorylation cycles of KaiC in the absence of KaiA and KaiB is based on the Rust model of ordered multisite phosphorylation for the KaiABC oscillator (Rust et al., 2007). The model is a three-dimensional system of ordinary differential equations that captures the temporal dynamics of KaiC in four different forms: unphosphorylated (U), singly phosphorylated at the T432 site (T), singly phosphorylated at the S431 site (S), and doubly phosphorylated at both the S431 and T432 sites (D). Our model equations are as follows:

$$\frac{dT}{dt} = k_{UT}U + k_{DT}D - k_{TU}T - k_{TD}T$$

$$\frac{dS}{dt} = k_{US}U + k_{DS}D - k_{SU}S - k_{SD}S$$

$$\frac{dD}{dt} = k_{TD}T + k_{SD}S - k_{DT}D - k_{DS}D$$

$$U = [\mathrm{KaiC}] - T - S - D$$

$$k_{XY} = k_{XY}^{B} + \left(k_{XY}^{M} - k_{XY}^{B}\right) f\left([\mathrm{Mg}]\right)$$

$$f\left([\mathrm{Mg}]\right) = \frac{[\mathrm{Mg}]^n}{(K_A)^n + [\mathrm{Mg}]^n}$$

where k_{XY} are the rate constants for transitions from state X to state Y, [KaiC] = 3.4 μM is the concentration of KaiC, [Mg] is the concentration of magnesium, K_A is the concentration at which the effect of magnesium on the transition rates is half-maximal, and n is the Hill coefficient.

Because the total amount of KaiC is conserved, [KaiC] is constant and U can be obtained algebraically from the amounts of the other phosphoforms. The transition rates

consist of baseline rates k^{B}_{XY} in the absence of magnesium and k^{M}_{XY} at high magnesium concentrations. If $k^{M}_{XY} > k^{B}_{XY}$ ($k^{M}_{XY} < k^{B}_{XY}$), then magnesium promotes (inhibits) that transition. We fit these baseline rates assuming first-order kinetics for the two limiting cases of high and low magnesium concentrations, respectively. First, suppose that [Mg] is sufficiently high such that the magnesium effect has saturated, that is, $f([\mathrm{Mg}]) \approx 1$ and $k_{XY} \approx k^{M}_{XY}$. These experimental conditions are similar to those indicated in Fig. 2B of Rust et al. (2007), in which autonomous dephosphorylation of KaiC was observed after removing KaiA. Thus, we used the parameters that Rust et al. estimated from those data, namely, $k^{M}_{TU} = 0.21$, $k^{M}_{SU} = 0.11$, $k^{M}_{DS} = 0.31$, and $k^{M}_{DT} = k^{M}_{UT} = k^{M}_{US} = k^{M}_{SD} = k^{M}_{TD} = 0\ \mathrm{h}^{-1}$. Second, suppose [Mg] = 0 and $k_{XY} = k^{B}_{XY}$. By fitting our experimental data on the autonomous phosphorylation of KaiC in the absence of magnesium (Suppl. Figure A.1), we obtained the parameters $k^{B}_{TU} = 0.5412$, $k^{B}_{SU} = 0.0530$, $k^{B}_{DS} = 0.1853$, $k^{B}_{DT} = 0$, $k^{B}_{UT} = 0.3623$, $k^{B}_{US} = 0.0267$, $k^{B}_{SD} = 0.0601$, and $k^{B}_{TD} = 0.1269\ \mathrm{h}^{-1}$. These parameters were found by minimizing the total least-squares error between the model and data for T-KaiC, S-KaiC, and ST-KaiC using MATLAB's constrained nonlinear optimization routine *fmincon* with the default interior point algorithm. We set parameter lower bounds of 0 and initial parameter guesses equal to the values of $k^{0}_{XY} + k^{A}_{XY}$ given in Rust et al. (2007).

To determine the remaining parameters K_A and n, we fit the model to the experimental hourglass data. In the simulations, we set [Mg] = 0 mM during the light periods and to the values 0.05, 0.1, 0.5, 1.0, and 5.0 mM during the dark periods. We used *fmincon* to minimize the total least-squares error between the model and data for P-KaiC. With an initial guess of $K_A = 0.43$ and $n = 2$, the optimized parameters were $K_A = 0.0298$ and $n = 1.1470$. Setting $n = 1$ and optimizing K_A alone yields $K_A = 0.0256$ and less than a

1% increase in the error function. We selected the more parsimonious model with Michaelis-Menten kinetics for magnesium (n = 1) and show the hourglass simulation results for K_A = 0.0256.

Adenosine diphosphate (ADP) inhibits the kinase reaction involved in KaiC phosphorylation, and periodic changes in the ATP/ADP ratio can entrain the KaiABC oscillator (Rust et al., 2011). To incorporate the effect of the ATP/ADP ratio into our model, we followed Rust's model (Rust et al., 2011) and added a multiplicative term to the transition rates for the phosphorylation (but not the dephosphorylation) reactions:

$$k_{XY} = \frac{[\mathrm{ATP}]}{[\mathrm{ATP}] + K_{rel}\,[\mathrm{ADP}]}\,\left(k_{XY}^{B} + \left(k_{XY}^{M} - k_{XY}^{B}\right) f\left([\mathrm{Mg}]\right)\right)$$

where XY = *UT, US, SD*, and *TD*, and K_{rel} = 1 is the relative affinity for ADP versus ATP in the kinase reactions. Because darkness leads to a drop in the ATP/ADP ratio, we simulated an hourglass experiment with ATP = 100%/ADP = 0% during the light periods when magnesium is absent and ATP = 30%/ADP = 70% during the dark periods when magnesium is present.

3.4. Results

3.4.1 Magnesium Ions Are Necessary for the Dephosphorylation of KaiC

To obtain insight into how the allosteric effect of the A-loop induces a conformational change in the active site of KaiC phosphorylation, we analyzed the crystal structure of the KaiC hexamer from a cyanobacterium, *Synechococcus elongatus*. The E318 residue in the

active site is known as a catalytic base, which activates T432 for the phosphoryl transfer reaction by taking a proton from the hydroxyl group in T432 (thereafter H^{Thr}; Hayashi et al., 2004; Valiev et al., 2003). In the active form of the KaiC hexamer, a magnesium ion holds E318 to prevent free rotation and movement, which are necessary to activate T432 for the phosphoryl transfer reaction (Figure 3.1a). All distances between the oxygen atom in the carboxyl group of the E318 side chain (thereafter O^{Glu}) and the oxygen atom in the hydroxyl group of the T432 side chain (thereafter O^{Thr}) in the 6 subunits of the KaiC hexamer are longer than the hydrogen bonding distance (~3 Å; Figure 3.1b). With these distances and structural restrictions, E318 is unable to activate T432 for the phosphoryl transfer reaction in the presence of magnesium. We hypothesized that the O^{Glu} can move or rotate freely in the absence of magnesium and can activate T432 for the phosphoryl transfer reaction by taking the H^{Thr}. To test this hypothesis, in vitro reactions with KaiC alone were established with various magnesium concentrations, and the KaiC phosphorylation state of each reaction was monitored. In general, KaiC alone is dephosphorylated in the reconstituted in vitro reaction, which has a comparatively high (5 mM) magnesium concentration (Kim et al., 2008; Nakajima et al., 2005). In the KaiC-alone in vitro reaction with various magnesium concentrations, the intensity of the dephosphorylation was reduced when the magnesium concentration was decreased, ultimately reaching a minimum in the absence of magnesium (Figure 3.1c; Supplemental Figure S2a). Although KaiC did not show phosphorylation under these reaction conditions, the phosphatase activity disappeared completely in the absence of magnesium. Therefore, KaiC needs magnesium to be dephosphorylated and is possibly a magnesium-dependent phosphatase, which uses magnesium as a reaction center (Shi, 2009).

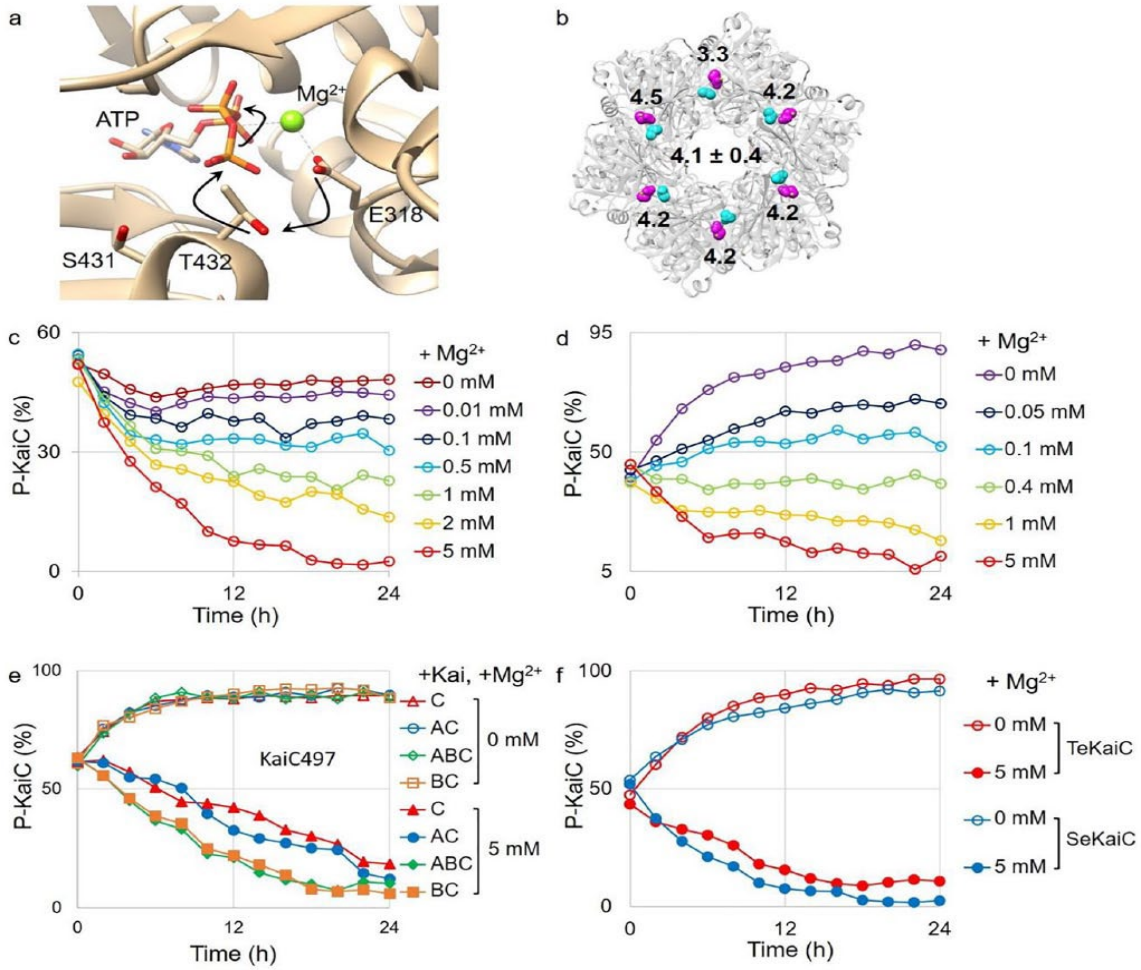


Figure 3.1 Magnesium regulates the phosphorylation and dephosphorylation of KaiC. (a) Crystal structure of an active site in the KaiC hexamer (PDB ID: 1U9I). The phosphate group on T432 was removed for clearance. Black arrows indicate the electron movements in the phosphoryl transfer reaction. (b) Distances (in angstroms, Å) between the oxygen atom in E318 and the oxygen atom in T432 are labeled on the crystal structure of the KaiC hexamer. Black represents E318, and gray represents T432. (c) Phosphorylation state of KaiC in the in vitro reaction with many different magnesium concentrations. Magnesium concentrations are labeled on the right of the graph. (d) Phosphorylation state of KaiC in the absence of ethylenediaminetetraacetic acid with many different magnesium concentrations. Magnesium concentrations are labeled on the right of the graph. (e, f) Phosphorylation state of the KaiC mutant and other species of cyanobacteria. Open markers indicate the absence of magnesium. Closed markers indicate the presence of magnesium (5 mM). KaiC497 was mixed with KaiC497 alone (▲), KaiA (•), KaiB (■), and KaiA and KaiB (♦) (e). Phosphorylation state of KaiC from *Thermosynechococcus elongatus* (TeKaiC) and from *Synechococcus elongatus* (SeKaiC) (f).

3.4.2 EDTA Inhibits the Phosphorylation of KaiC in the Absence of KaiA and Magnesium

Because KaiC alone did not show phosphorylation in the absence of magnesium, we hypothesized that at least one of the components in the reaction mixture possibly inhibits the kinase activity of KaiC. For the original in vitro oscillator developed by Kondo (Nakajima et al., 2005), EDTA is used as a metal chelator; however, EDTA is not present as a natural component in cyanobacteria. We removed EDTA from the in vitro reaction to determine whether the kinase activity was recovered. In the absence of magnesium and EDTA, KaiC was phosphorylated to the same level as that observed when KaiC was incubated with KaiA in vitro (Figure 3.1d; Supplemental Figure S2b). KaiC was still dephosphorylated with higher magnesium concentrations in the absence of EDTA. The removal of EDTA did not affect the dephosphorylation of KaiC. To confirm the inhibition of kinase activity by EDTA, we added different concentrations of EDTA to the KaiC-alone in vitro reactions in the absence of magnesium. The intensity of the phosphorylation was decreased by increasing the EDTA concentration in the reaction mixture (Supplemental Figure A.3). Therefore, the deficiency of magnesium turns on the kinase activity of KaiC, while EDTA inhibits its kinase activity.

3.4.3 Magnesium Regulation of KaiC Phosphorylation Is Universal

KaiC497 is a KaiC mutant with 23 residues (residues 497-519) deleted from the A-loop, maintaining a dephosphorylation state even in the presence of KaiA due to the lack of the KaiA binding site (Kim et al., 2008). We checked the phosphorylation level of KaiC497 to test the magnesium regulation of KaiC phosphorylation in this mutant. KaiC497 alone spontaneously dephosphorylates with a high magnesium concentration while phosphorylating without magnesium (Figure 3.1e; Supplemental Figure A.4). The addition of KaiA or KaiB did not affect KaiC phosphorylation at either concentration. Therefore, the magnesium regulation of KaiC phosphorylation is a downstream regulatory element of the A-loop for the phosphorylation and dephosphorylation of KaiC.

We also tested the magnesium effect with another cyanobacterial species, *Thermosynechococcus elongatus*, which is mainly used for the structural study of the cyanobacterial circadian clock (Chang et al., 2015; Vakonakis and LiWang, 2004). Again, KaiC from *T. elongatus* behaves in the same manner as that from *S. elongatus* (Figure 3.1f; Supplemental Figure A.5). Therefore, the magnesium regulation of KaiC phosphorylation can be considered a universal mechanism shared by the two species.

3.4.4 Molecular Dynamics Simulation Reveals That E318 Activates T432 for the Phosphorylation of KaiC in the Absence of Magnesium

To provide structural insight into the effect of magnesium on the regulation of KaiC phosphorylation, we performed molecular dynamics simulations of the crystal structure of the KaiC hexamer, which has the A-loop in the buried conformation. The overall backbone conformation of KaiC did not significantly change in the simulations performed with or without magnesium (Figure 3.2a; Supplemental Figure A.6). However, the distances between O^{Glu} and O^{Thr} were significantly longer than 3 Å when the simulations were performed with magnesium (Figure 3.2b; Supplemental Figure A.7a). In the simulations performed without magnesium, 1 of the 6 pairs of O^{Glu}-O^{Thr} distances was found to be sufficiently short (less than ~3 Å) to allow O^{Glu} to interact with H^{Thr} (Figure 3.2c; Supplemental Figure A.7b). This interaction enables T432 to be activated for phosphorylation. Thus, these molecular dynamics simulations suggest that magnesium interacts with O^{Glu} and keeps it away from H^{Thr}, which leads to the suppression of KaiC phosphorylation. In contrast, in the absence of magnesium, O^{Glu} can move and/or rotate more freely toward H^{Thr}, which enables residue T432 to be activated for phosphorylation.

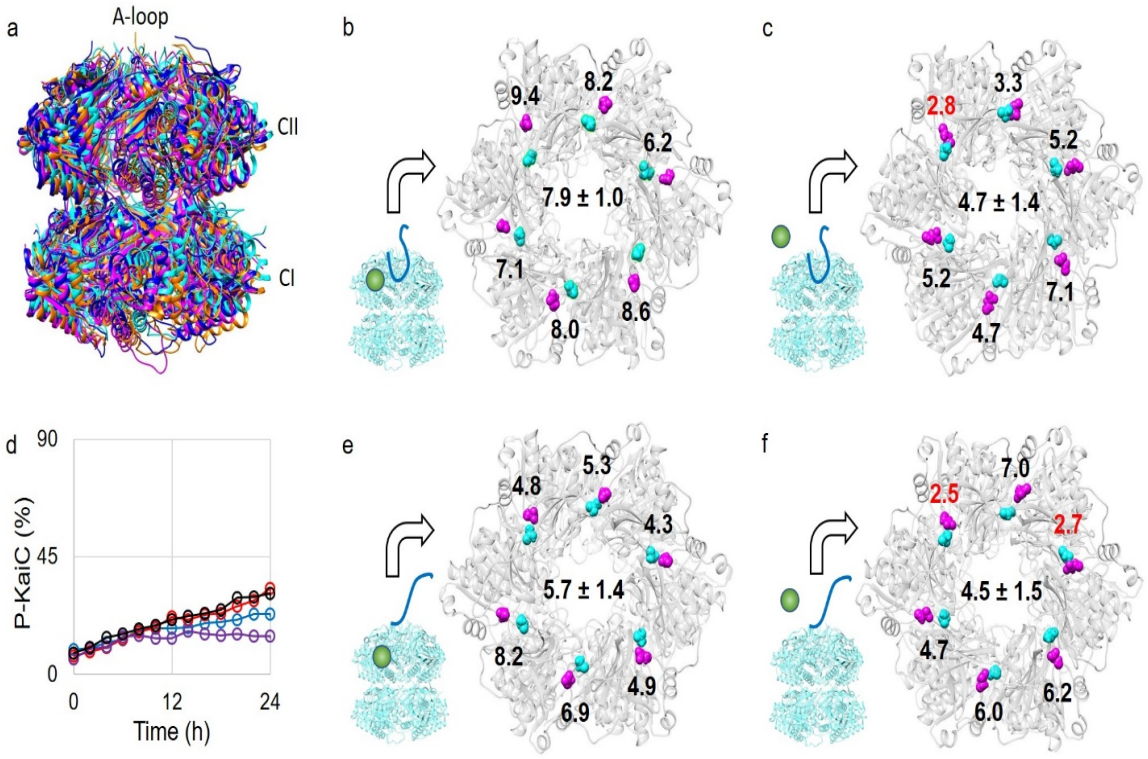


Figure 3.2 Molecular dynamics (MD) simulations of the KaiC hexamer. (a) Four MD simulation structures are superimposed. The "buried" conformations are shown in magenta (with magnesium) and cyan (without magnesium). The "exposed" conformations are shown in orange (with magnesium) and blue (without magnesium). The 3 domains of KaiC are labeled at the position. (b,c,e,f) MD simulation structures of the KaiC hexamer. Magenta represents E318, and cyan represents T432. The distances (in angstroms, Å) between the oxygen atom in E318 and the oxygen atom in T432 are labeled on the structure. Distances shorter than 3 Å are labeled in red. The average distance of all 6 subunits is labeled at the center of the structure. The combinations of the A-loop conformation and magnesium (see the cartoon representations) were the buried conformation with magnesium (b), the buried conformation without magnesium (c), the exposed conformation with magnesium (e), and the exposed conformation without magnesium (f). (d) Phosphorylation state of KaiC E318 D. Four different reaction mixtures were prepared to measure the phosphorylation state: KaiC alone (Δ), KaiC and KaiA (□), KaiC and KaiB (×), KaiC, KaiA, and KaiB (○).

The molecular dynamics simulations reveal that certain O^{Glu}-O^{Thr} distances are shorter than 3 Å but longer than 2 Å in the absence of magnesium. We hypothesized that if E318 is mutated to D318, this mutant would not be able to activate T432 for phosphorylation because of the shortness of the side chain. Indeed, KaiC E318D cannot

phosphorylate in the absence of magnesium (Figure 3.2d; Supplemental Figure S8). Interestingly, the addition of KaiA cannot activate the kinase activity of KaiC E318D (Figure 3.2d; Supplemental Figure S8). Therefore, the O^{Glu}–O^{Thr} distance is an important factor for KaiC phosphorylation and is regulated by magnesium.

3.4.5 Magnesium Is a Downstream Regulator of the A-Loop Conformation in the Circadian Oscillation of KaiC Phosphorylation

We hypothesized that magnesium regulation of KaiC phosphorylation is correlated with the conformation of the A-loop. A molecular dynamics simulation of KaiC was previously reported with an exposed A-loop conformation that is mimicked by removing the entire A-loop (residue 487 to 518) from KaiC (Egli et al., 2013). When the A-loop is in the exposed conformation, the flexibility of the 422-loop in KaiC is increased in the presence of magnesium, but the distance information between O^{Glu} and O^{Thr} has not been reported (Egli et al., 2013). To understand the relationship between the A-loop conformation and magnesium regulation, we performed molecular dynamics simulations with an A-loop deletion mutant KaiC in both the presence and absence of magnesium. As in the simulations performed with the A-loop, the overall backbone conformation of KaiC did not significantly change in the absence of the A-loop (Figure 3.2a; Supplemental Figure S6). All O^{Glu}–O^{Thr} distances were longer than 3 Å in the presence of magnesium (Figure 3.2e; Supplemental Figure S.7c). In contrast, the distances in the two subunits were shorter than 3 Å in the absence of magnesium (Figure 3.2f; Supplemental Figure A.7d). We suggest that this rare chance for activation helps explain why phosphorylation in the cyanobacterial circadian oscillator is so slow. These results also suggest that the distances between O^{Glu} and O^{Thr} were significantly affected not by the conformation of the A-loop but by the presence or absence of magnesium (Figure 3.2b, c, e, and f). However, the

conformation of the A-loop does enhance the probability of phosphorylation in the absence of magnesium, as we found more subunits that were within the hydrogen bonding distance when the A-loop was in the exposed conformation (Figure 3.2f) than in the buried conformation (Figure 3.2e). The average O^{Glu}–O^{Thr} distance of all subunits was the shortest when the A-loop was exposed in the absence of magnesium (Figure 3.2f) and the longest when the A-loop was buried in the presence of magnesium (Figure 3.2b). Therefore, the phosphorylation and dephosphorylation of KaiC is regulated by the presence or absence of magnesium, which is an effect downstream of the A-loop conformation.

We tested this finding using the KaiC E487A mutant, which shows constitutive phosphorylation without KaiA (Kim et al., 2008). The A-loop conformation in the mutant KaiC seems to stay exposed by breaking the H-bond network formed between the A-loops in the KaiC hexamer (Kim et al., 2008). The binding affinity of the regulatory magnesium ion should be significantly lower in this mutant, keeping KaiC in the hyperphosphorylated state even in a high magnesium concentration. To test this hypothesis, we constructed the in vitro reaction with KaiC E487A in a high magnesium concentration. Indeed, KaiC E487A was able to stay hyperphosphorylated in 20 mM magnesium (Supplemental Figure A.9).

The possible molecular mechanism of KaiC phosphorylation is proposed in Figure 3.3. When the A-loop in KaiC is exposed by binding KaiA, magnesium is released from the active site of KaiC, and O^{Glu} moves and rotates toward H^{Thr} to activate T432 for the phosphoryl transfer reaction (Figure 3.3). While the A-loop is buried after sequestration of KaiA by KaiB, magnesium is tightly bound to O^{Glu}, making it unable to activate T432 for phosphorylation, and KaiC undergoes dephosphorylation (Figure 3.3).

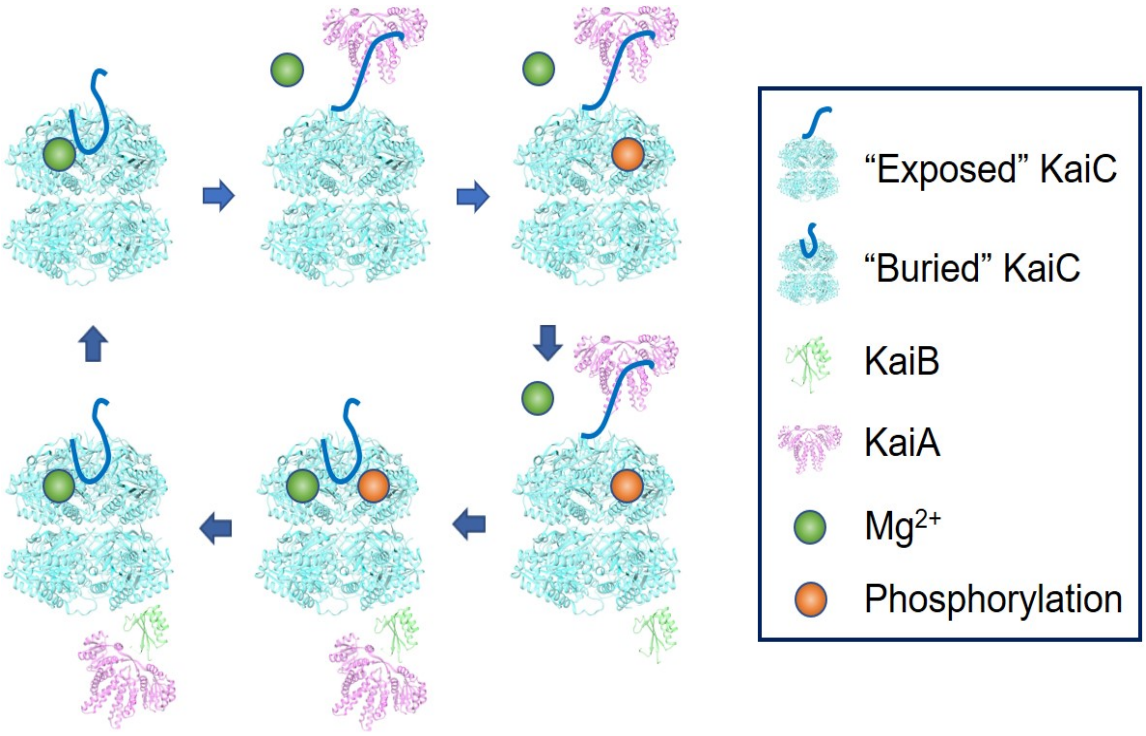


Figure 3.3 Molecular mechanism of the circadian oscillator in cyanobacteria. KaiC is fully dephosphorylated, the A-loop (solid line) is "buried," and the magnesium ion is bound on E318 in the active site (upper left). KaiA is bound to the A-loop to keep it in the "exposed" conformation, and magnesium is released from the active site (upper center). KaiC is fully phosphorylated (upper right). KaiB is bound on the CI domain of KaiC to sequester KaiA from the A-loop (lower right). A-loop turns to the buried conformation by dissociation of KaiA, and magnesium is bound on E318 in the active site (lower center). KaiC is fully dephosphorylated (lower left), and KaiB and KaiA are dissociated from KaiC (upper left).

3.4.6 The Circadian Oscillator Has Possibly Evolved from an Hourglass to a Self-Sustained Oscillator in Cyanobacteria

This magnesium regulation of KaiC phosphorylation enables the construction of an hourglass-type in vitro reaction with KaiC alone that is suggestive of the evolutionary trajectory of the circadian clock in cyanobacteria. Previous genetic analysis indicated that KaiC is the oldest protein among the oscillator components and that KaiB and KaiA were added later, respectively (Dvornyk et al., 2003; Tauber et al., 2004). Based on the evolutionary information of the oscillator components, a reasonable hypothesis was

proposed: the prokaryotic timekeeping system evolved from an hourglass timer to a self-sustained oscillator in response to selective pressures (Johnson et al., 2017; Ma et al., 2016). Before the appearance of KaiB or KaiA, day/night alterations of the environment caused the phosphorylation state of KaiC to oscillate as an hourglass. In other organisms, magnesium concentration is altered by the natural light/dark cycle and provides feedback to the circadian clock (Feeney et al., 2016). Thus, we hypothesized that the KaiC-alone timekeeping system may operate as an hourglass timer driven by the influx and efflux of magnesium, assuming the magnesium concentration was altered by an environmental signal such as the light/dark cycle in the ancient cyanobacteria. If we assume that the environmental magnesium concentration in the habitat of cyanobacteria oscillated daily as a result of evaporation during the day and condensation during the night, then the KaiC-alone hourglass could have been used as a timekeeping system in the ancient cyanobacteria before establishing the current magnesium homeostasis system.

To test the possibility of an hourglass timer in vitro, we constructed an in vitro reaction with KaiC alone. By altering the magnesium concentration every 12 h, KaiC showed a robust phosphorylation and dephosphorylation cycle with a 24-h period, although the variation of magnesium concentrations (0.5-5 mM) was somewhat greater than that observed under the physiological condition (Figure 3.4a; Supplemental Figure A.10). We still observed the phosphorylation and dephosphorylation cycle in the low magnesium concentration range (0.05-0.1 mM), although the amplitude was significantly decreased compared with that of the KaiABC oscillator. Simulations of a mathematical model of KaiC alone (see the Materials and Methods section) can reproduce the hourglass timer (Figure 3.4b). In cyanobacteria, light alters ADP concentration (Rust et al., 2011; Simons,

2009) in addition to magnesium concentration. Increasing ADP can induce dephosphorylation of KaiC in cyanobacteria and entrain the self-sustained KaiABC circadian oscillator (Rust et al., 2011). This ADP effect may enhance the amplitude of the phosphorylation rhythm in the hourglass timer by inducing robust dephosphorylation at night. When we applied the ADP effect in simulations of the mathematical model of the KaiC-alone hourglass timer, robust oscillations were observed across the entire range of magnesium concentrations (Figure 3.4c). The existence of the hourglass timer provides evidence for the hypothesis that the molecular evolution of the cyanobacterial circadian clock started from a KaiC-alone hourglass timer. To further test steps along an evolutionary path to an oscillator, we constructed an in vitro reaction with KaiB and KaiC, which was proposed to be a damped oscillator (Johnson et al., 2017). Many different magnesium concentrations were screened for the signature of a damped oscillator, but all the phosphorylation patterns were similar to the KaiC-alone reaction (Supplemental Figure A.11). The in vitro reaction with KaiB and KaiC also behaved as an hourglass timer (Figure 3.4d; Supplemental Figure A.12). It is possible that the KaiBC timekeeping system in each species may be at a different stage of evolution. Thus, whether the mechanism is an hourglass timer, a damped oscillator, or a self-sustained oscillator is species-dependent. In this study, we found an hourglass KaiBC timer using both KaiB and KaiC from *S. elongatus*, but a KaiBC damped oscillator might be discovered using KaiB and KaiC from a different species of bacterium. Finally, we checked the magnesium effect on the self-sustained circadian oscillator composed of KaiA, KaiB, and KaiC with many different magnesium concentrations. In the self-sustained oscillator, KaiC phosphorylation was

observed as a stable oscillation with a circadian period (~24 h) over a wide range of magnesium concentrations (Figure 3.4e; Supplemental Figure A.13).

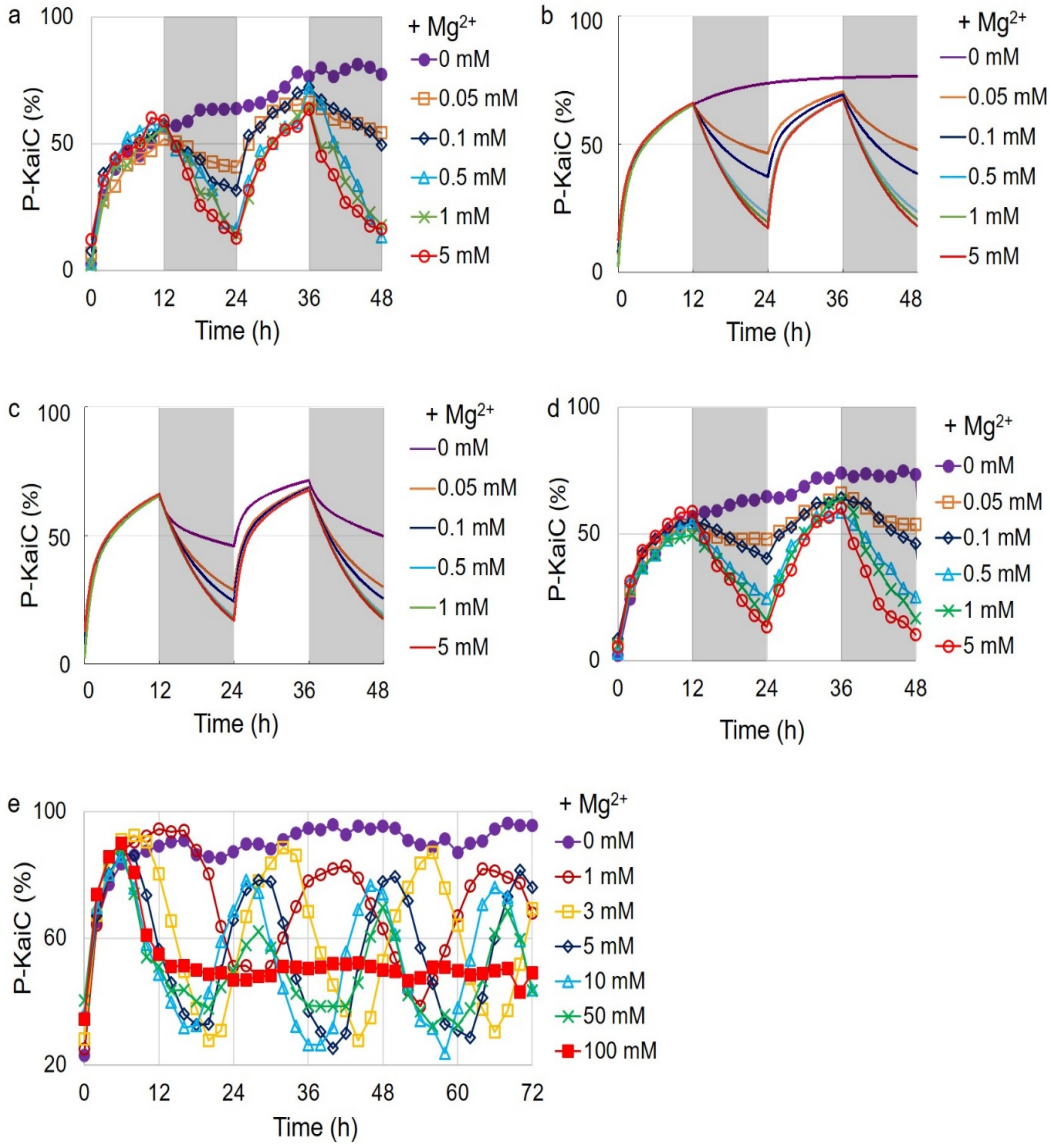


Figure 3.4 Molecular evolution of the circadian clock in cyanobacteria. (a) Hourglass timer of KaiC alone in vitro. The magnesium concentration was altered every 12 h (low in white and high in gray). The magnesium concentrations in gray are labeled on the right of the graph. (b) Simulations of a mathematical model of the hourglass KaiC timer. All other features are the same as those in (a). (c) Simulations of a mathematical model of the hourglass KaiC timer with ATP/ADP effect. All other features are the same as those in (b). (d) Hourglass-type oscillation of KaiBC in vitro oscillator. All other features are the same as those in (a). (e) Magnesium effect on the KaiABC self-sustained oscillator. Magnesium concentrations are labeled on the right of the graph.

3.5. Discussion

We successfully demonstrate the phosphorylation of wild-type KaiC without KaiA. Until now, it was believed that KaiC needed KaiA or a mutation on the A-loop to undergo phosphorylation (Kim et al., 2008; Nishiwaki et al., 2004). Because of this limitation, although the KaiC-alone hourglass was proposed before as a major step in the evolutionary pathway of the circadian oscillator, no experimental proof was reported (Axmann et al., 2009; Ma et al., 2016). We present a possible pathway for the evolution by removing EDTA, an unnatural reagent commonly used in reactions, from the hourglass reaction mixture. The inhibition of the phosphorylation of KaiC by EDTA was not observed in the presence of KaiA. Currently, the detailed mechanism of the inhibition of kinase activity by EDTA is unknown. We thought that the examination of the A-loop conformation could serve as a basis for elucidating the effect of EDTA on KaiC phosphorylation.

The general consensus on the mechanism of the phosphoryl-transfer reaction is that the magnesium ion acts as a cofactor to neutralize the negative charges on the γ-phosphate of ATP to facilitate the nucleophilic phosphoryl-transfer reaction. Our findings on the activation of KaiC kinase activity run counter to this general behavior. However, there are other examples of bacterial autokinases that respond in a similarly unusual way to changes in magnesium concentration. In PhoQ, which is involved in magnesium homeostasis in bacteria, the autokinase and the autophosphatase activities are predominant in low and high magnesium concentrations, respectively (Montagne et al., 2001). Although the detailed reaction mechanism at the atomic level is not yet elucidated, the magnesium ion regulates the autokinase activity of PhoQ in the same way as KaiC. In either case, the addition of EDTA inhibits the autophosphorylation in low magnesium concentration (Castelli et al.,

2000). Therefore, we cannot exclude the possibility that a trace amount of magnesium ions from the impurities of the reagents may act as a cofactor for the phosphoryl-transfer reaction. EDTA may strip magnesium ions from the active sites, resulting in the inhibition of kinase activity. Indeed, 2 magnesium ions are found within some KaiC crystal structures. One is located at the same position that we are claiming here as a "regulatory magnesium," and the other is located near the γ-phosphate of ATP (Supplemental Figure A.14). However, even if the kinase activity is inhibited in absolute zero magnesium concentration, our findings here are still valid because absolute zero magnesium is impossible in cyanobacteria.

The magnesium concentration in cyanobacteria may be affected by fluctuating environmental conditions such as evaporation and condensation, because cyanobacteria can live even in the small amount of moisture found in a desert. Although magnesium concentration did not have a large effect on the self-sustained period, it is still possible that day/night magnesium alternations play a role in entraining the KaiABC oscillator because small changes in the self-sustained period can have a large effect on the entrained phase (Granada et al., 2013). We also hypothesize that signaling pathways other than magnesium, such as Quinone signaling through KaiA (Kim et al., 2012), evolved to enable effective entrainment of the self-sustained oscillator. For example, CikA, a major input pathway protein (Kaur et al., 2019; Schmitz et al., 2000), directly interacts with the KaiABC oscillator to entrain circadian rhythms in cyanobacteria (Tseng et al., 2017). The step-by-step evolution from an hourglass timer permitted the development of a robust and entrainable circadian clock system in this single-celled organism.

3.6. Conclusion

Until recently, adding KaiA was the only way to activate the kinase activity of wild-type KaiC. We found that magnesium regulates KaiC phosphorylation downstream of the A-loop conformation, which is the master regulator of KaiC phosphorylation. By modulating magnesium concentration, the phosphorylation state of KaiC can be regulated without KaiA and KaiB. In ancient cyanobacteria, the KaiC-alone hourglass timekeeping system, which evolved before the current homeostasis system was established, may be used for the regulation of gene expression in the fluctuating magnesium environment. The current self-sustained circadian oscillator possibly evolved from the KaiC-alone hourglass controlled by magnesium.

CHAPTER 4

MUTATION IN KaiB MODULATES THE PERIOD OF CYANOBACTERIAL CIRCADIAN OSCILLATOR

4.1 Abstract

The cyanobacteria species *Synechococcus elongatus* PCC 7942 (hereafter, *S. elongatus*) is the simplest organism with a stable and sturdy circadian clock replicated in the test tube. The clock's central oscillator portion consists of three proteins: KaiA, KaiB, and KaiC, with KaiC being the critical player undergoing autophosphorylation and autodephosphorylation cycles over a ~24-hour time period. The mechanism of oscillator protein KaiB is not well defined, but mutations in any one of its amino acids can disrupt the clock by shortening its period in vivo. We performed similar experiments in vitro to compare the results. We found a similar shortening effect, but the length of periods is not the same as in vivo. These findings need a more detailed study to find out the cause of variations and also the role of KaiB in circadian oscillation.

4.2 Introduction

The Cyanobacteria circadian clock is composed of three Kai protein KaiA, KaiB, and KaiC, which can be purified and replicated in the test tube in the presence of ATP and other constant conditions to operate for an infinite time (Mihalcescu, Hsing, & Leibler, 2004). The Proteins involved in the circadian clock interact with other clock proteins to generate 24-hour KaiC phosphorylation (M. Ishiura et al., 1998; Nakajima et al., 2005).

In *S.elongatus,* the circadian period can be tracked in vivo via the luciferase gene's insertion for gene expression. While monitoring the luminescence from *S. elongatus*

genetically mutated colonies, several rhythmic genes were isolated. Three genes, KaiABC were identified as essential proteins for generating a circadian oscillation (M. Ishiura et al., 1998). Amino acid substitutions in KaiA, KaiB, or KaiC alter the period length, phase, wave amplitude, or waveform of the circadian clock, while most *KaiB* mutations showed a short period phenotype (Ishiuria et al., 1998; Uzumaki et al., 2004; Iwasaki et al., 2002). To see the function of mutated KaiB protein on the KaiC oscillations, we performed in vitro experiments with two-point mutations in KaiB, L11F, and R74W. We found a shorter period, a similar product as in vivo, but the shortening time was different.

4.3 Results

KaiB mutants L11F and R74W shortens the oscillation period of KaiABC. We propose from these results that KaiB might play an essential role in the period of the circadian clock oscillator. The mutant R74W showed a period of 16h, but previous in vivo experiments demonstrated the period shortened to 22h (Masahiro Ishiura et al., 1998) (Figure 4.1)

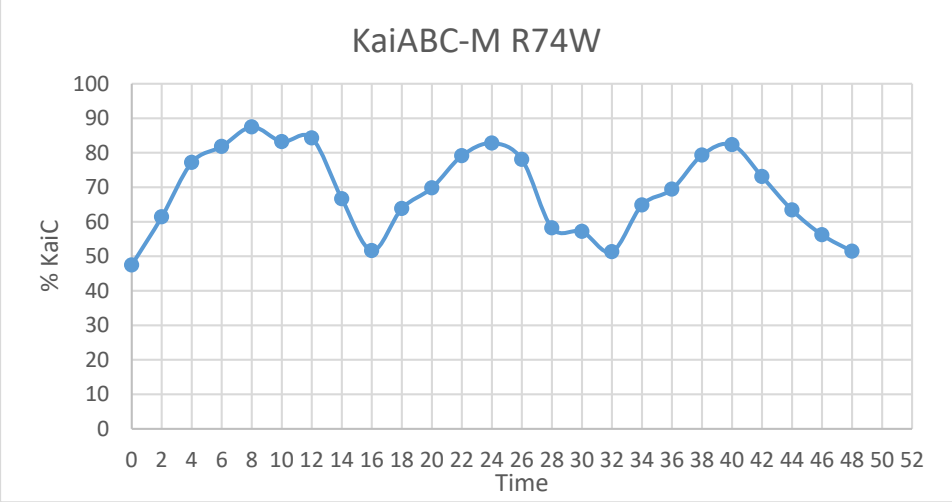


Figure 4.1 The circadian oscillation of KaiABC with mutant KaiB R74W.

The mutant L11F also showed a period of 16h, which is the same as the mutant R74W, but the period length varies from the in vivo, which was observed to be 21h (Masahiro Ishiura et al., 1998) (Figure 4.2).

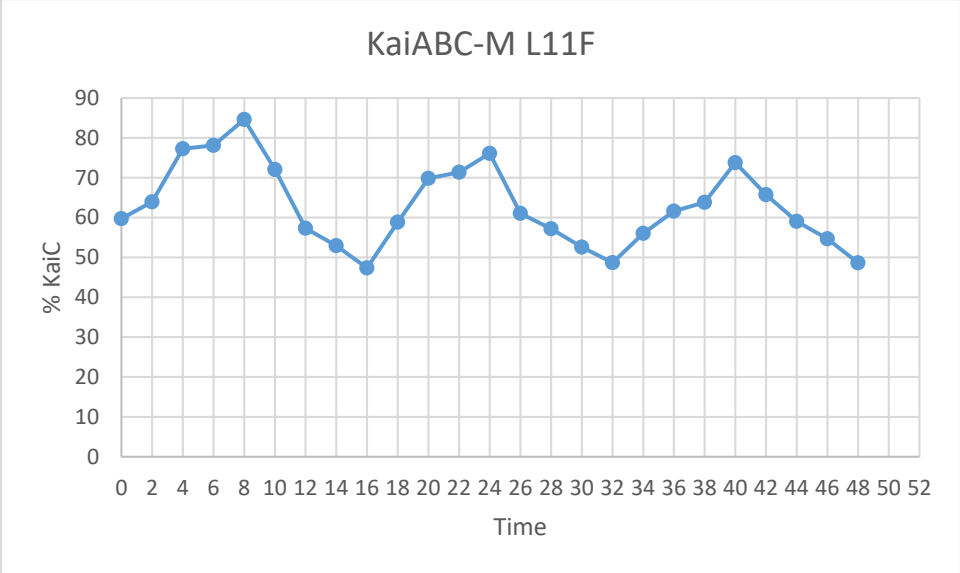


Figure 4.2 The circadian oscillation of KaiABC with mutant KaiB L11F.

4.4 Conclusion

Previous in vivo experiments have shown that KaiB mutants can shorten the period of the cyanobacterial circadian clock. We have also produced the same result in vitro, but the length of period shortening is not precisely the same as the in vivo data. Further experiments need to conduct to figure out whether the disparity in the amount of period shortening is due to structural and conformational changes or not.

CHAPTER 5

SUMMARY

The elegant 24-hour rotation rate on Earth is one attribute that makes our world so welcoming to life. But the periodic disturbances and changes are everywhere to adapt; organisms develop a timekeeping system called the circadian clock, which drives gene expression and physiological behavior.

The circadian clock was present only in eukaryotes for a long time. But the intensive study had shown that it does in a varied class of organisms, cyanobacteria being the clock model. S.Elangatus clock has been studying for a very long period, and fascinating observation was made as gene expression control, three-dimensional structures of the oscillator proteins – KaiA, KaiB, and KaiC – , oscillatory mechanism of KaiC phosphorylation in vivo and replicated circadian clock in the test tube but certain areas still need to be explored.

Reconstructing the KaiABC oscillator in vitro is a valuable way of understanding the circadian clock timekeeping mechanism and entrainment aspects. Many unknown variations in metabolites and protein interactions may affect the clock oscillation, phase, and KaiC phosphorylation period.

This dissertation discusses how CikA, a histidine kinase protein, affects the circadian oscillation period by modulating the effect of KaiA on the KaiC phosphorylation in vitro. We observe CikA binds with KaiB, and it remains the KaiC phosphorylation turned on, due to which it delays the period of KaiC dephosphorylation. So we assume CikA can be involved in the phase delay of the circadian clock. It can enable the entrainment of cyanobacterial circadian rhythms if introduced as an Input component. Then we studied

magnesium's effect on the KaiC phosphorylation and observed the intensity of the dephosphorylation reduced with decreased magnesium concentration. To understand the relationship between the A-loop conformation and magnesium regulation, we performed molecular dynamics simulations. We confirmed that magnesium regulates KaiC phosphorylation downstream of the A-loop conformation, the master regulator of KaiC phosphorylation. This study concluded that the magnesium held KaiC phosphorylation state without KaiA and KaiB by modulating its concentration. Chapter 4 discusses the effect of KaiB oscillator component mutations on the period of its oscillations in vitro. We performed two KaiB mutations and observed the shorter period of oscillation with both.

We conclude by saying it is crucial to investigate each clock component, their protein mutations, and their interactions to solve the hidden complexities of the developmental biological chronometer.

APPENDIX A

MAGNESIUM REGULATES THE CIRCADIAN OSCILLATOR IN CYANOBACTERIA

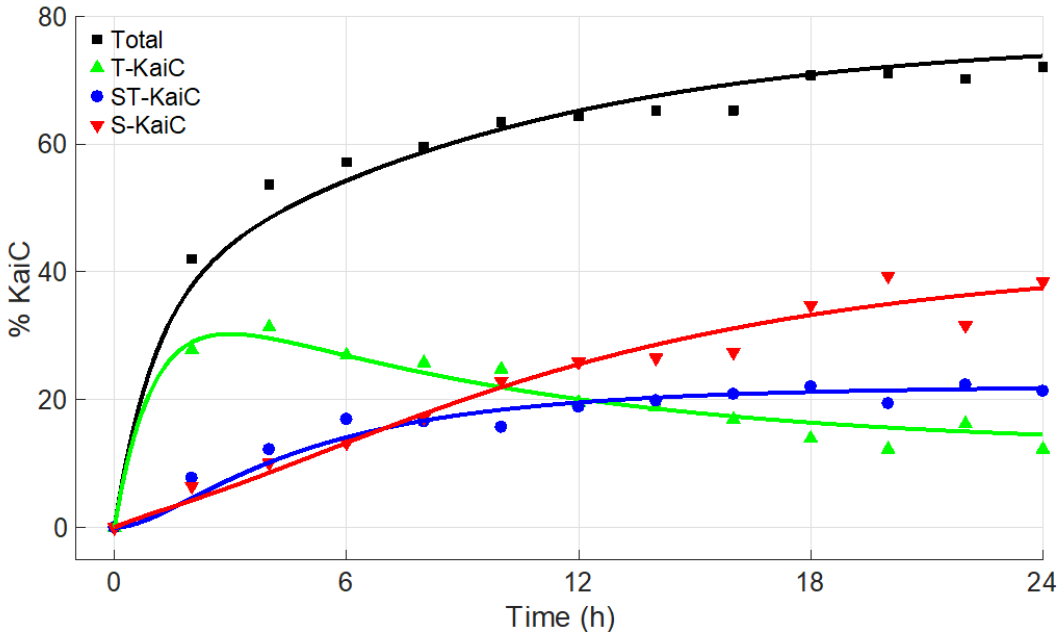
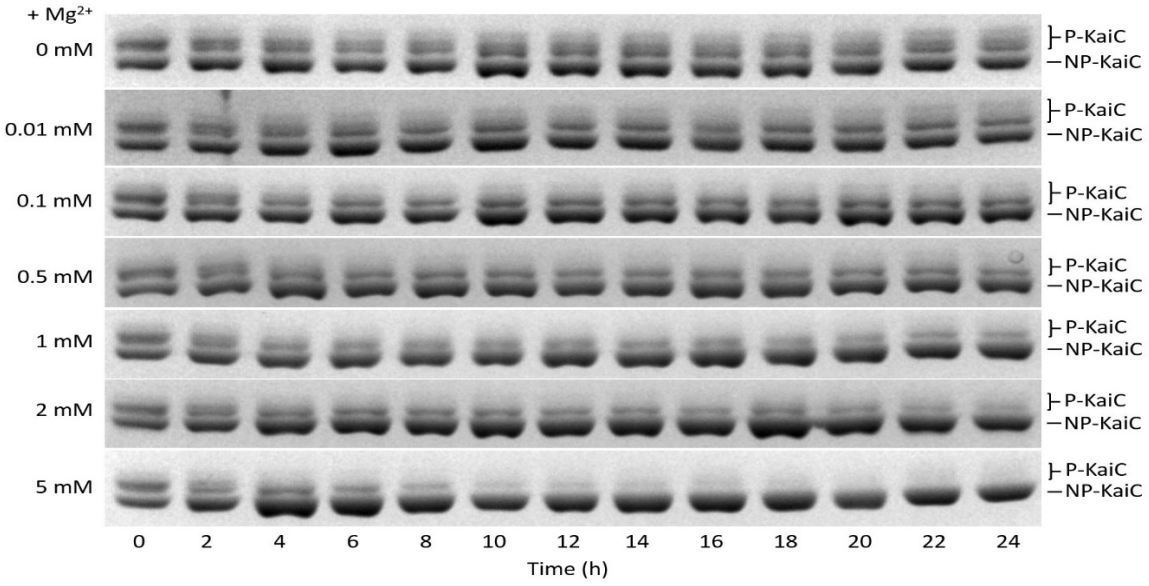


Figure A.1 Mathematical modeling of autonomous phosphorylation of KaiC in the absence of magnesium**.** Experimental data points on the amount of T-KaiC (green triangles), ST-KaiC (blue circles), and S-KaiC (red triangles) were used to fit the model parameters *kBXY* representing the baseline transition rates between different phosphoforms in the absence of magnesium. Model simulations with the optimized parameter values are shown for T-KaiC (green curve), ST-KaiC (blue curve), S-KaiC (red curve), and the total amount of phosphorylated KaiC (black curve).

a)



b)

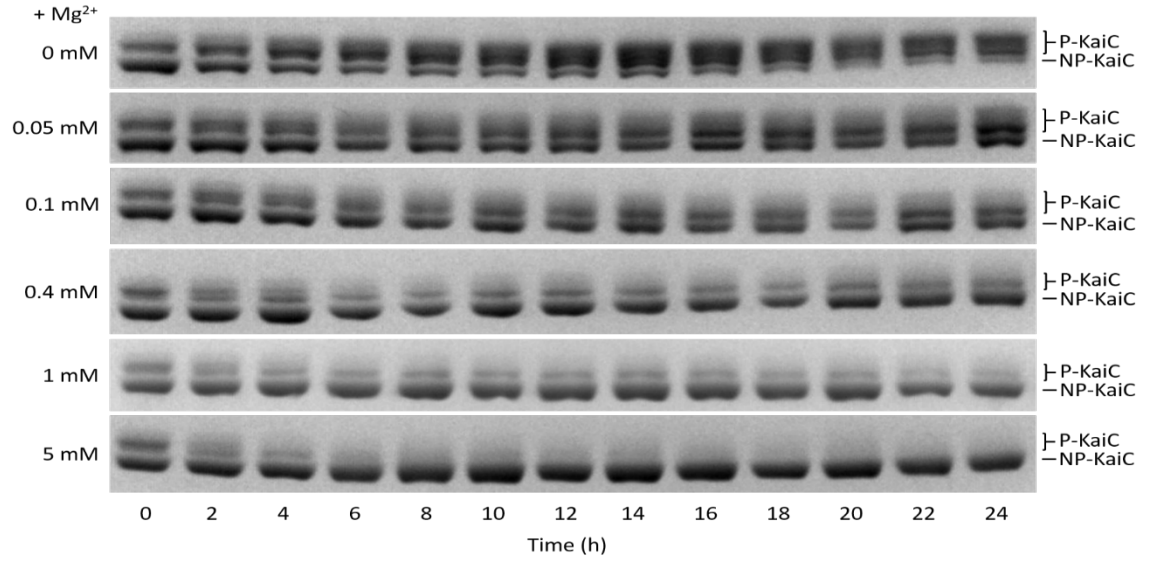


Figure A.2 (**a**) The phosphorylation state of KaiC in the presence of EDTA. The densitometry analysis of the bands is shown in Figure 3.1(c). (**b**) The phosphorylation state of KaiC in the absence of EDTA. The densitometry analysis of the bands is shown in Figure 3.1(d)**.**

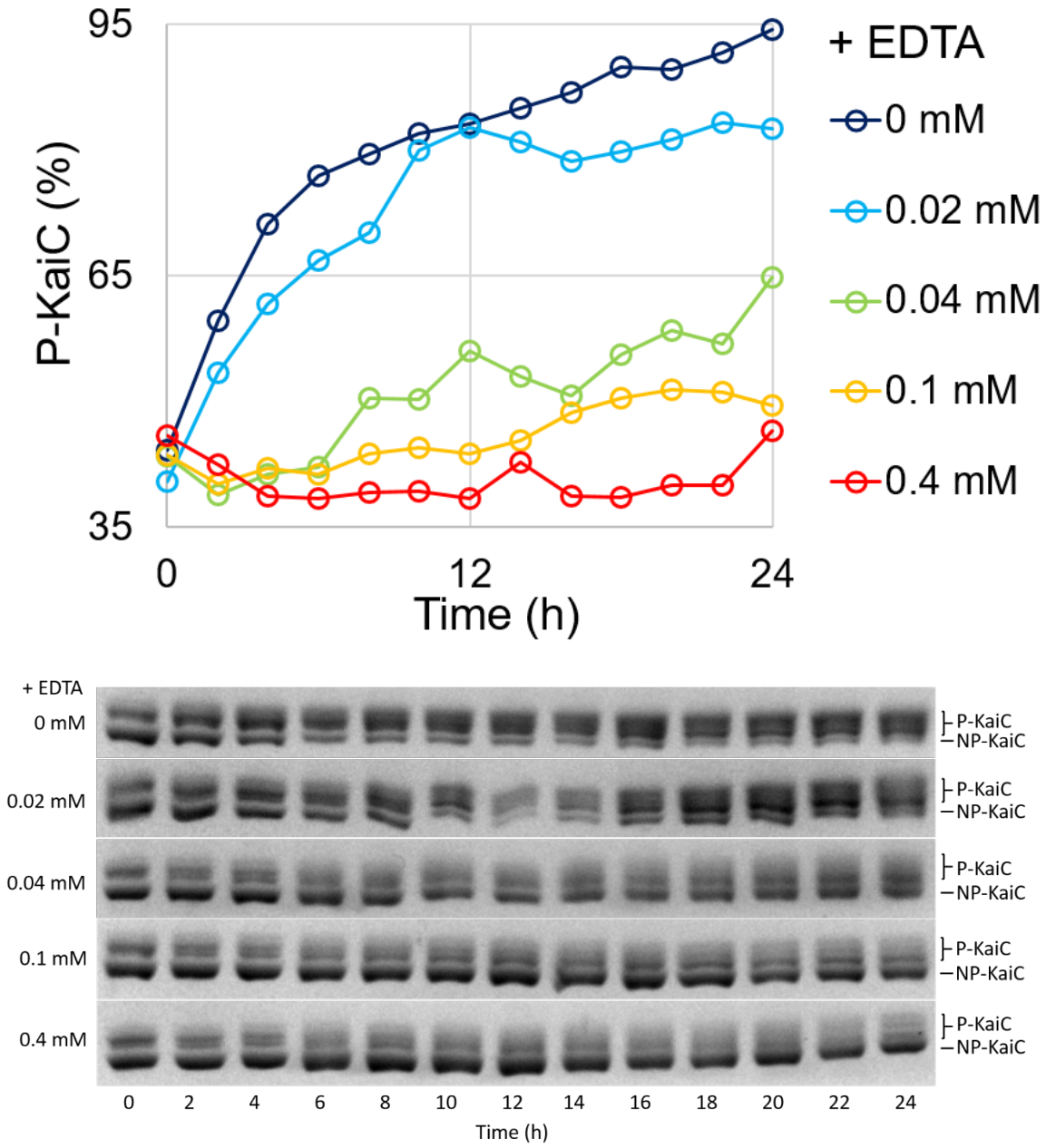


Figure A.3 The phosphorylation state of KaiC in the absence of magnesium**.** The densitometry analysis of the bands is shown in upper panel. Phosphorylation state of KaiC in the absence of magnesium with many different EDTA concentrations. EDTA concentrations are labeled on the right of the graph.

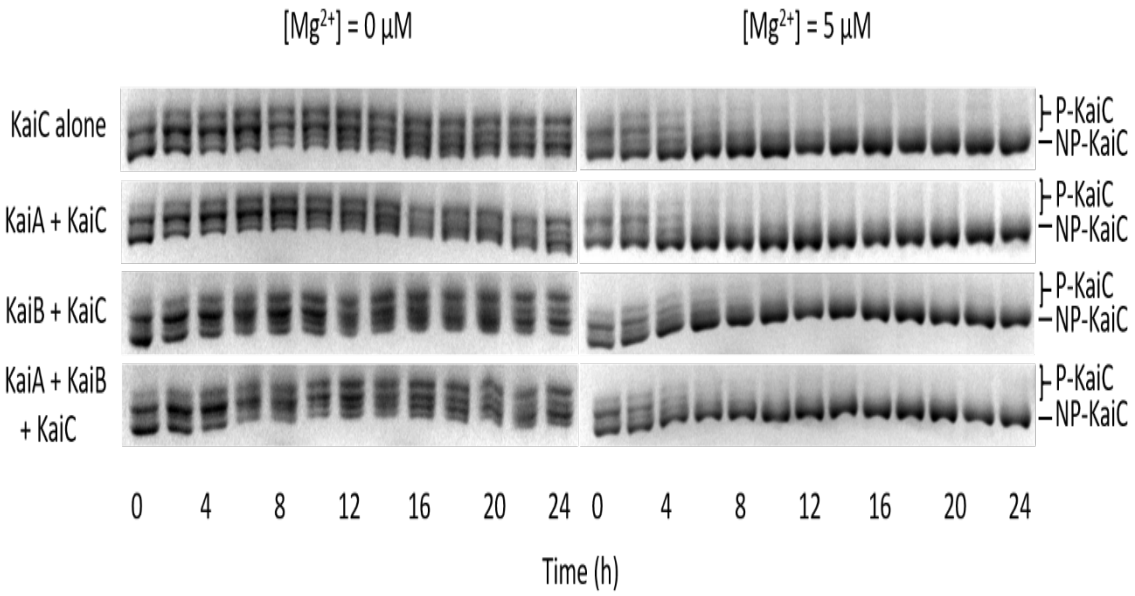


Figure A.4 The phosphorylation state of KaiC497**.** The densitometry analysis of the bands is shown in Figure 3.1(e).

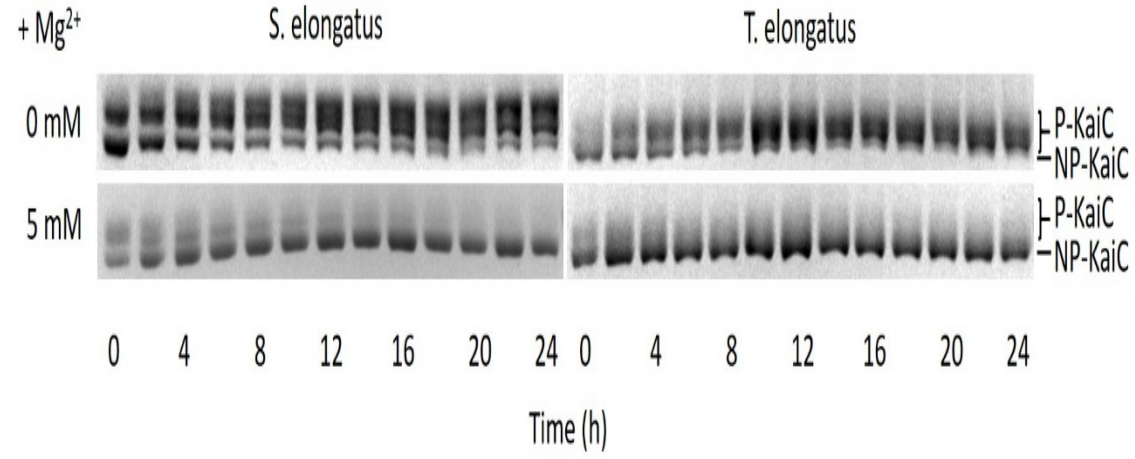


Figure A.5 The phosphorylation state of KaiC from T. elongatus**.** The densitometry analysis of the bands is shown in Figure 3.1(f).

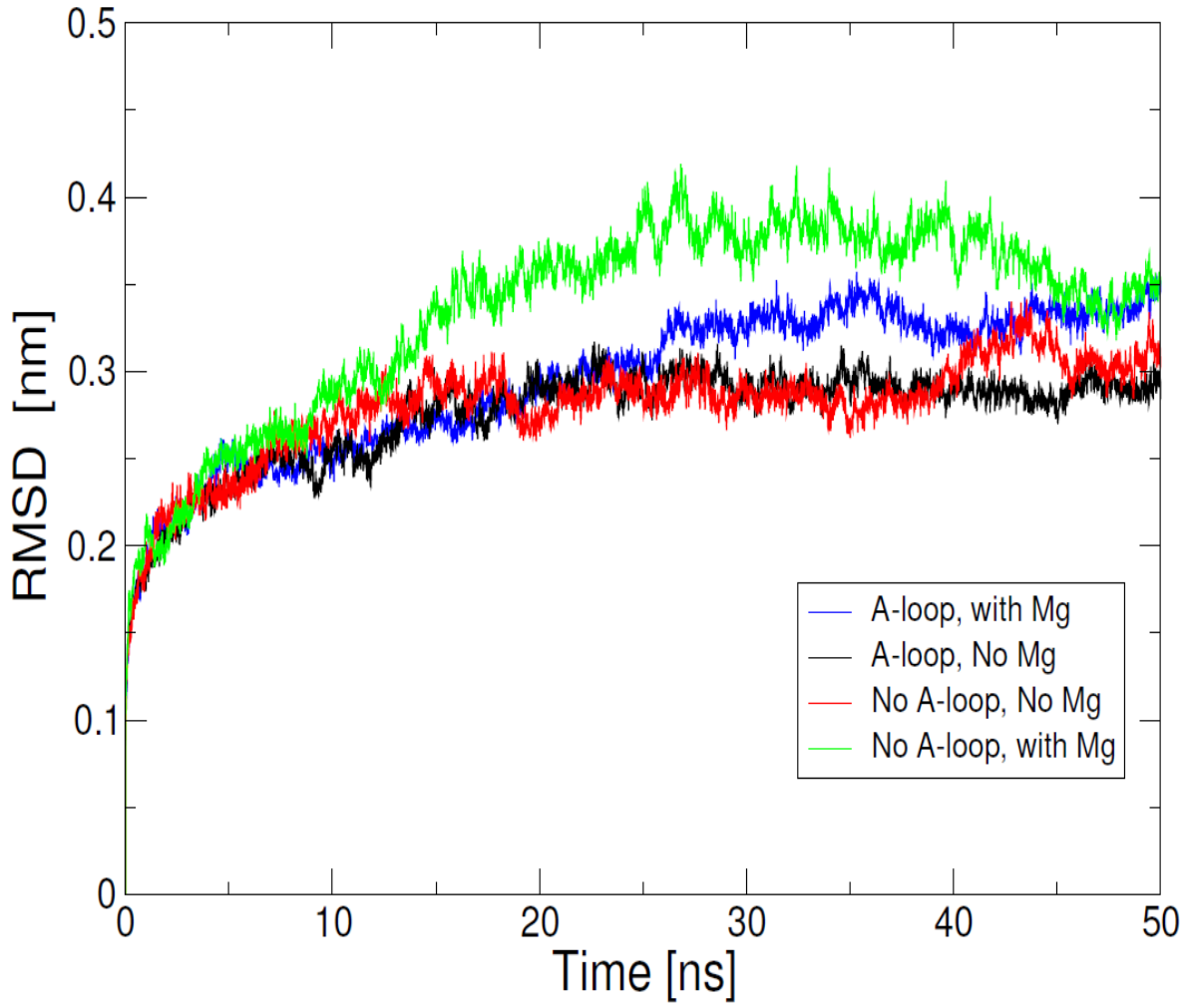


Figure A.6 Root mean square deviation (RMSD) of backbone atoms relative to the initial conformation of the KaiC hexamer**.**

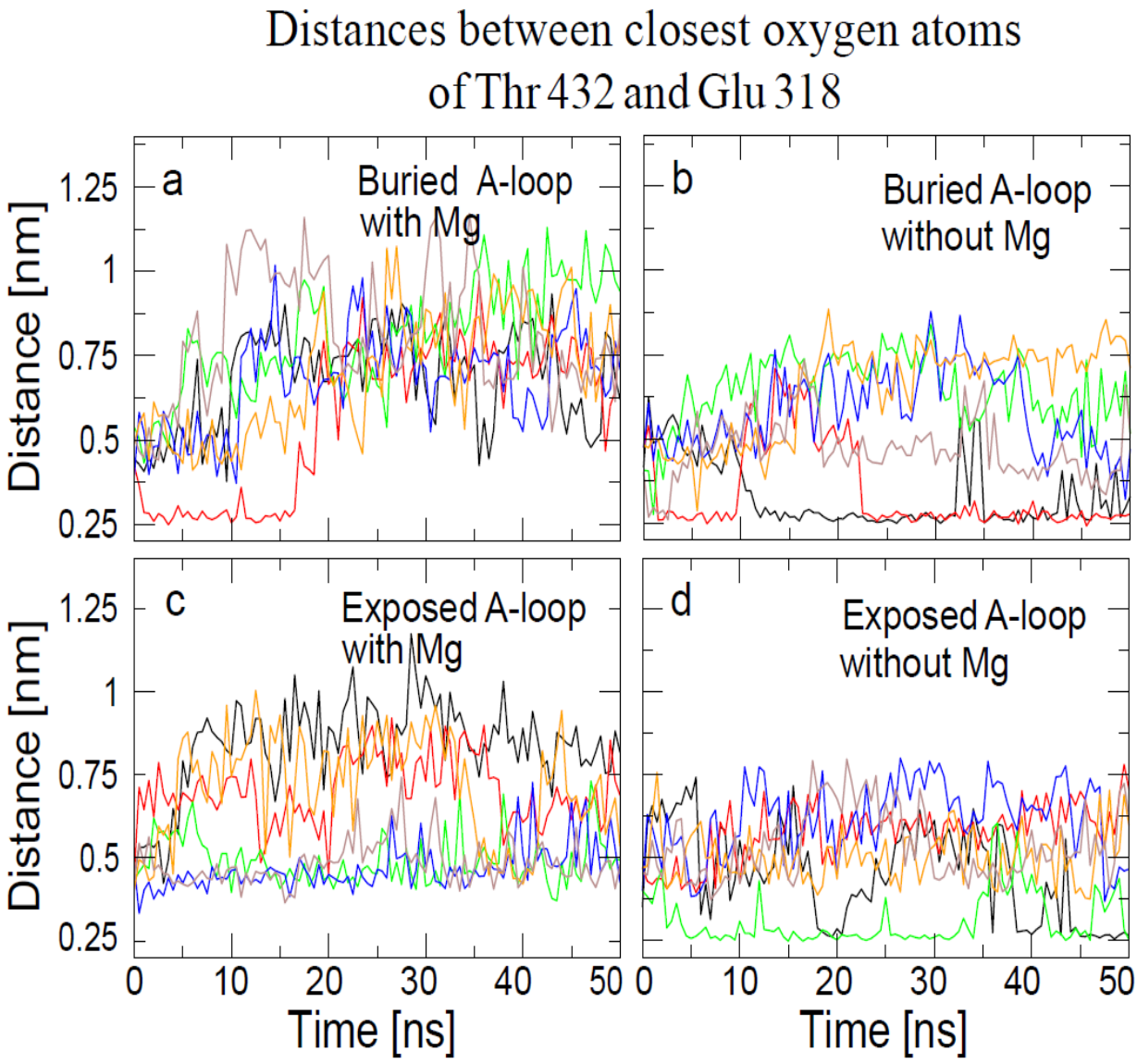


Figure A.7 Distances between closest oxygen atoms of Thr432 and Glu318 as a function of time**.** The A-loop of the KaiC protein is included and deleted from simulations in top (a-b) and bottom (c-d) panels, respectively. Simulations in left (a, c) and right (b, d) panels are performed with and without magnesium, respectively. The six color lines in each panel are the distance result for the six neighboring pairs of T432 and E318 in KaiC hexamer.

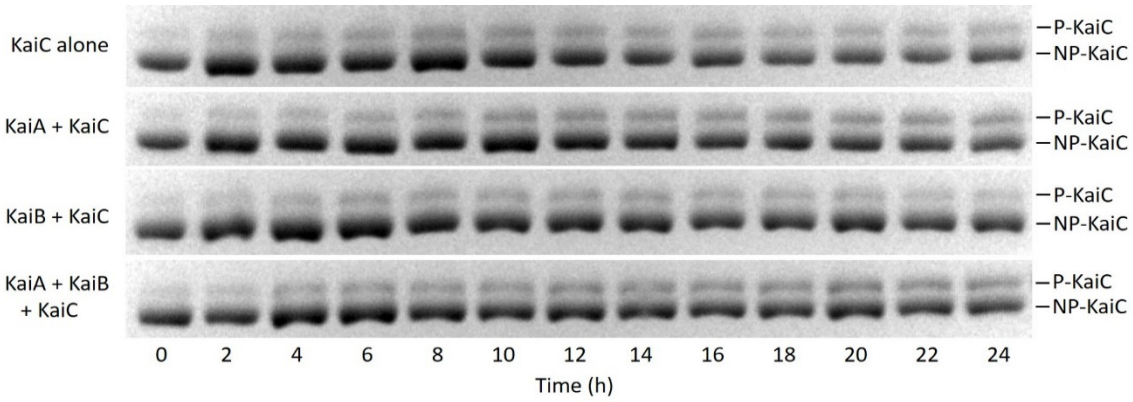


Figure A.8 The phosphorylation state of KaiC E318D**.** The densitometry analysis of the bands is shown in Figure 3.2(d).

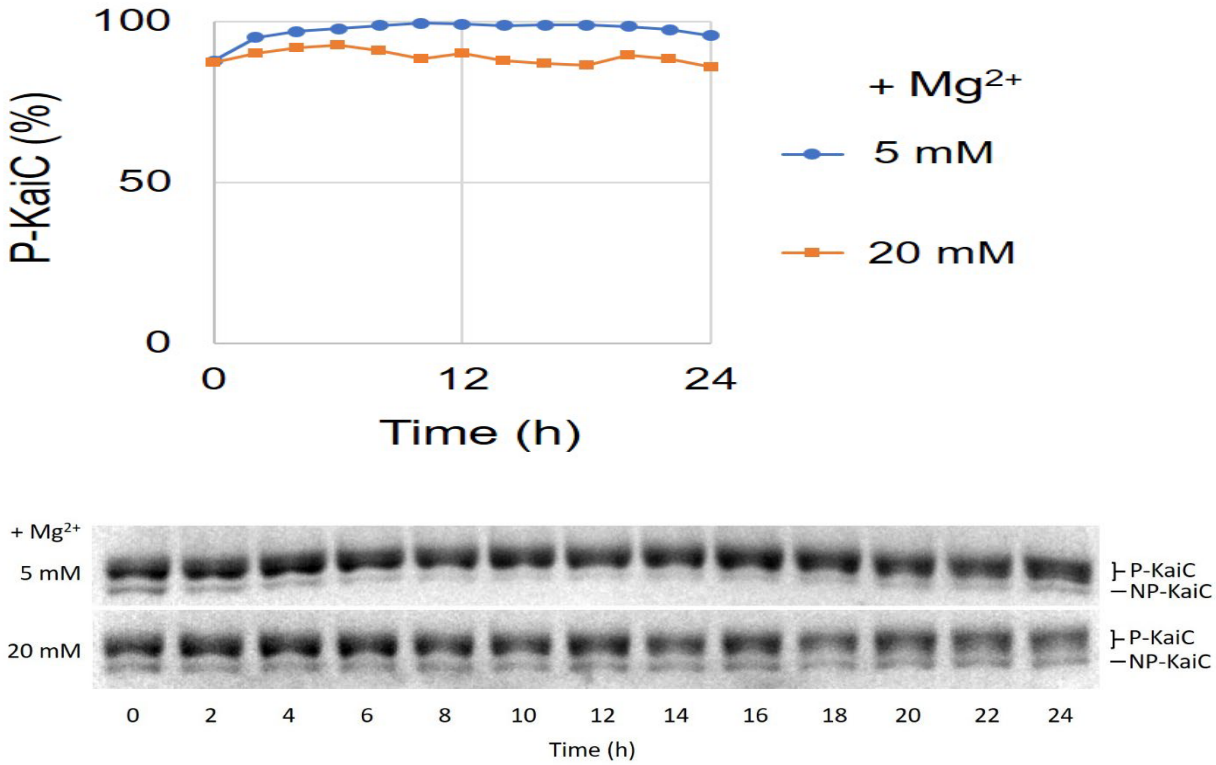


Figure A.9 The phosphorylation state of KaiC E487A in the high magnesium concentration**.** The densitometry analysis (upper panel) and SDS-PAGE (bottom panel) of KaiC E487A in 20 mM magnesium concentration

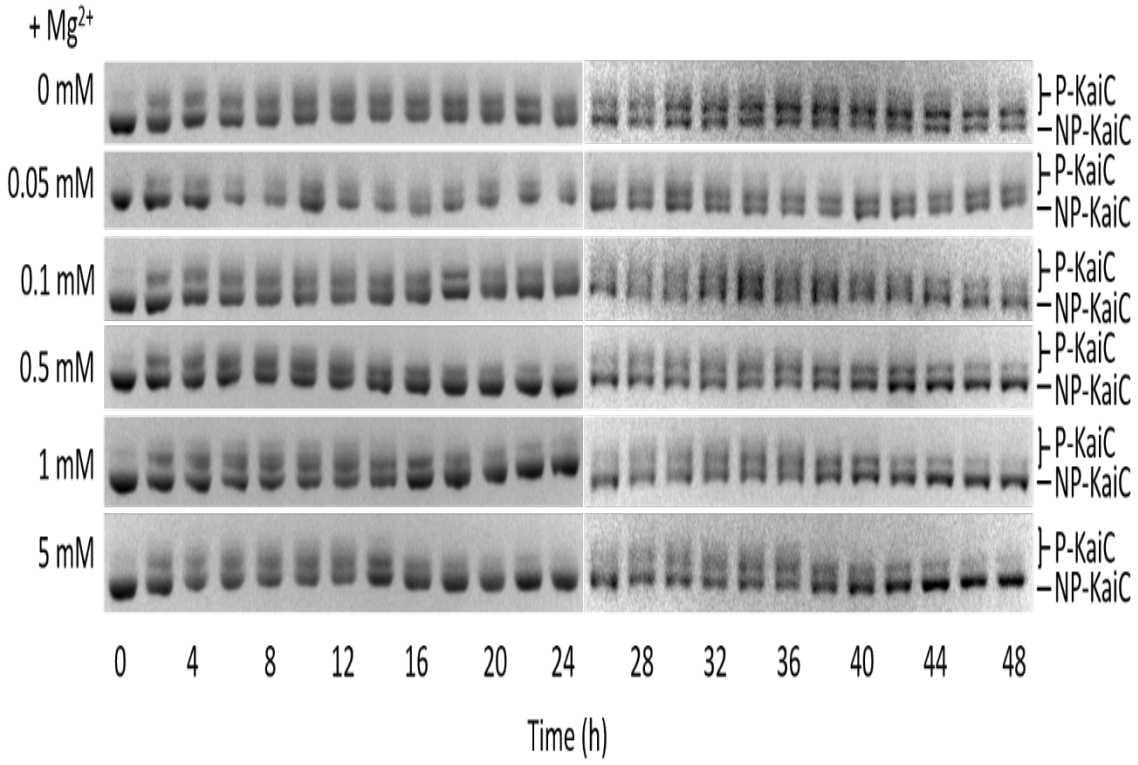


Figure A.10 The phosphorylation state of the hourglass timer of KaiC**.** The densitometry analysis of the bands is shown in Figure 3.4(a).

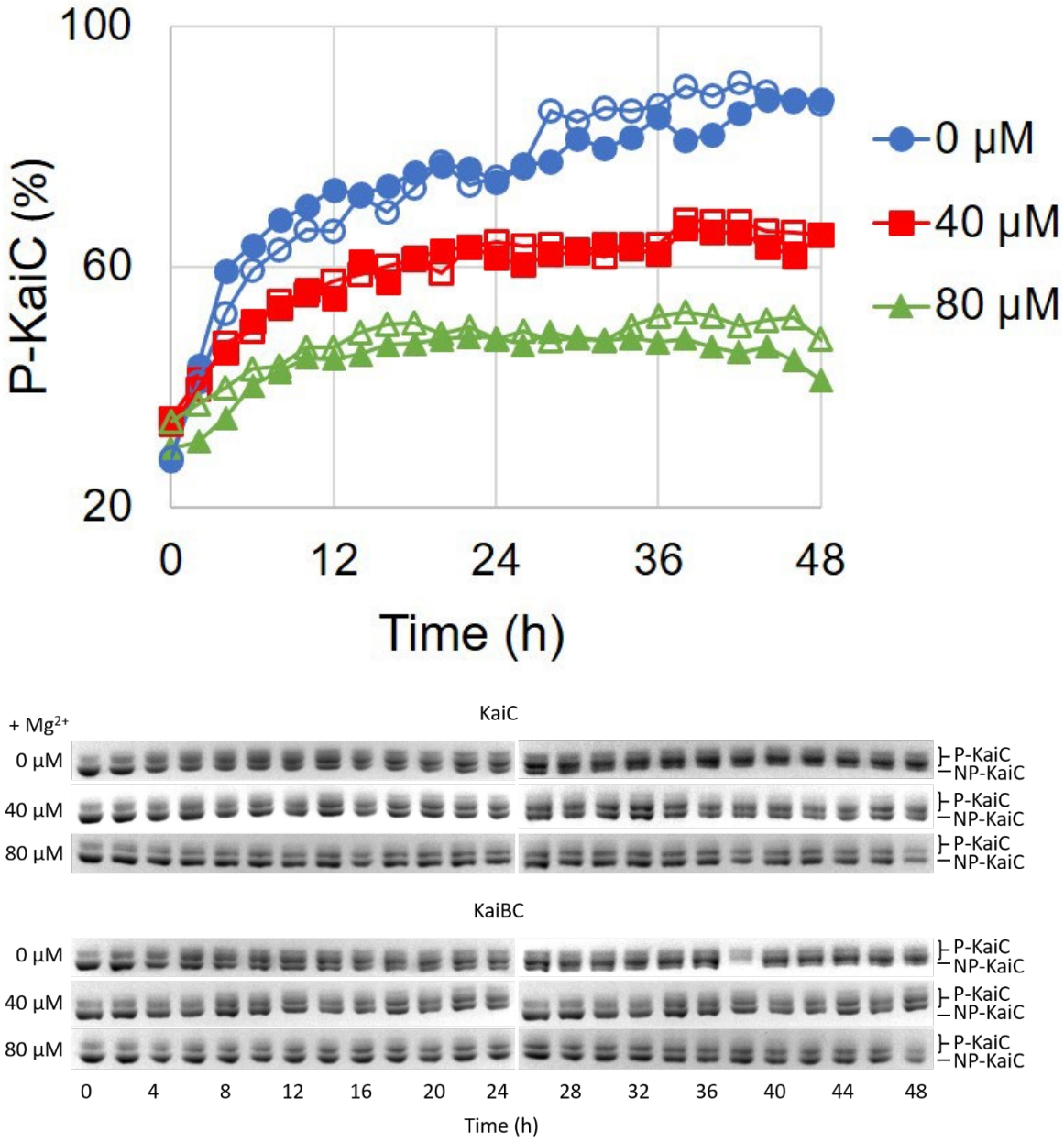


Figure A.11 The phosphorylation state of KaiC**.** Time course of phosphorylation of KaiC alone (solid) and KaiBC (open) in various magnesium concentrations.

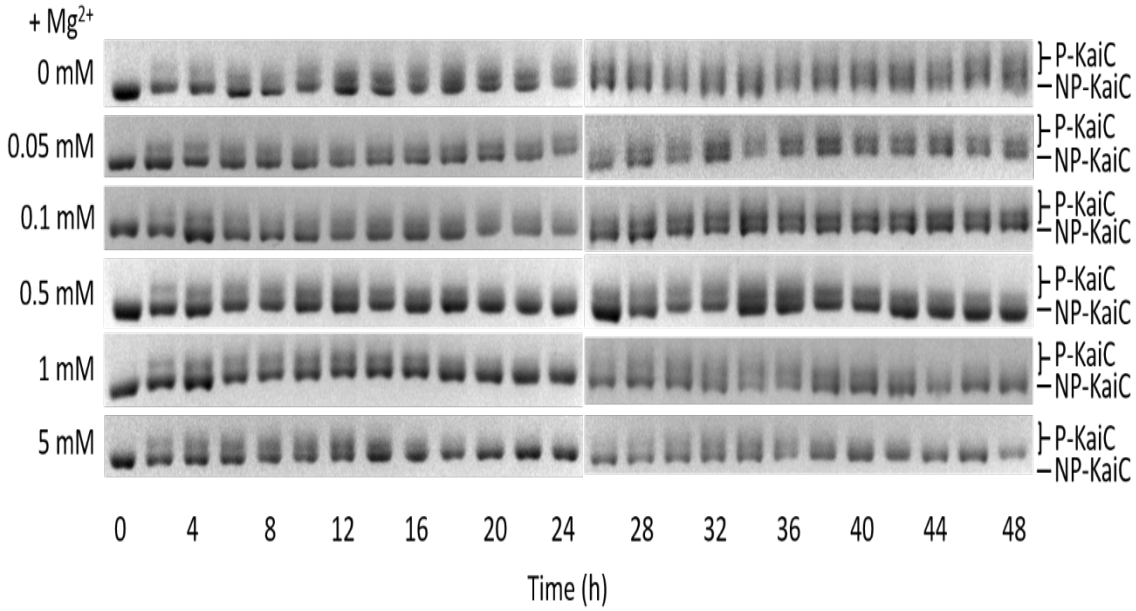


Figure A.12 The phosphorylation state of the hourglass timer of KaiBC**.** The densitometry analysis of the bands is shown in Figure 3.4(d).

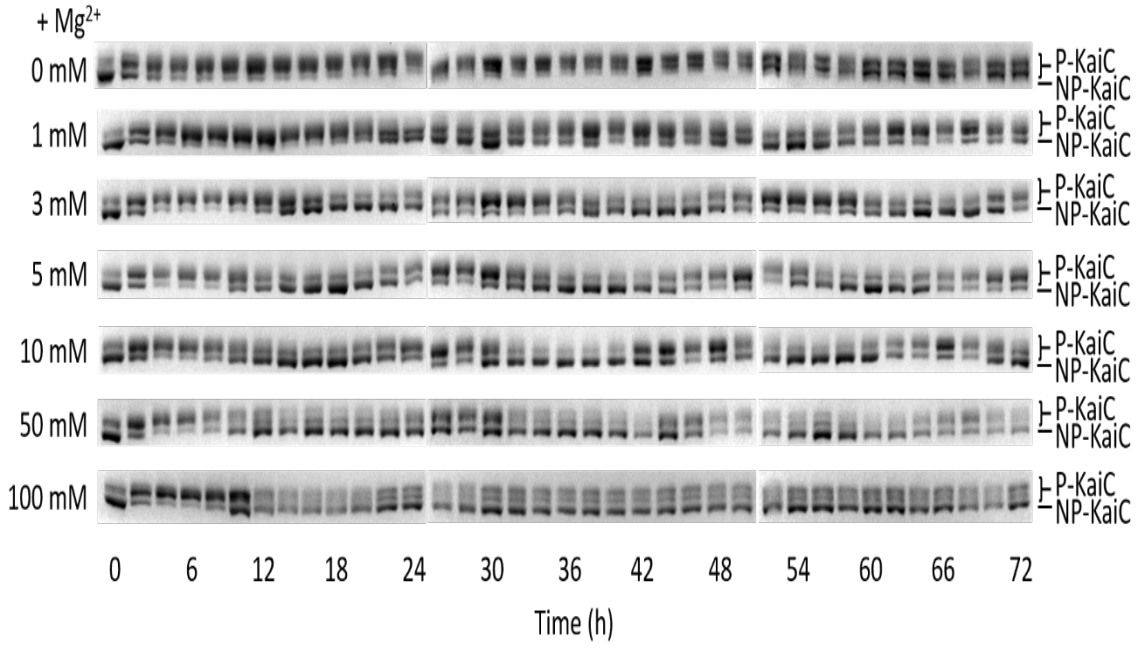


Figure A.13 The phosphorylation state of KaiABC in vitro oscillator**.** The densitometry analysis of the bands is shown in Figure 3.4(e).

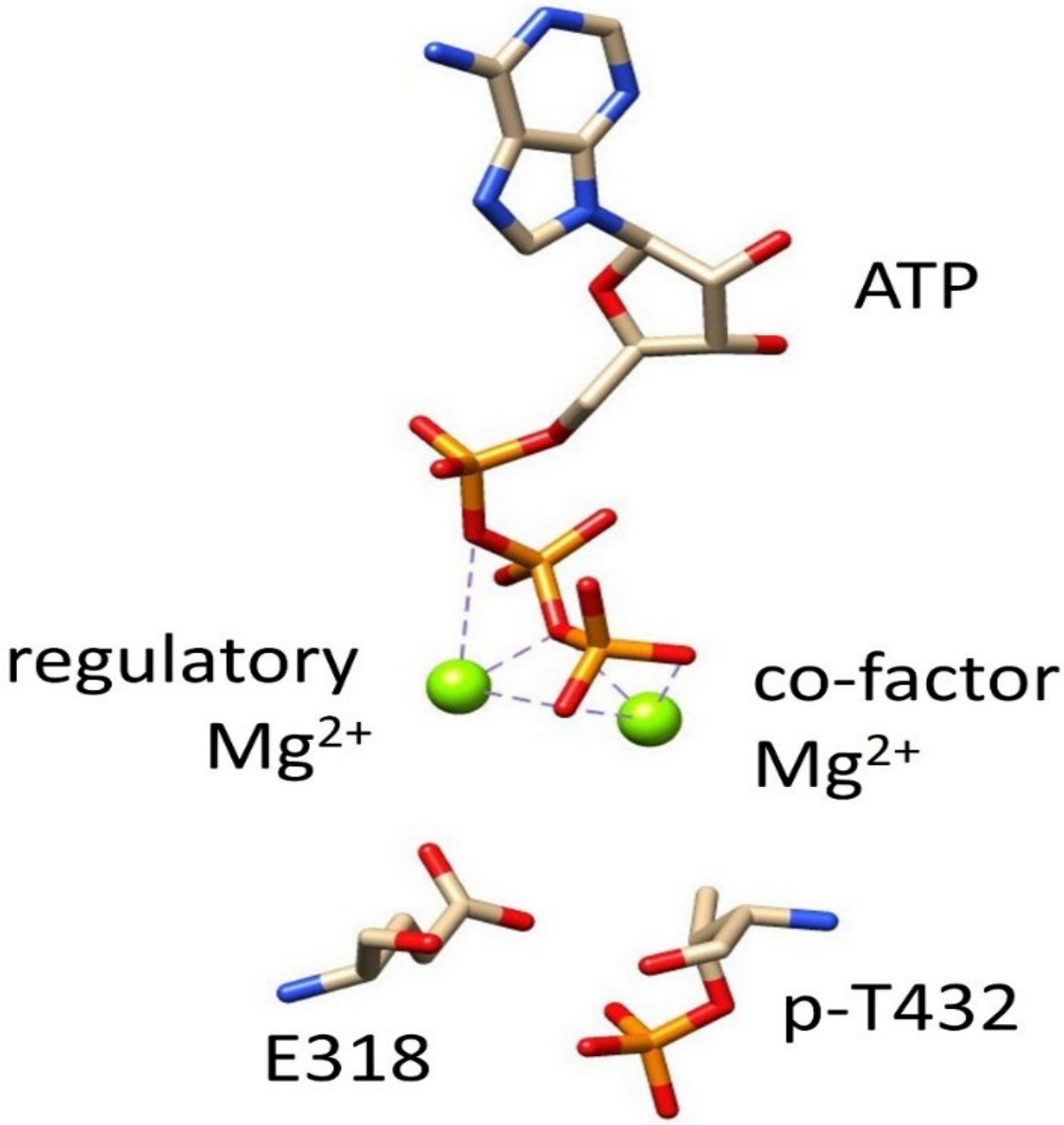


Figure A.14 The active site structures of KaiC S431A mutant (PDB ID: 3k0a)**.** The structures with two magnesium ions bound can also be found in KaiC S431D (PDB ID: 3k09) and KaiC S431E/T432E (PDB ID: 3s1a).

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