(Form 3)

Summary of Doctoral Thesis

Damien Stephane SIMON

Disassembly Mechanism of Circadian Clock Proteins in Cyanobacteria

Circadian clock systems are time keeping systems that enable organisms to adapt to daily environmental changes with a period of approximately 24 h. Biological clockworks are seen ubiquitously in prokaryotes and eukaryotes, and share the following three characteristics. First, the clock systems generate an autonomous oscillation with an approximately 24-hour period even without any external cues (self-sustained oscillation). Second, the period length of the oscillation is little affected by temperature (temperature compensation), even though individual reaction steps that constitute the clock system could be temperature dependent. Q_{10} is one measure of the temperature dependence, indicating how many times reaction rates or frequencies are accelerated by raising the temperature by 10°C (Segel I. H. *Enzyme kinetics: behavior and analysis of rapid equilibrium and steady-state enzyme systems.* New York: Wiley, 1975). The Q_{10} value of the circadian clock systems is almost unity. Third, external stimuli such as light and temperature shift the phase of the oscillator to synchronize it with the phase of the environment (synchronization). Since the discovery of clock genes and clock proteins (Gekakis *et al. Science* **280**, 1564-1569, 1998; Ishiura *et al. Science* **281**, 1519-1523, 1998), scientists from a wide range of disciplines have devoted considerable effort to elucidating the mechanisms underlying the above three physiological properties of the circadian clocks (Partch *J. Mol. Biol.* **432**, 3426-3448, 2020; Akiyama *Cell. Mol. Life Sci.* **69**, 2147-60, 2012).

The simplest of the circadian clock systems known so far is the one from cyanobacteria, since its oscillator can be reconstituted *in vitro* simply by mixing three clock proteins, KaiA, KaiB and KaiC, together with ATP (Nakajima *et al. Science* **308**, 414-415, 2005). KaiC reveals rhythmic changes in its ATP hydrolysis (ATPase) activity (Terauchi *et al. PNAS* **104**, 16377-16381, 2007) and phosphorylation state (Nakajima *et al. Science* **308**, 414-415, 2005) with the period of approximately 24 h through cyclic assembly and disassembly among the three Kai proteins (Akiyama *et al. Mol. Cell* **29**, 703-716, 2008). Although the assembly of Kai complexes such as KaiB-KaiC and KaiA-KaiB-KaiC has been studied intensively so far (Rekha *et al. PLoS One* **6**(8), 1-19, 2011; Snijder *et al. Science* **355**, 1181-1184, 2017), little is known about how these

excessively stable complexes dissociate autonomously. This has prevented us from understanding the mechanism in the night-to-dawn phase and drawing an accurate picture of the whole circadian cycle.

Another reason for focusing on disassembly process is that the property of each Kai protein may be more reflected in the three properties of the whole clock system via the dissociation process. While the assembly of free Kai proteins is a process mainly influenced by the probability of their encounter in solution, the autonomous disassembly is an event triggered by internal states such as structures, post-translational modifications, and ligand exchanges of the Kai proteins within the complexes. Among the three Kai proteins, KaiC is the only one that can change such the internal states with a high degree of freedom. Therefore, this thesis focuses on how the internal states of KaiC are communicated to the entire Kai-protein clock system through the regulation of the complex disassembly.

According to the previous study (Mukaiyama *et al. Sci. Rep.* **8**, 8803, 2018), the phosphorylated KaiC in its post ATP-hydrolysis state preferentially assembles into the complex with KaiA and KaiB. In the assembly phase, KaiC first binds KaiB to form the KaiB-KaiC complex, then KaiA is trapped by KaiB bound to KaiC, and finally the KaiA-KaiB-KaiC complex is formed (Snijder *et al. Science* **355**, 1181-1184, 2017). Since the direct interaction between KaiA and KaiB is extremely weak under conditions where KaiC is not present (Mutoh *et al. Genes Cells* **15**, 269-280, 2010), there is no doubt that it is the KaiB-KaiC interaction that determines the stability of these complexes and the timing of their dissociation. Thus, I first investigated the dissociation dynamics of KaiB-KaiC complex, and then examined the detailed mechanism of dissociating KaiA-KaiB-KaiC complex based on the findings from the binary complex.

This thesis is structured in five chapters. Chapter I describes a historical background on the discovery of circadian clocks and a general introduction to the circadian clock system in cyanobacteria.

In Chapter II, I focused on the disassembly of KaiB-KaiC complex in the absence of KaiA (Simon *et al. Biophys. Physicobiol.* **19**, e190008, 2022). The free KaiB and free KaiC produced by dissociation are immediately reassembled into the KaiB-KaiC complex, because of its high stability. This has made it difficult to experimentally observe the disassembly process of the KaiB-KaiC complex. I thus developed a buffer-exchange method to replace bulk nucleotides (ATP and ADP) with a non-hydrolysable analogue of ATP, so that the free KaiC resulted from the dissociation would not form the post ATP-hydrolysis state with the high affinity for free KaiB. The size exclusion

chromatography (SEC) and SDS-PAGE technique were used to monitor the disassembly process of the KaiB-KaiC complex. The apparent disassembly rate of wild-type KaiC ($KaiC^{WT}$) was unusually slow (2.1 ± 0.3 d^{-1}) as compared with that of the assembly process (approximately 12 d^{-1}) (Mukaiyama *et al. Sci. Rep.* **8**, 8803, 2018; Terauchi *et al. Proc. Natl. Acad. Sci. USA* **104**, 16377-16381, 2007; Abe *et al. Science* **349**, 312-316, 2015). Then, I extended this experimental system to a variety of KaiC mutants showing different cycle frequencies. Interestingly, the apparent disassembly rate of the KaiB-KaiC complex was moderately correlated to the frequency of the KaiC phosphorylation cycle in the presence of KaiA and KaiB. Furthermore, the dissociation dynamics of the KaiB-KaiC complex was perfectly temperature-compensated (Q_{10} = 1.1 ± 0.2). Taken together, the slow and temperature-compensated change in the internal state of KaiC is an important factor that can affect not only the timing of disrupting the KaiB-KaiC interaction, but also the properties of the Kai-protein clock system.

In Chapter III, I investigated the temporal evolution of KaiC-bound nucleotide in order to gain insights into its internal states responsible for the dissociation process. For this mean, I used high performance liquid chromatography (HPLC). The established method would enable to reveal how the exchange of bound nucleotides in KaiC is related with the disassembly process of the KaiB-KaiC interaction in the clock system.

In Chapter IV, I studied a KaiA-assisted mechanism of accelerating the autonomous slow disassembly of KaiB-KaiC complex (Furuike *et al. PNAS* **119**, e2119628119, 2022). The experimental system in Chapter II was slightly modified so that the effect of KaiA could be detected. KaiA was added to the KaiB-KaiC complex pre-formed without the non-hydrolysable ATP analogue, and the dissociation kinetics was traced by SEC, Native-PAGE, phosphorylation assay, and ATPase assay. The added KaiA was temporary trapped as the KaiA-KaiB-KaiC complex, but the KaiB-KaiC interaction in the ternary complex was disrupted faster than that in the binary complex. The results were well explained by the auto-catalytic mechanism; the intrinsically slow KaiB-KaiC dissociation within one ternary complex results in the release of one free-KaiA molecule, which attacks another ternary complex to dissociate it instantly and to generate another free-KaiA molecule. In this way, the number of free-KaiA molecules increases autocatalytically by a factor of two with each step. This results in a sudden and dramatic acceleration of the slowly initiated dissociation.

Finally, I conclude this thesis in Chapter V. Although much attention has been focused on the assembly of the KaiB-KaiC and KaiA-KaiB-KaiC complexes, little has been known so far about the disassembly mechanism of these Kai complexes. This thesis

uncovered that the change in the nucleotide-bound state of KaiC is one of the main internal factors that promote the autonomous disassembly of the Kai-protein complexes, and that the rate and temperature dependence of KaiC ATPase are thus inherited to the system-level characteristics. Although clock proteins themselves are not conserved universally, the formation of the clock-protein complexes in dusk-to-night phase and their dissociation in dawn-to-morning phase are common to a wide variety of organisms ranging from bacteria to mammals. Therefore, the findings and conclusion in this thesis about the cyanobacterial clock system should contribute significantly to our understanding of the circadian clocks in other organisms.