Master allostery in clock protein KaiC orchestrates circadian rhythm

Allostery, a biological (or ubiquitous) event in which a chemical process occurring at one site of a protein or enzyme affects the catalytic activity of another site, is the basis of non-linear cellular responses. The circadian rhythm, a major example of such regulatory phenomena, is a biological timekeeping system that enables organisms to adapt to environmental fluctuations caused by Earth's rotation. Efforts have been dedicated to understanding the mechanisms by which activities and structures of molecules, complexes, and cells autonomously oscillate with a 24-hour period. However, the precise relationship between protein allostery and rhythmicity remains to be elucidated.

The core oscillator of circadian rhythms in cyanobacteria consists of three clock proteins, KaiA, KaiB, and KaiC (Fig. 1(a)). The three Kai proteins repeatedly associate and disassociate *in vitro* in the presence of adenosine triphosphate (ATP). KaiC, a component with the highest molecular mass among the three, orchestrates the assembly states of KaiA and KaiB through autonomous activation and deactivation of its own catalytic activities.

The cyanobacterial circadian rhythm is generated by two reaction cycles in KaiC (Fig. 1(b)). The KaiC hexamer hydrolyzes ATP in its N-terminal domain (C1) to determine the period length of the rhythm (C1-ATPase cycle). In its C-terminal domain (C2), phosphorylation and dephosphorylation of S431 and T432 configure the cycle with a circadian period (C2-phospho cycle) (ST→SpT→pSpT→pST: S for S431, T for T432, p for phosphorylated states). Although the active sites of C1 and C2 are 4 nm distant, they function cooperatively through mutual allosteric regulations. The importance of this cooperativity is evident from the fact that the loss of function of either C1 or C2 results in arrhythmicity, but the mechanisms of the rhythm emergence via C1-C2 coupling and the transmission of temporal information to KaiA and KaiB are still unresolved. To address this issue, we conducted comprehensive structural analyses of KaiC.

To gain insights into the structural basis of the rhythmicity, we crystallized KaiC in different phosphorylation states and obtained electron density maps at 2.2–3.1 Å resolutions through diffraction experiments at SPring-8 **BL44XU** [1][2][3]. Our structure library consisting of 13 KaiC coordinates covered the entire C2-phospho cycle via KaiC-ST, KaiC-SpT, KaiC-pSpT, and KaiC-pST. Similarly, for C1, we identified ATP, nucleophilic water molecules, and adenosine diphosphate (ADP) in the active sites and confirmed that pre- and post-hydrolysis states in C1-ATPase cycle were captured.

The KaiC structure library captured three conformational changes in C1 and C2. First, a secondary structural transition associated with the C2-phospho cycle was identified (C2-PSw, phosphorylation switch) (Fig. 2(a)). The upstream region of the phospho-sites adopted helical and coiled conformations due to differences in the volumes and charges around S431/pS431 [1]. This dynamic structural change was also confirmed in the solution phase during the *in vitro* oscillation [3]. The C2-phospho cycle through the biochemical 4-state was nearly a conformational switch between the two structural states.

The second was a whole domain positional rearrangement in which the neighboring C1 shifts systematically at the protomer-protomer interfaces (C1-domain slide) (Fig. 2(b)). This was related to the oscillation of the ATP hydrolysis activity, through ADP-ATP exchange process and the regulation of lytic water positions (Fig. 2(c)) [2].

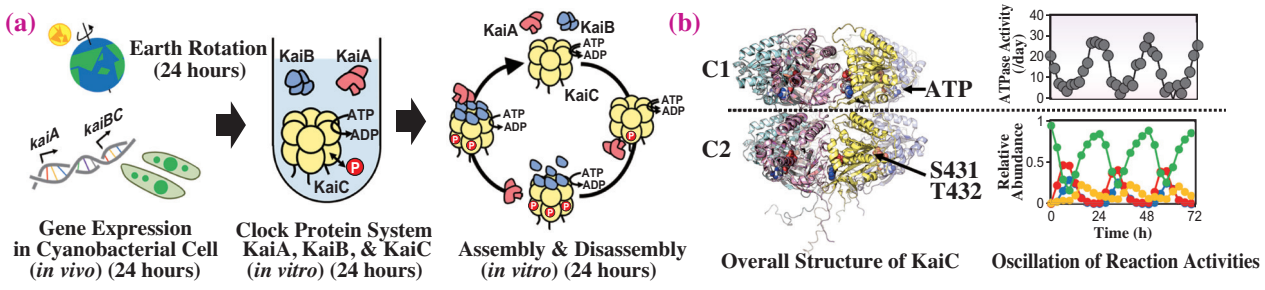

Fig. 1. Clock protein KaiC mastering the cyanobacterial circadian rhythm. **(a)** Cross-scale relationships between the oscillation *in vitro*, the rhythm *in vivo*, and Earth's rotation. **(b)** The overall architecture of KaiC and the oscillations of states in C1 and C2. KaiC-ST, KaiC-SpT, KaiC-pSpT, and KaiC-pST, are distinguished by green, red, blue, and orange, respectively.

Third, the above structural alternations in C1 and C2 were mediated by the hydrogen bond rearrangements in their interface (Fig. 2(d)). The neutral Q394 was the key residue for the functional and structural C1-C2 communication, which is accomplished by switching its hydrogen bonding partner between the basic R217 and the acidic E214 (ERQ triad) [1].

KaiC integrates elementary reactions of C1-ATPase cycle and C2-phospho cycle through these secondary, tertiary, and quaternary structural arrangements to generate the circadian rhythm. This observation was experimentally verified through the examination of a mutant with the single phosphorylation at S432 (KaiC-SV). Despite the mutation of T432 into valine, KaiC-SV still retained a rhythmicity based on C2-PSw. Furthermore, its prolonged period could be *allosterically* shortened by activating C1-ATPase (Fig. 2(e)) [1].

The C1-C2 allostery is indeed a core of the clock that transmits the time information throughout the cell. The disassembly of KaiA-KaiB-KaiC complexes formed during the cycle is essentially suppressed with an extremely low dissociation rate constant [4]. The cue for the disassembly is provided by the activation of ATP hydrolysis accompanied by C1-domain slide [2]. Moreover, the circadian rhythm is known as a temperature-insensitive phenomenon, in which the period length is almost constant in the range of physiological temperature, and this ability referred to as temperature compensation also relies on C1-C2 allostery [5].

For understanding biological phenomena such as the circadian rhythm that span a wide range of spatio-temporal scales (from fast atomic dynamics to slow complex disassembly dynamics), it is important to observe the structural changes of biomolecules underlying the cellular events. Slight structural changes in the active sites can be particularly significant in these systems, so it is still necessary to solve the crystal structures as demonstrated in this study.

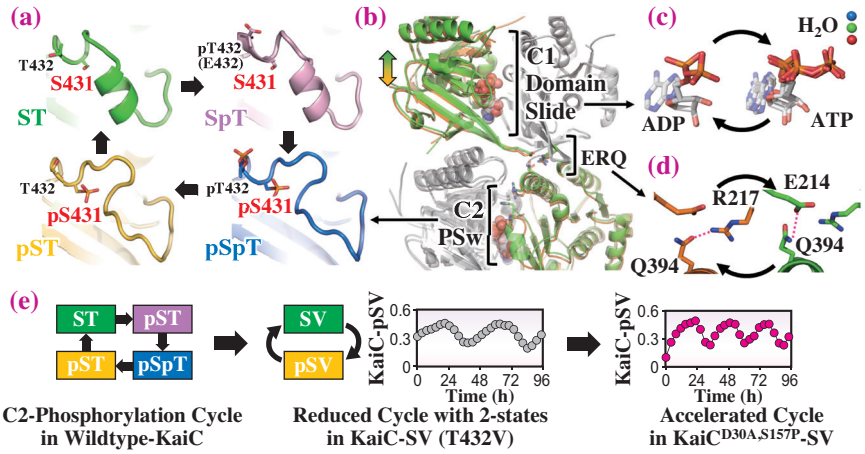

Fig. 2. Master allostery integrating C1-ATPase cycle and C2-phospho cycle in KaiC. **(a)** Helical and coiled structures found at the upstream region of S431. **(b)** Structural coupling between C1-domain slide and C2-phosphorylation switch. The direction of C1-domain slide is indicated by an arrow. **(c)** ATP, nucleophilic water molecules, and ADP found in C1-ATPase cycle. The positional rearrangements of the waters are represented by red (high activity), green (middle activity), and blue (low activity) spheres. **(d)** Hydrogen-bond switch among E214, R217, and Q394. **(e)** Scheme of the experiments verifying the identified allostery. The period length of KaiC-SV oscillation was 47 hours in the presence of KaiA and KaiB. The mutations, D30A and S157P introduced into C1, resulted in the reduction of the period length down to 29 hours.

Yoshihiko Furuike[a,b,*], Atsushi Mukaiyama[a,b,c] and Shuji Akiyama[a,b]

[a] Research Center of Integrative Molecular Systems (CIMoS), Institute for Molecular Science
[b] Department of Functional Molecular Science, SOKENDAI, The Graduate University for Advanced Studies
[c] Department of Bioscience and Biotechnology, Fukui Prefectural University

*Email: furuike@ims.ac.jp

References

[1] *Y. Furuike, A. Mukaiyama, D. Ouyang, K. Ito-Miwa, D. Simon, E. Yamashita, T. Kondo and S. Akiyama: Sci. Adv.* ***8*** *(2022) eabm8990.*
[2] *Y. Furuike, A. Mukaiyama, S. Koda, D. Simon, D. Ouyang, K. Ito-Miwa, S. Saito, E. Yamashita, T. Nishiwaki-Ohkawa, K. Terauchi, T. Kondo and S. Akiyama: Proc. Natl. Acad. Sci. USA* ***119*** *(2022) e2119627119.*
[3] *A. Mukaiyama, Y. Furuike, E. Yamashita and S. Akiyama: Biochem. J.* ***479*** *(2022) 1505.*
[4] D. Simon *et al.*: Biophys. Physicobiol. **19** (2022) e190008.
[5] Y. Furuike *et al.*: Comm. Phys. **5** (2022) s42005-022-00852-z.