Visualization of circadian ticking of cyanobacterial clock protein KaiC in real time

Circadian clocks are endogenous timing systems for various organisms to adapt to dairy alterations in external environments. The cyanobacterium *Synechococcus elongatus* PCC7942 is one of the simplest organisms known to possess a circadian oscillator consisting of the three clock proteins KaiA, KaiB, and KaiC [1]. KaiC is rhythmically phosphorylated and then dephosphorylated *in vivo* without a transcriptional-translational negative feedback mechanism, which has been proposed to underlie eukaryotic circadian clocks. In addition, Nakajima *et al.* have shown that the phosphorylation cycle of KaiC is reconstituted simply by co-incubating the three Kai proteins in the presence of ATP [2]. KaiC possesses autokinase/autophosphatase activities. KaiA stimulates the auto-phosphorylation of KaiC, whereas KaiB promotes the auto-dephosphorylation of KaiC by attenuating the effect of KaiA. During the KaiC phosphorylation cycle, the three Kai proteins are assembled into hetero-multimeric complexes and then disassembled in a rhythmic manner [3]. The frequency of the phosphorylation cycle of KaiC is closely correlated with the rate of ATP hydrolysis exhibited by KaiC only [4]. The ATPase activity of KaiC, which is extremely low and is kept constant in a range of physiological temperatures, plays a central role in generating the temperature-compensated circadian period of the Kai oscillator.

KaiC is a dumbbell-shaped molecule composed of tandemly duplicated N-terminal (C1) and C-terminal (C2) domains. Six protomers are assembled into a hexamer to attain a double-doughnut shape. A KaiC mutant having only a C1 domain forms a C1 ring and exhibits approximately 70% of the ATPase activity of full-length KaiC. KaiC has two phosphorylation sites, i.e., Ser431 and Thr432, in the C2 domain, and both residues are phosphorylated and then dephosphorylated in a programmed sequence during the phosphorylation cycle as follows: $KaiC^{S/pT} \rightarrow KaiC^{pS/pT} \rightarrow KaiC^{pS/T} \rightarrow KaiC^{S/T}$ (where 'S' represents Ser431, 'pS' represents phosphorylated Ser431, 'T' represents Thr432, and 'pT' represents phosphorylated Thr432). The phospho-mimicking mutant of $KaiC^{S/T}$ exhibits a higher ATPase activity than the phospho-mimicking mutant of $KaiC^{pS/pT}$, indicating a functional coupling between ATPase and the phosphorylation state through the KaiC hexamer. Hence, the determination of the structural change of KaiC interlocked with ATPase and the phosphorylation state is of great importance.

Taking advantage of the fact that the ATPase activity is closely coupled with the phosphorylation state, we investigated the structure of KaiC in solution along the reaction sequence of the phosphorylation cycle. Auto-dephosphorylation reaction was induced by a temperature jump from 'on ice' to 30°C, and the structural transition was monitored using time-resolved small-angle X-ray scattering (SAXS) at beamline **BL45XU** [5]. Concomitantly with the shift in the phosphorylation state, we observed a gradual increase in the apparent radius of gyration (R_g) estimated from the measured SAXS curves. As a result of the detailed Guinier analysis, a substantial increase in R_g by 5% was observed in the $KaiC^{pS/pT} \rightarrow KaiC^{pS/T}$ transition. The 5% increase in R_g indicates the occurrence of a

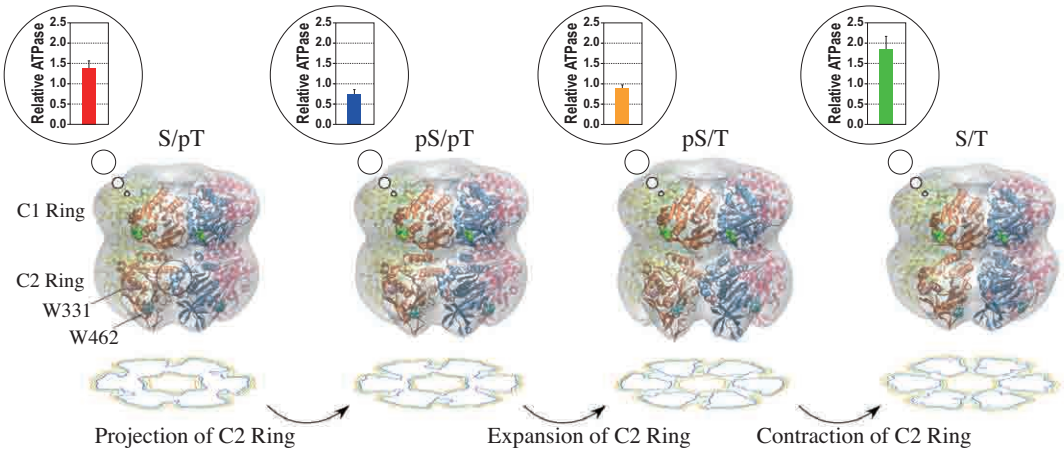

Fig. 1. Expansion and contraction motions of C2 ring of KaiC interlocked with ATPase activity. The protomer-protomer arrangement of the known X-ray structure of KaiC was refined against the SAXS data under the assumption of P6 symmetry. The ATPase activity of each phosphorylation state is plotted relative to that of KaiC-WT at 30°C.

large conformational change in the KaiC hexamer.

The structural transition of the KaiC hexamer was further monitored by time-resolved fluorescence spectroscopy. KaiC intrinsically has three tryptophan (Trp) residues, which are useful for monitoring local structural changes. One Trp residue is located in the C1 ring, whereas the other two are located in the C2 ring. We observed a gradual change in the fluorescence intensity during auto-dephosphorylation, and then identified that the observed change was mainly attributed to the change in the local environments around the two Trp residues in the C2 ring.

To visualize the structural change, we built low-resolution SAXS models of the KaiC hexamer by the rigid-body refinement of a known X-ray crystal structure (Fig. 1). The global shape remains almost unchanged in the transition from $KaiC^{S/pT}$ to $KaiC^{pS/pT}$, whereas the radius of the C2 ring is dramatically enlarged in the subsequent transition from $KaiC^{pS/pT}$ to $KaiC^{pS/T}$. The expanded C2 ring is partly contracted in the transition from $KaiC^{pS/T}$ to $KaiC^{S/T}$, and further contracted in the subsequent transition from $KaiC^{S/T}$ to $KaiC^{S/pT}$. Our model implies that KaiC ticks through expanding and contracting motions of the C2 ring.

The Trp fluorescence coming from KaiC displays a circadian oscillation in the presence of both KaiA and KaiB (Fig. 2(a)), providing direct evidence of the dynamic structural ticking of KaiC in solution. The observed oscillation in the fluorescence intensity can be well explained by a combination of the fluorescence intensity of the each phosphorylation state and its abundance, supporting the expanding and contracting motions of the C2 ring during circadian oscillation.

The dynamic structural change of the KaiC hexamer revealed in this study provides notable insights into the cyanobacterial circadian clock. The ATPase activity of KaiC is less sensitive to the C2 ring expansion in the transition from $KaiC^{pS/pT}$ to $KaiC^{pS/T}$, and the partial contraction of the C2 ring in the transition from $KaiC^{pS/T}$ to $KaiC^{S/T}$ results in a significant activation of ATPase (Fig. 1). These observations suggest that ATPase activity is not as simple as an on/off (contraction/expansion) switching process. Our results may reflect the auto-inhibitory mechanism proposed in a previous study [4]. Additionally, the timing of the accumulation of $KaiC^{pS/T}$ with the expanded C2 ring is coincident with that of the accumulation of the ternary complex with KaiA and KaiB [3]. Considering that KaiA and KaiB bind to the C2 ring of KaiC, the conformational ticking of KaiC serves as a timing cue for assembly/disassembly with KaiA and KaiB (Fig. 2(b)).

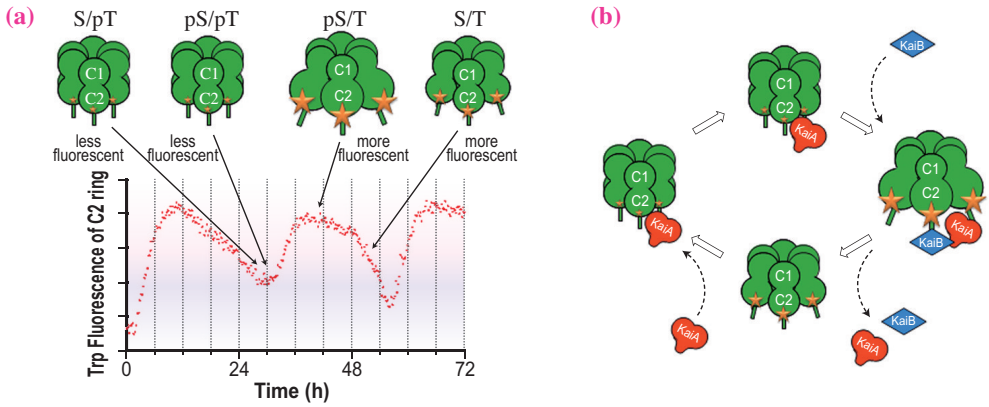

Fig. 2. Circadian ticking of KaiC. **(a)** Trp fluorescence intensity in the presence of KaiA, KaiB, and KaiC. **(b)** Schematic presentation of Kai protein oscillator. The dynamic structural change of the C2 ring of KaiC serves as a timing cue for recruiting KaiA and KaiB.

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