Real-time Small-angle X-ray Scattering Observation of Assembly and Disassembly Dynamics of Cyanobacterial Periodosome

Circadian clocks are endogenous timing systems enabling a variety of living organisms to adapt daily alternations of environments on earth. Cyanobacterium *Synechococcus elongatus* PCC 7942 is one of the simplest organism known to possess a self-sustained circadian oscillator composed of three clock proteins termed KaiA, KaiB, and KaiC [1]. KaiA enhances the autophosphorylation of KaiC, whereas KaiB induces the autodephosphorylation of KaiC by antagonizing KaiA activity. Recently, Nakajima *et al.* succeeded in reconstructing the KaiC phosphorylation cycle *in vitro* solely by incubating KaiA, KaiB, and KaiC in the presence of ATP [2]. This pioneering work suggests that the Kai proteins are assembled and disassembled into heteromultimeric Kai complexes (periodosome) to effect a circadian switching of KaiC activity from autophosphorylation to autodephosphorylation and *vice versa*.

In order to elucidate the oscillatory mechanism, much attention has been focused on the structure of the Kai periodosome. To date, the crystal structures of the individual Kai proteins have already been determined independently [3]. However, a potential relationship between the assembly/disassembly dynamics and the KaiC phosphorylation cycle is still poorly understood because of the difficulty in unraveling the underlying mechanisms solely from the static crystal structure of individual clock components. Although it is important to characterize Kai complexes along a reaction coordinate, a blind search for crystallization conditions of oscillatory-transient complexes is not necessarily a promising strategy.

To take a structural snapshot of the Kai periodosome, we followed the assembly/disassembly dynamics of a ternary mixture containing KaiA, KaiB, and KaiC in real-time using small-angle X-ray scattering (SAXS) at beamline **BL45XU** [4]. As shown in Fig. 1, weight-averaged molecular weight (MW^{app}) estimated from forward scattering intensity of measured SAXS curves increased dramatically upon mixing three Kai proteins and peaked at 9 h. After a monotonous decrease from 9 to 18 h, MW^{app} revealed a robust oscillation with a period of 24.4 h. Interestingly, MW^{app} was phase-delayed by approximately 6.6 h (6.6/24.4 = ~π/2) relative to the phosphorylated KaiC fraction ($Phos^{app}$), whereas the period of MW^{app} was nearly identical to that of $Phos^{app}$ (23.8 h). The phase delay of π/2 implies that $Phos^{app}$ is closely related to the time derivative of MW^{app}, and that there is a relationship between reactant and product. The current observations therefore suggest that the assembly/disassembly of the Kai periodosome is not under steady-state conditions, but evidently driven by the phosphorylation status of KaiC.

To investigate the relationship between the Kai complex structure and KaiC phosphorylation, we conducted titration SAXS experiments [4,5] for binary mixtures of KaiA and KaiC, and KaiB and KaiC. Titration data sets of both binary pairs were fitted uniquely to a theoretical curve for a 1:1 binding scheme. The dissociation constant (K_d) of the phosphorylated KaiA:^{P}KaiC complex (4.7 μM) was approximately 40-fold larger than that of the dephosphorylated KaiB:^{NP}KaiC complex (0.12 μM), indicating a greater affinity of KaiB for ^{NP}KaiC than of KaiA for ^{P}KaiC.

On the basis of the binding scheme and K_d, the scattering curves of the binary complexes were estimated by global fitting on the titration data set. Low-resolution envelopes were restored from the deconvoluted SAXS curves (Figs. 2(a) and 2(c)), onto which the crystal structure of each Kai protein was uniquely superimposed (Figs. 2(b) and 2(d)). Our complex models clearly demonstrate that KaiA binds a

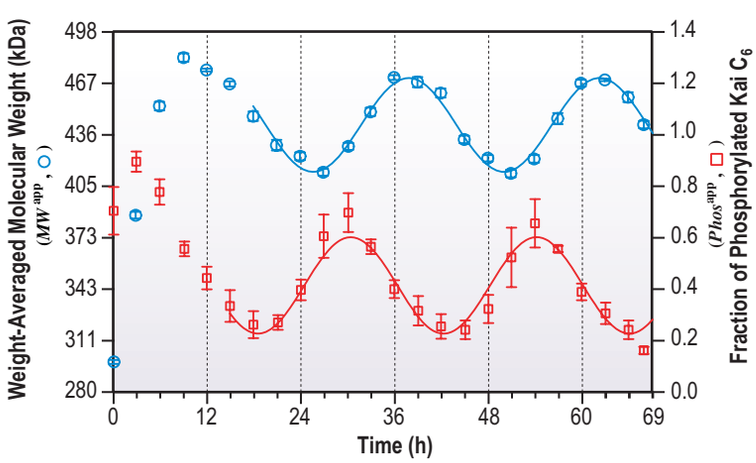

Fig. 1. Assembly/disassembly dynamics of ternary mixture containing KaiA, KaiB, and KaiC in the presence of ATP. Weight-averaged molecular weight (MW^{app}, blue open circle) is phase-delayed by π/2 relative to phosphorylated KaiC fraction ($Phos^{app}$, red open square). The solid lines represent sine-wave functions fitted to the experimental data.

broad interface ranging from the C2 domain to the tail of ^{P}KaiC. Interestingly, KaiB shares a common docking site of the C2 domain with KaiA.

These binary interactions offer a reasonable explanation for the assembly/disassembly of the Kai periodosome in a phosphorylation-dependent manner. Upon mixing three Kai proteins, KaiA immediately binds KaiC to form the KaiA:KaiC complex, thereby enhancing the autophosphorylation of KaiC. KaiB then approaches the KaiA:^{P}KaiC complex to inhibit KaiA activity. Because of the greater affinity of KaiB for the common docking site of the C2 domain than KaiA, KaiB can displace KaiA of the KaiA:^{P}KaiC complex, and thereby switching KaiC activity from autophosphorylation to autodephosphorylation.

It must be noted that the Kai periodosome assembles and disassembles robustly also *in vivo* [1,2]. This is astonishing because assembly/disassembly processes under physiological conditions should be affected to some extent by crowding and higher viscosity. From the crystal structure [3], dual phosphorylation sites of KaiC are localized to the C2 domain. Thus, the resistances against crowding and viscosity will be achieved in the Kai complexes by controlling the timing of assembly/disassembly of KaiA and/or KaiB though phosphorylation-dependent structural changes of the C2 domain of pacemaking KaiC.

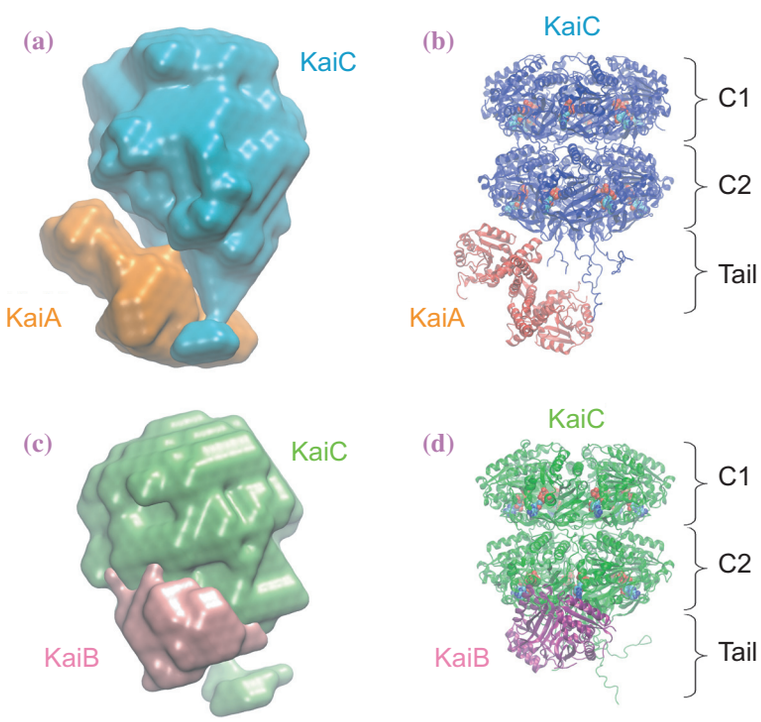

Fig. 2. Low-resolution models of Kai complexes. **(a)** Low-resolution envelope of KaiA:^{P}KaiC complex restored from SAXS data. **(b)** Superimposed crystal structures of KaiA and KaiC to low-resolution model of KaiA:^{P}KaiC complex. **(c)** Low-resolution envelope of KaiB:^{NP}KaiC complex restored from SAXS data. **(d)** Superimposed crystal structures of KaiB and KaiC to low-resolution model of KaiB:^{NP}KaiC complex.

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