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REVIEW

Structural and dynamic aspects of protein clocks: how can they be so slow and stable?

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Abstract KaiC is a core protein of the cyanobacterial Kai oscillator, which persists without transcription–translation feedback. In the presence of KaiA and KaiB, KaiC reveals rhythmic activation/inactivation of its ATPase and autokinase/autophosphotase activities over approximately 24 h. Since the in vitro reconstruction of the Kai oscillator, the structures and functions of the Kai proteins have been studied extensively. Each protein's crystal structure and low-resolution views of Kai complexes have been reported. In addition, newer data are emerging on dynamic aspects such as assembly/disassembly of the Kai components and a ticking motion of KaiC, which is probably coupled to its slow, temperature-compensated ATPase activity. The accumulated evidence offers an ideal opportunity to revisit a fundamental question regarding biological circadian clocks: what determines the temperature-compensated 24 h period? In this review, I summarize the current understanding of the Kai oscillator's molecular mechanism and discuss emerging ideas on protein clocks.

Keywords Circadian clock · Cyanobacteria · KaiC · Complex · SAXS · EM

Introduction

The Greek philosopher Aristotle defined time as motion with respect to a concept of before and after (Aristotle, *Physics*, Book IV, Chapter 11). In fact, motion has been considered an attribute of time since ancient times, as we have measured time by following the motions of the sun (a sundial), the flow of water (a water clock), the fall of sand (an hourglass), and the rotation of meshed gears (a watch). The time-keeping system reviewed here is much smaller than these examples: a biological nano-clock residing in living cells.

Circadian clocks are endogenous timing mechanisms that enable organisms to adapt to daily alterations in the environment [1]. These time-keeping mechanisms, found ubiquitously in prokaryotes and eukaryotes, share the following three characteristics. First, the clock system reveals a self-sustained oscillation with an approximately 24 h period even without any external cues. Second, the clock's oscillatory period depends little on the ambient temperature. Temperature compensation is a remarkable characteristic of circadian clocks because any underlying elementary biological reactions should be more or less temperature dependent. Third, the phase of the oscillator is reset in response to periodical changes in ambient temperature, light, and humidity so as to synchronize its oscillatory status with daily fluctuations in the environment. These three are the characteristics of the circadian clock oscillators as distinguished from other bacterial oscillators (see ref [2] references are therein) such as spatial oscillators for cell division, DNA segregation, and cell polarity.

Since the first isolation of clock mutants in the fly [3], the molecular mechanisms of circadian clocks have been studied extensively in many living organisms [4, 5]. The appearance of circadian rhythmic behaviors in eukaryotic systems is generally interpreted in terms of a transcriptional and translational oscillatory (TTO) model, in which the transcription of core clock genes is under negative feedback control by its translation product (clock protein). The cycle consists of a wide spectrum of biological events: transcription of the clock gene in the nucleus; translation of the

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transcript in the cytoplasm; refolding and hetero- or homo-complexation of the clock proteins; nuclear transport of phosphorylated clock proteins; and degradation of the clock proteins. The period of the TTO model likely emerges from the accumulation of a series of these biological reactions occurring over a wide range of spatial and temporal scales.

In contrast, the central oscillator of the cyanobacterium *Synechococcus elongatus* PCC 7942 (*S. elongatus*) consists of three clock proteins, KaiA, KaiB, and KaiC [6], and persists even in the absence of transcription–translation feedback [7]. KaiA enhances autophosphorylation of KaiC [8, 9], whereas KaiB inhibits KaiA activity to promote autodephosphorylation of KaiC [10, 11]. KaiC co-incubated in vitro with both KaiA and KaiB undergoes repeated phosphorylation and dephosphorylation on a circadian cycle [13–15]. Astonishingly, the period of this in vitro protein clock is temperature compensated, as in an in vivo clock [7, 15]. Whereas the TTO model is supported by a complex series of reactions, the cyanobacterial oscillator composed of only three Kai proteins is the simplest known form of circadian clock.

Five years after the in vitro reconstruction of the Kai oscillator [15], an oscillatory process persisting without transcription–translation feedback was found in human red blood cells [16]. This finding suggests that the self-sustained protein clock in the prokaryotic cyanobacterium might not be exceptional. The complementary coexistence of a transcription–translation-free oscillator with a transcription–translation-based oscillator is thought to make the overall rhythm more robust against cellular noise [17]. Thus, it is anticipated that transcription–translation-free oscillators will soon be found in other living organisms, which is expected to attract attention from not only chronobiologists but also biophysicists, structural biologists, and protein chemists.

The Kai oscillator reconstituted in vitro provides a practical means of studying the detailed molecular mechanisms by which protein clocks generate 24 h oscillation. Consequently, the structure and function of the three Kai proteins have been extensively studied. The results revealed that although the Kai system appears to be simpler than the TTO system, the mechanisms underlying its temperature-compensated circadian rhythm are far from simple. Considering the growing attention to transcription–translation-free oscillators, I will review our present understanding of the molecular mechanism of the cyanobacterial Kai oscillator and then discuss emerging ideas on protein clock systems.

Individual Kai proteins

The structure of each Kai protein has been studied extensively over the last decade. The crystal structure of *S. elongatus* KaiA is a 65.3 kDa homodimer (Fig. 1a), the

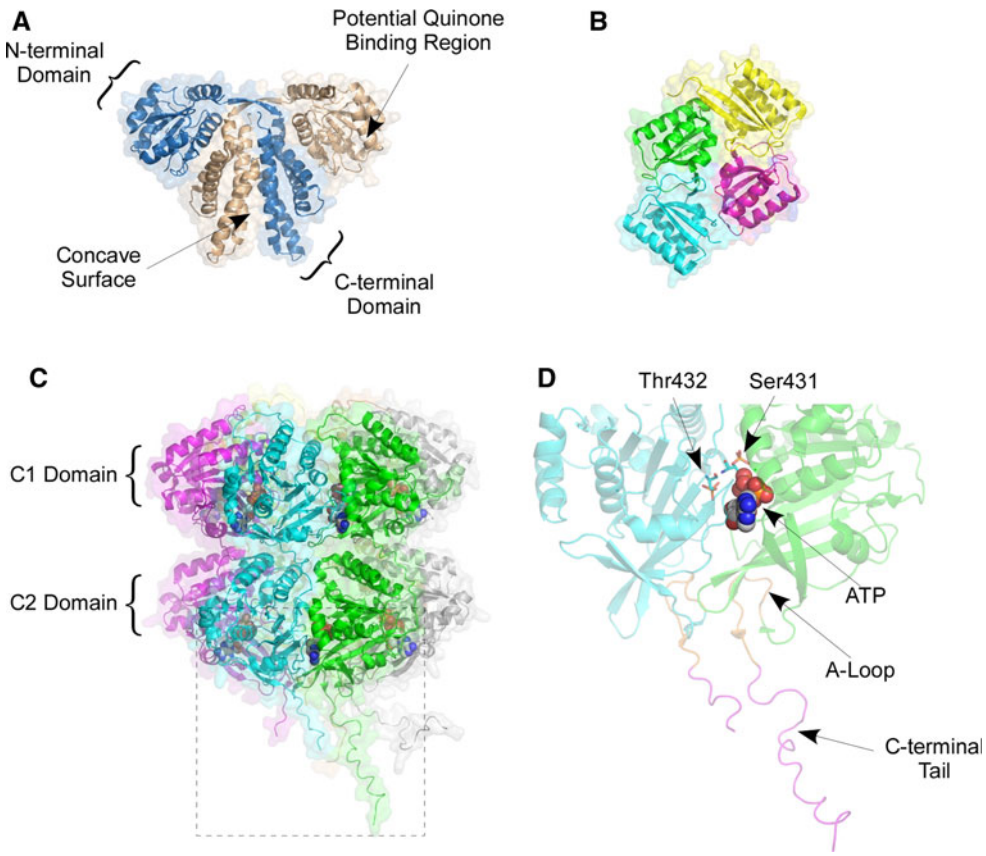

Fig. 1 Crystal structures of Kai proteins. **a** Crystal structure of *S. elongatus* KaiA [18]. **b** Crystal structure of *T. elongatus* KaiB [28]. **c** Crystal structure of *S. elongatus* KaiC [35]. **d** Expanded view of dotted square in (**c**). A-loop and C-terminal tail are *orange* and *magenta*, respectively

subunits of which are composed of an N-terminal and a C-terminal domain [18]. An earlier nuclear magnetic resonance (NMR) study by LiWang's group reported that a truncated N-terminal half of KaiA exhibits folding similar to that of two-component response regulators [19]. Although this similarity suggests that it functions as an input pathway to the Kai oscillator, the conserved Asp residue required for the phosphoryl transfer reaction was not observed, making it difficult to identify its physiological role. The same group later confirmed that the N-terminal domain of KaiA acts as a module that senses environmental changes in the redox state and then modulates the KaiC phosphorylation cycle [20]. The atomic structures of KaiA from *Thermosynechococcus elongatus* BP-1 (*T. elongatus*) [21, 22] and from *Anabaena* sp. PCC7120 [23], which lacks the N-terminal domain, were determined by X-ray crystallography and NMR. The C-terminal domain of KaiA is responsible for its dimerization and forms a concave surface [21] to recruit the C-terminal region of KaiC [24–26].

S. elongatus KaiB, which consists of 102 amino acid residues (11.4 kDa), is the smallest of the three Kai proteins. Crystallographic studies have revealed the detailed structure of KaiB from *Synechocystis* PCC6803 [27], *T. elongatus* [28, 29] (Fig. 1b), and *Anabaena* PCC7120 [23]. KaiB and the N-terminal input domain of histidine kinase (SasA) have markedly similar amino acid sequences, and both bind to KaiC [30], but a later NMR study revealed little similarity in their tertiary folding [31]. In the absence of both KaiA and KaiC, KaiB from *S. elongatus* [24], *Synechocystis* PCC6803 [27], and *T. elongatus* [28] were observed to remain homotetramers (45.6 kDa) in solution by small-angle X-ray scattering (SAXS), dynamic light scattering, and analytical ultracentrifuge (AUC) techniques. Size-exclusion chromatography (SEC) results suggested that KaiB from *Anabaena* PCC7120 has a homodimeric conformation (22.8 kDa) [23]. A recent electron spin resonance (ESR) study using site-directed spin labeling indicated a conformational relaxation of the tetrameric *T. elongatus* KaiB upon a sharp increase in temperature [32]. Neither physiological ligands or cofactors of KaiB were identified, except for trimethylglycine [27], which is used as a precipitant for crystallization.

S. elongatus KaiC is the largest Kai protein, having 519 amino acid residues (58.0 kDa). An earlier study using AUC and electron microscopy (EM) provided the first glimpse of *S. elongatus* KaiC's hexameric ring shape ($58.0 \times 6 = 348$ kDa) [33], and its three-dimensional hexagonal pot shape was visualized in a later EM study of *T. elongatus* KaiC [34]. Egli and colleagues reported the detailed atomic structure of *S. elongatus* KaiC [35] (Fig. 1c). Each protomer of KaiC consists of tandemly duplicated N-terminal (C1) and C-terminal (C2) domains and a C-terminal tail. The monomeric KaiC is assembled into a double-doughnut-shaped homohexamer in an ATP-dependent manner [33, 34], while an ATP molecule is incorporated into every C1–C1 and C2–C2 interface (for a total of 12 ATPs). A truncated KaiC C1 domain (KaiC-C1) undergoes ATP-dependent hexamerization, as full-length KaiC does, but the C2 domain alone does not [33, 36]. The ring architecture composed of the C2 domains (C2 ring) is thus less stable than that composed of the C1 domains (C1 ring). This C2 ring with marginal stability includes major phosphorylation sites for Ser431 and Thr432 (Fig. 1d), both of which are repeatedly phosphorylated and then dephosphorylated in the following order [9, 14, 37]: $KaiC^{S/pT} \rightarrow KaiC^{pS/pT} \rightarrow KaiC^{pS/T} \rightarrow KaiC^{S/T} \rightarrow KaiC^{S/pT}$ ($KaiC^{X/pY}$:KaiC with amino acids X and phosphorylated-Y at 431 and 432, respectively). T426 located near S431 is thought to be a minor possible phosphorylation site sharing a phosphate group with S431 [37, 38]. Stepwise repositioning of the C2 domains was demonstrated by time-resolved SAXS and Trp fluorescence techniques [39]. The C-terminal loop (residues 498–519), which is important for KaiA binding [26], is adjacent to a hairpin loop structure called the A-loop (residues 488–497) [35] (Fig. 1d). KaiC mutants lacking an A-loop and C-terminal tail are of particular interest because they remain phosphorylated even without KaiA [40], suggesting the importance of the A-loop in balancing autokinase and autophosphotase activity in KaiC.

Formation of heteromultimeric Kai complexes

Although the structure of individual Kai proteins was studied extensively, the core mechanism underlying the circadian periodicity remained poorly understood, partly because of the practical difficulties in deducing it from the static structures of the individual proteins. It was as if we had detailed knowledge of the designs of a clock's gears but did not know how they meshed with each other in order to tick.

Iwasaki and colleagues first noted direct inter-protein associations among the three Kai proteins both in vivo and in vitro [41]. A later study by Nishiwaki et al. [13] indicated a correlation between the composition of the Kai complexes and the status of the dual phosphorylation site. For a full understanding of the proteins' periodic activities, it is necessary to determine the atomic structure of each Kai complex and sort them according to the reaction coordinate of the cycle. However, there is no universal, straightforward way to visualize the atomic structure of the Kai complexes, whose composition and stoichiometry change continuously in a time-dependent manner. One practical method is to prepare isotope-labeled Kai proteins with truncated domains for analysis by conventional hetero-nuclear NMR. An earlier NMR study reported the

structure of a KaiAC subcomplex (Fig. 2a), in which a peptide fragment mimicking the C-terminal tail of *T. elongatus* KaiC binds to the concave surface of *T. elongatus* KaiA [26].

Another practical option is to make a slight compromise with respect to the spatial resolution. Because the crystal structure of each Kai protein is known, the essence of each complex structure is expected to be resolved by docking the known crystal structure of individual Kai proteins onto the low-resolution shape of the entire Kai complex. Both single-particle EM and SAXS/small-angle neutron scattering (SANS) offer a way to visualize the overall size and shape of proteins and elementary domains. Single-particle EM enables real-space imaging of individual objects with moderate spatial resolution, but exceptional care should be taken when fixing biological objects so as not to induce non-physiological deformations. On the basis of negatively stained EM images of the *T. elongatus* KaiAC complex, Pattanayek et al. [25] suggested two different geometries for KaiA binding to the KaiC hexamer. One is the tethered

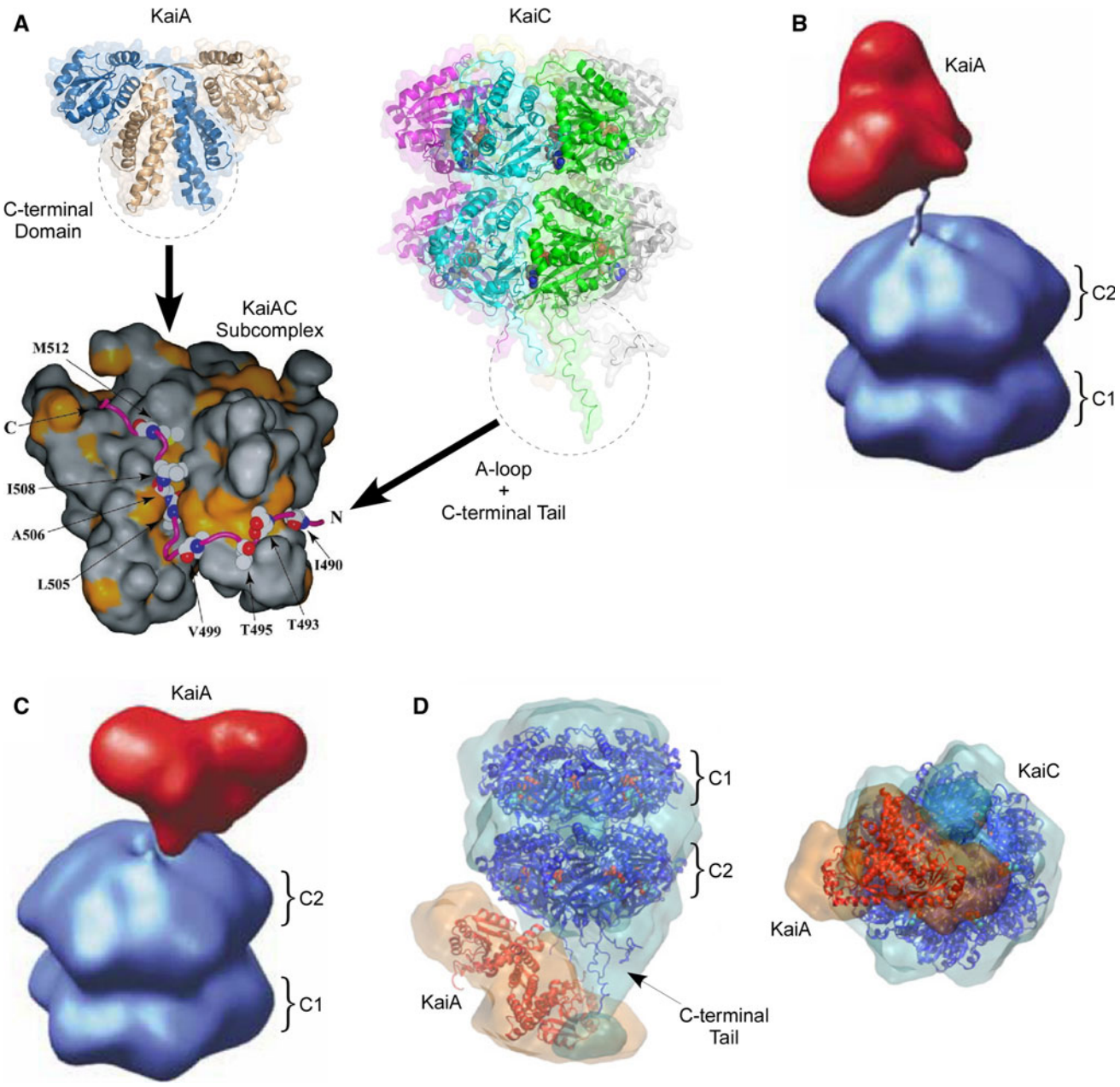

Fig. 2 Structural models for KaiAC complex. **a** NMR-based *T. elongatus* KaiAC subcomplex (adapted from Ref. [26]). **b** EM-based *S. elongatus* KaiAC complex (tethered model) [25]. **c** EM-based *S. elongatus* KaiAC complex (engaged model) [25]. (**b**, **c**) Adapted from Ref. [29]. **d** Two orthogonal views of SAXS-based *S. elongatus* KaiAC complex (adapted from Ref. [24])

model, in which KaiA binds to the tip of the elongated C-terminal tail approximately 35 Å away from the C2 ring (Fig. 2b). The other is the engaged model, in which KaiA binds directly to the C2 domain of the hexameric barrel (Fig. 2c). A closer interaction between KaiA and KaiC was demonstrated in the *S. elongatus* KaiAC complex by SAXS [24] (Fig. 2d). SAXS can restore the molecular shape under physiological solution conditions with good time resolution [42]; however, minor heterogeneities, if any, cannot be seen directly owing to signal averaging over all the particles included in the illuminated volume. In the SAXS model, KaiA is located near the bottom of the C2 ring, thereby establishing an interaction via an association interface ranging from the C2 domain to the C-terminal tail. The SAXS model is rather similar to the tethered model in terms of the orientation of KaiA relative to KaiC, but they differ from each other in terms of the distance between KaiA and KaiC. If KaiA is entrapped in both the A-loop (residues 488–497) and the C-terminal loop (residues 498–519), as in the *T. elongatus* KaiAC subcomplex [26] (Fig. 2a), the C-terminal domain of KaiA inevitably occupies a position adjacent to the C2 ring of KaiC, allowing direct interactions in the full-length KaiAC complex.

Notably, the KaiBC complex is more stable than the KaiAC complex. The binding affinity between KaiB and KaiC was first examined by surface plasmon resonance (SPR) analysis [43] and later confirmed in solution by a quantitative titration experiment to be 40 times that between KaiA and KaiC [24]. Thus, one could generally expect the KaiBC complex to crystallize more easily than the KaiAC complex. However, no studies have yet reported its successful crystallization. This may be attributed in part to minor heterogeneities in the stoichiometry observed under EM [29]. Two structural models were proposed for a major constituent of the KaiBC complex possessing a molecular mass of ~400 kDa [24] on the basis of SAXS [24] and EM studies [29]. These two models exhibit the same molecular mass and selective binding of KaiB to the C2 ring, but they differ in the oligomeric state of the bound KaiB. Whereas KaiB bound onto the KaiC hexamer remains a homotetramer in the SAXS model (KaiBtC) [24] (Fig. 3b), it is modeled as two separate KaiB homodimers in the EM model ($KaiBd_2C$) [29] (Fig. 3a). Note that the SAXS model of the KaiBC complex assumes that KaiB remains a homotetramer in both the free and bound forms [24]. The EM-based KaiBC model suggests that the tetramer/dimer equilibrium shifts toward the dimer, especially in the presence of KaiA and/or KaiC; i.e., a post-binding split of the KaiB homotetramer into two KaiB homodimers on the KaiC hexamer occurs.

A pioneering biochemical study suggested direct but weak interaction between KaiA and KaiB [41]. Although this binary interaction was not confirmed in subsequent biophysical studies using SAXS [24] and fluorescence [29], Mutoh et al. [32] recently reported an ESR signal that indicates a direct association between *T. elongatus* KaiA and KaiB. Because it is not surprising that some difference exists between the stabilities of the *S. elongatus* and *T. elongatus* KaiAB complexes, it would be worth reinvestigating the *S. elongatus* KaiAB complex with much more sensitive techniques.

No three-dimensional models of the KaiABC complex are currently available. Asymmetric binding was proposed on the basis of a visual inspection of two-dimensional EM images [29] that showed one KaiC hexamer, two KaiB dimers, and one KaiA dimer ($KaiABd_2C$). Further EM, SAXS, or crystallographic analyses coupled to AUC or other techniques will be essential for addressing the three-dimensional structure of the ternary complex.

Assembly and disassembly dynamics of Kai complexes

Kageyama and coworkers made pioneering investigations of the dynamics of the Kai complexes both in vitro [43] and

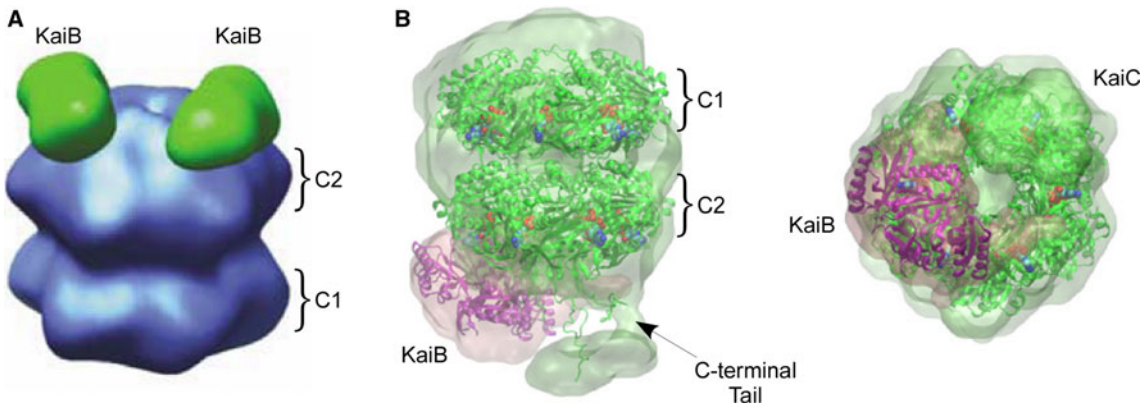

Fig. 3 Structural models for KaiBC complex. **a** EM-based *T. elongatus* KaiBC complex (adapted from Ref. [29]). **b** Two orthogonal views of SAXS-based *S. elongatus* KaiBC complex (adapted from Ref. [24])

in vivo [44] using SEC and pull-down analyses. They found that as the phosphorylation state of KaiC oscillates periodically, the heteromultimeric Kai complexes undergo repeated circadian assembly and disassembly. Their biochemical estimation [43], as well as visual inspection of 2D EM images [45], suggested that most of the KaiC hexamer (~80%) do not interact with either KaiA or KaiB during the cycle and that the complexes form in the order KaiC → KaiAC → KaiBC → KaiABC → KaiC.

The assembly/disassembly dynamics of the in vitro Kai oscillator were first detected in real time with SAXS [24]. The forward scattering intensity, $I(0)$, is an excellent measure of the weight-averaged molecular mass of all particles contained in the system [46]. Thus, its time evolution reveals the dynamic changes in not only the amount of the complexes but also their stoichiometries. The experimentally determined $I(0)$ value revealed robust circadian oscillation (Fig. 4d) but was inconsistent with the large proportion of the free KaiC hexamer [24]. The discrepancy suggests that most of the KaiC hexamers are involved in inter-protein interactions with KaiA and/or KaiB with a higher order of complex stoichiometry.

The discrepancy is likely of methodological origin. Any analytical procedure other than real-time measurement could lead to under- or over-estimation of the complex stoichiometry as well as the amounts. The reason is that complexes already formed at the beginning of the analysis have every chance of being further assembled or disassembled at every step of the concentration and dilution procedures until the assay itself is completed. In fact, a smaller fraction of free KaiC was suggested in a mathematical model [47] and in another experiment [48].

The poor match between the experimental $I(0)$ trace and a model in which every KaiC hexamer forms first-order complexes (KaiAC, KaiBC, and KaiABC) clearly indicates a higher order of complex stoichiometry [24]. A qualitative examination of the SAXS data suggested that the KaiC hexamer can be complexed with at most two KaiA dimers and/or two KaiB tetramers ($KaiA_2Bt_2C$). This estimation is an upper limit of the complex stoichiometry assuming that all the Kai molecules follow an ordered assembly/disassembly cycle. Therefore, it does not exclude Kai complex interactions having a moderately complicated stoichiometry, such as $KaiA_2BtC$, although it does exclude the assembly/disassembly dynamics of only first-order complexes (KaiAC, KaiBC, and KaiABC). Some EM images seem to provide a glimpse of a large ternary complex [45], which it is hoped resembles higher-order ternary complexes [24]. However, more recent EM observations suggested a ternary complex with a low stoichiometry ($KaiABd_2C$) [29].

Despite the argument on the complex stoichiometry, both SAXS [24] and EM studies [45] indicated robust circadian oscillations in the amounts and compositions of the Kai

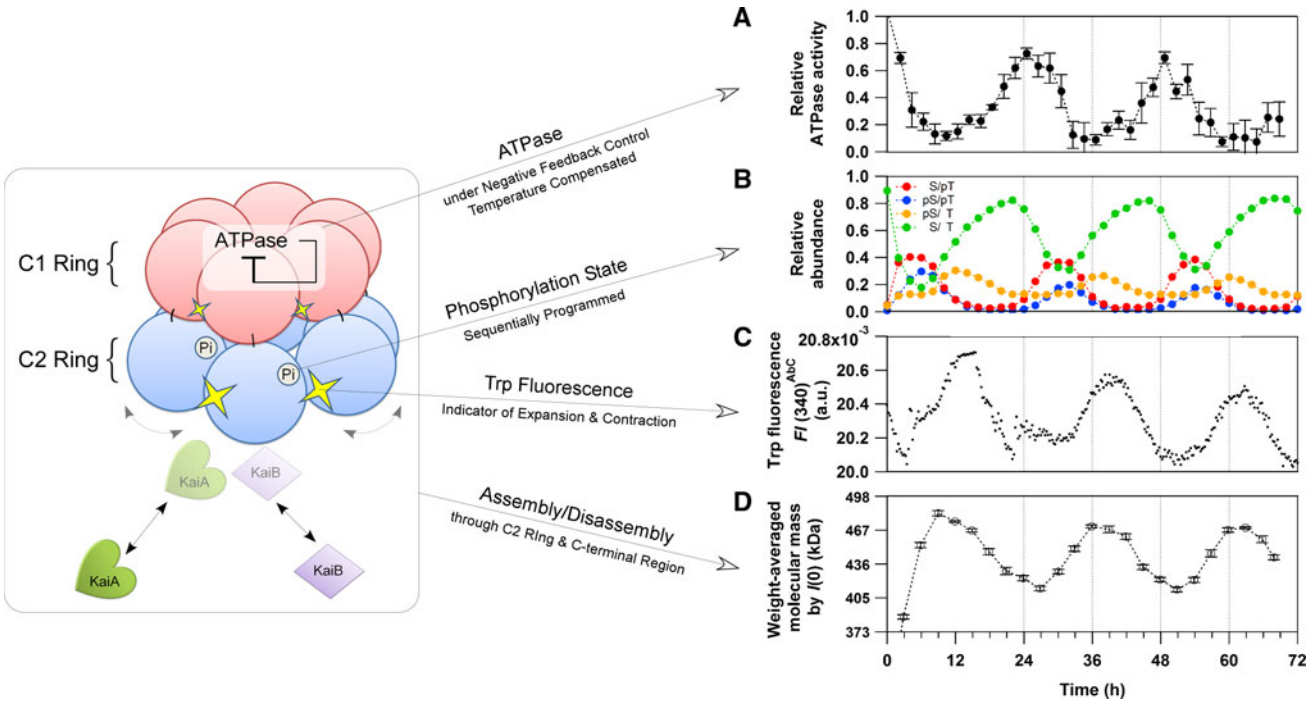

Fig. 4 Expansion and contraction of KaiC interlocked with ATPase, phosphorylation state, and assembly/disassembly of Kai complexes in presence of KaiA and KaiB. **a** ATPase activity. **b** Relative abundance of four phosphorylation states (*red* $KaiC^{S/pT}$, *blue* $KaiC^{pS/pT}$, *orange* $KaiC^{pS/T}$, *green* $KaiC^{S/T}$). **c** Circadian fluctuation of Trp fluorescence in the C2 domain. **d** Weight-averaged molecular mass of Kai complexes from $I(0)$ [24]. Adapted from Ref. [39]

complexes. In particular, the circadian oscillation of $I(0)$ is an astonishing observation (Fig. 4d) because the proteins or subunits are generally assembled and disassembled on a much faster time scale. If two reactant molecules of a simple bimolecular reaction are assumed to be uniform spheres, the diffusion-controlled rate constant is given by [49]

$$k = 4\pi N_A (D_A + D_B)(R_A + R_B) = \frac{2RT}{3\eta}\frac{(R_A + R_B)^2}{R_A R_B} \approx \frac{8RT}{3\eta} \quad (1)$$

where N_A is the Avogadro constant, R is the gas constant, T is the absolute temperature, and η is the solvent viscosity. The collision rate is weakly dependent on the size of the reactant molecules, given a similarity in their size ($R_A \approx R_B$), because the slower diffusion of large particles is compensated for by their greater collision area. According to Eq. 1, the calculated value for k in water at 30°C is 8.4×10^9 M^{-1} s^{-1}. Assuming a physiological concentration of 1.0×10^{-6} M for each reactant molecule, a 95% reaction is achieved in 2.3 ms. For realistic protein molecules, the reaction advances only when the reactant molecules collide in a specific configuration. Thus, this conformational constraint or an additional rotational search for the reactive configuration within the encountered complex reduces the actual association rate. Even then, the rate of general inter-protein association is on the order of 10^6–10^9 M^{-1} s^{-1} [50], and the reactions are expected to be complete (95% reaction) in 19 ms to 19 s. The ordered assembly/disassembly observed for the Kai oscillator is much slower than these examples. Furthermore, the period of the Kai oscillator is significantly resistant to changes in the solvent viscosity [24], despite its potential effect on the collision frequency itself (Eq. 1). These considerations suggest that the circadian assembly and disassembly of the Kai complexes is limited by an unknown slow elementary step other than association/dissociation processes.

Ticking of core clock KaiC

It appears that KaiC probably plays a role in the assembly/disassembly dynamics of the Kai oscillator. To recruit or eject KaiA and/or KaiB with circadian timing, KaiC must undergo slow conformational changes in a phase-dependent manner. These conformational changes were recently tracked and visualized by complementary time-resolved SAXS and fluorescence techniques.

At 30°C, the autokinase/autophosphotase balance in KaiC is inclined toward autophosphotase. Thus, incubation of phosphorylated KaiC at 30°C induces the auto-dephosphorylation reaction, which can be monitored by SAXS and fluorescence in real time to obtain structural information. Furthermore, by applying sodium dodecyl sulfate polyacrylamide gel electrophoresis [13] or mass spectrometry [14] to an aliquot taken from the reaction solution, the fractional concentration of each of the four phosphorylation states can be routinely determined. Using this structural and stoichiometric information, I constructed a model for the conformational ticking of KaiC in solution [39] by rigid-body refinement of a known X-ray crystal structure [35] against the experimental SAXS curves (Fig. 5).

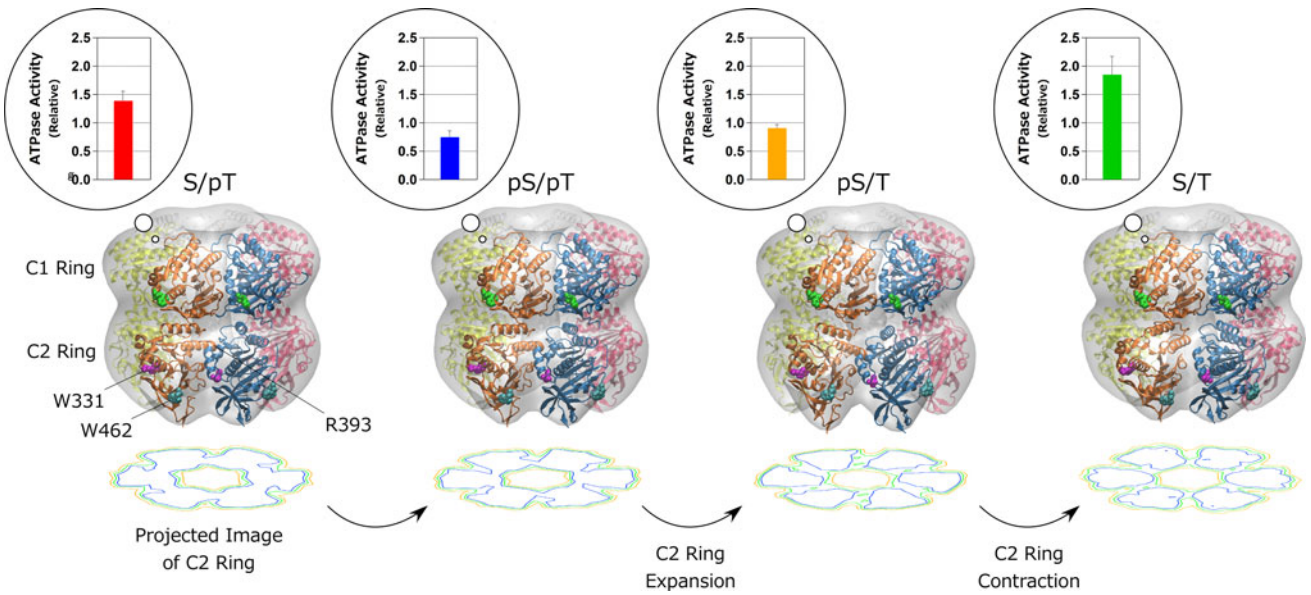

Fig. 5 Expansion and contraction of C2 ring interlocked with ATPase in C1 ring. ATPase activity of KaiC is given as a relative value. Adapted from Ref. [39]

Every C2 domain of KaiC$^{S/pT}$ is located near a neighboring C2 domain, producing a stable hexameric C2 ring on the C1 ring scaffolding. The radius of gyration (R_g), which is a measure of the overall shape of the KaiC hexamer, remains nearly unchanged in the transition from KaiC$^{S/pT}$ ($R_g = 45.4 \pm 0.6$ Å) to KaiC$^{pS/pT}$ ($R_g = 45.6$ Å). A dramatic shape change occurs in the subsequent step from KaiC$^{pS/pT}$ ($R_g = 45.6$ Å) to KaiC$^{pS/T}$ ($R_g = 48.0$ Å), in which the C2 domains are rearranged outward and become more loosely packed. This rearrangement is further supported by lower fluorescence quenching of Trp331 at the C2–C2 interface in KaiC$^{pS/T}$ (the most fluorescent configuration), which expands the C2 ring in KaiC$^{pS/T}$ noticeably. The expanded C2 ring is partially contracted in the subsequent step from KaiC$^{pS/T}$ ($R_g = 48.0$ Å) to KaiC$^{S/T}$ ($R_g = 46.9 \pm 0.4$ Å) and is further contracted in the least fluorescent configuration, KaiC$^{S/pT}$ ($R_g = 45.4 \pm 0.6$ Å), by repacking of the C2 domains. These expansions and contractions of KaiC can be tracked in real time with Trp fluorescence (Fig. 4c).

The expansion and contraction of the C2 ring are important timing cues for the assembly/disassembly dynamics. The timing that maximizes the fraction of KaiC$^{pS/T}$, which carries the expanded C2 ring, is associated with the timing required to accumulate large ternary complexes [13, 24, 39] (Fig. 4b–d). KaiA and/or KaiB bind with KaiC$^{pS/T}$ to form ternary Kai complex in response to the expansion of the C2 ring; the ternary complex is then probably disassembled in response to its partial contraction.

It is important to correlate the expansion and contraction of the C2 ring [39] with the results of other structural studies. Pattanayek et al. [29] observed a number of KaiBC particles under negative-stain EM and found that the central pore of the C2 ring is larger than that of the C1 ring. They explained the enlargement plausibly as a consequence of expanding the A-loop and C-terminal tail folded near the edge of the C2 channel into a more extended conformation. Alternatively, the C2 ring expansion suggested by a SAXS study [39] may contribute to the channel enlargement of the KaiBC complex. Of course, care should be taken with the latter interpretation because pore enlargement itself does not necessarily indicate expansion of the C2 ring (separation of the C2 protomer).

An X-ray crystallographic study visualized the detailed structure of phospho-mimicking KaiC mutants [38]. Mutation-induced structural changes in the crystalline phase were far less dramatic than the expansion and contraction of the C2 ring in solution and were localized near the dual-phosphorylation site in the C2 ring. Lattice packing is one plausible explanation. Protein structural units with certain switching activities or certain flexible linkers could be confined in the crystal lattice as a conformational state that is within the accessible conformational space, but that is very minor in solution. The discrepancy between the characteristics of the crystalline phase and those in solution provides important clues to the dynamic nature of the C2 ring.

A recent NMR study by Chang et al. [51] also pointed out the dynamic nature of the C2 ring in *T. elongatus* KaiC. They used methyl-transverse relaxation-optimized spectroscopy (TROSY) technique to probe dynamics of $\delta 1$ methyl group of isotopically labeled Ile residues. On the basis of the TROSY and gel-filtration experiments using the respective phospho-mimicking KaiC mutants, they suggested a rigidity of the C2 ring drops in the following order: KaiC$^{pS/T}$ > KaiC$^{pS/pT}$ > KaiC$^{S/T}$, KaiC$^{S/pT}$. This observation is quite interesting, because the earlier *S. elongatus* KaiC$^{pS/T}$ model carrying the expanded C2 ring (Figs. 4, 5) has rather evoked an image of a looser and more dynamic protomer–protomer C2-interface than other phosphorylation states. The R_g value is a model-independent parameter serving as a good measure of compactness [42], and thus it should be safe in concluding a less compact conformation of *S. elongatus* KaiC$^{pS/T}$ (Fig. 2c in Ref. [39]). The view of less compact but more rigid KaiC$^{pS/T}$ is apparently puzzling. This apparent discrepancy may be canceled partly by hypothesizing a notable deformation of each C1 and/or C2 protomer into a less compact structure while keeping a stable protomer–protomer interaction, otherwise will be attributed to the difference in the sample sources between *S. elongatus* and *T. elongates*. There seems some consensus on KaiC$^{pS/T}$ as the key state in the assembly/disassembly dynamics [13, 39, 51] and in coupling between the C1 (ATPase) and C2 (phosphorylation state) rings [39, 51], yet how the less compact but more rigid KaiC$^{pS/T}$ is realized and works remains puzzling.

Time scale for protein dynamics: count-up or once per day?

An important question remains: why do the dynamic expansions and contractions of the C2 ring (Figs. 4c, 5) take so much time? The motion of protein molecules spans a wide range of both temporal (10^{-14}–10^{4} s) and spatial (0.1–100 Å) scales (Fig. 6). According to three previous articles that reviewed protein dynamics [52–54], local motions such as bond stretching/bending and side chain fluctuations are the most rapid. The next fastest (10^{-9}–10^{0} s) are rigid-body motions of domains, subunits, and packed secondary structures. The slowest (10^{-7}–10^{4} s) includes large-scale motions such as refolding of unstructured polypeptide chains into a compact globular structure, assembly/disassembly of macromolecules, and opening/closing motions coupled to partial folding/unfolding. These examples indicate that both temporal and spatial

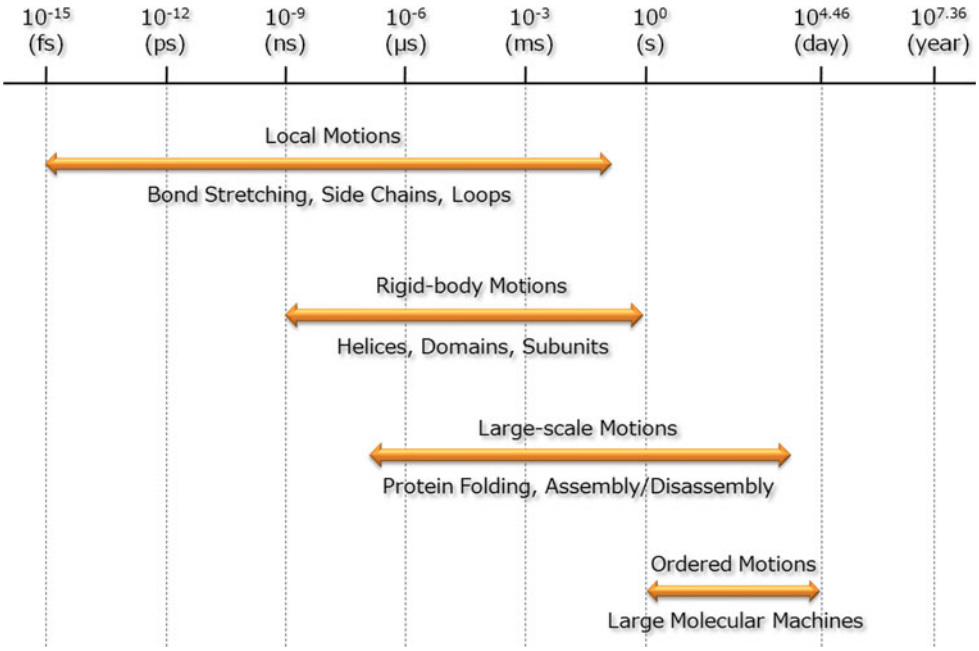

Fig. 6 Spatio-temporal scale of protein dynamics. Approximate time scales of motions were taken from Refs. [52–54]

hierarchies exist in protein dynamics. Thus, in the absence of a regulatory mechanism, a longer time suggests motion on a larger scale.

Given that the expansion and contraction of the C2 ring in KaiC are driven primarily by rigid-body rearrangements of the C2 domains, their dynamic transitions are expected to require far less than 1 s. The discrepancy with the observed time scale suggests that these transitions are both very slow and very stable. Two extreme possibilities can be hypothesized. One is a prescaler mechanism by which KaiC counts its own rapid functional motion up to circadian time. Assuming that circadian clocks have a precision of 15 min ($\sim$1% of 24 h), structural alternations of roughly 2 digits ($\sim$100) would be required for the KaiC hexamer. The expansion and contraction of the C2 ring could be a consequence of carrying, that is, the addition of a higher-order digit to the medium-scale structural changes when their timing equals or exceeds the counting in the lower-order digit of the rapid local structures. The methyl-TROSY technique [51] might enable tracking of the rapid motions of the large KaiC macromolecule and should facilitate evaluation of this scenario.

The other possibility is a robust but as yet unknown mechanism by which KaiC can exhibit a limited number of oscillations per day (e.g., one or two). However, any biological macromolecules in solution are continuously exposed to noise arising from collisions and fluctuations of water and other molecules. Thus, this possibility cannot be realized without assuming an intra-molecular reaction that is very slow and robust against ambient noise. Furthermore, in any case, the system must also achieve temperature compensation, as described below.

Temperature compensation

The temperature dependence of a simple elementary reaction having a reaction rate k independent of time can be approximated by the Arrhenius-like equation

$$k(T) = A(T)\exp\left(-\frac{E_a}{RT}\right) \tag{2}$$

where $A(T)$ is the pre-exponential factor, and E_a is the activation energy, which is assumed to be temperature independent here. The temperature dependency of $k(T)$ comes mainly from the E_a component, and $A(T)$ itself generally depends only weakly on temperature. In the limit of $E_a = 0$ (the so-called barrierless downhill transition), the reaction can be faster and less temperature dependent.

Q_{10} is another measure of the temperature dependence and is often used in biology. It indicates the factor by which the reaction is accelerated by raising the ambient temperature by 10°C:

$$Q_{10} = \frac{k(T+10)}{k(T)} = \exp\left\{\frac{E_a}{R}\frac{10}{T(T+10)}\right\} \tag{3}$$

Here, $A(T)$ is assumed to be equal to $A(T + 10)$. The above equation indicates that the value of Q_{10} is not strictly independent of the experimental temperature as long as the reaction follows Arrhenius-like behavior.

According to Eq. 2, a slow but slightly temperature-dependent reaction can be achieved if $A(T)$ is small enough and $E_a = 0$. What type of biological reaction is described by these conditions? Most biochemical reactions are widely believed to exhibit a temperature dependence of $Q_{10} = 2$–3; that is, $E_a = 55$–87 kJ mol^{-1} at 303.2 K. A

barrierless transition is often discussed with respect to the early phases of protein folding [55], but such reactions [large $A(T)$ and $E_a = 0$] should be fast ($> \sim 10^4\ s^{-1}$) and are considered to be limited by chain diffusion (i.e., to be temperature dependent, Eq. 1).

The following regulatory mechanisms can produce apparent temperature insensitivity. The first relies on extensive balancing among multistep reactions. For clarity, I use a Michaelis–Menten-type reaction as an example

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{ES} \xrightarrow{k_2} E + P \quad \text{Scheme 1}$$

$$E \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} \mathrm{IE} \quad \text{Scheme 2}$$

where E, S, ES, P, and IE correspond to the concentrations of the active enzyme, substrate, enzyme/substrate complex, product, and inactive enzyme, respectively. Under steady-state approximations for both ES and IE, the time dependence of the product concentration is given by

$$v = \frac{\mathrm{d}[P]}{\mathrm{d}t} = k_2[\mathrm{ES}] = \frac{k_2[S][E_0]}{K_\mathrm{I}K_\mathrm{m} + [S]} = \frac{A_2[S][E_0]}{K_\mathrm{I}K_\mathrm{m} + [S]} \exp\left(-\frac{E_\mathrm{a}^2}{RT}\right) \tag{4}$$

$$K_\mathrm{I} = 1 + \frac{k_3}{k_{-3}} = 1 + \frac{A_3}{A_{-3}} \exp\left\{-\frac{\left(E_\mathrm{a}^3 - E_\mathrm{a}^{-3}\right)}{RT}\right\} \tag{5}$$

$$K_\mathrm{m} = \frac{k_{-1} + k_2}{k_1} = \frac{A_{-1}}{A_1} \exp\left\{-\frac{\left(E_\mathrm{a}^{-1} - E_\mathrm{a}^1\right)}{RT}\right\} + \frac{A_2}{A_1} \exp\left\{-\frac{\left(E_\mathrm{a}^2 - E_\mathrm{a}^1\right)}{RT}\right\} \tag{6}$$

where K_m is the Michaelis constant, K_I is a constant determining the degree of inactivation, and A_i and E_a^i are the pre-exponential factor and activation energy, respectively, in the k_i's reaction step. Given that A_i is temperature insensitive and $E_\mathrm{a}^1 = E_\mathrm{a}^{-1} = E_\mathrm{a}^2$, K_m becomes temperature independent, as discussed elsewhere [56, 58]. Thus, there is a finite chance that K_I cancels out the temperature dependence of the E_a^2 component in Eq. 4 ($E_\mathrm{a}^3 \neq E_\mathrm{a}^{-3}$, indicating a temperature-dependent increase in K_I), so the reaction rate (v) becomes nearly temperature insensitive. Note that even in the absence of inactivation ($K_\mathrm{I} = 1, k_{-3} \to \infty$), the temperature dependency of E_a^2 part in Eq. 4 can be compensated for by the temperature-dependent increase in K_m ($E_\mathrm{a}^1 > E_\mathrm{a}^{-1}, E_\mathrm{a}^2$). By using this logic, temperature compensation can be implemented theoretically in most oscillator models [56]; for example, Mehra et al. [57] reproduced the temperature independence of the period in their mathematical model of the Kai oscillator.

The temperature compensation mechanism given by Kotov et al. [58], which may be a subcategory of the first, is based on control of the product concentration by a bienzyme or a chain of multienzymes. They showed that the concentration of an intermediate product in bienzyme-controlled or multienzyme-controlled systems can be nearly temperature independent if every reaction possesses a similar temperature dependence (similar activation energy).

The third model is based on negative feedback regulation. If the released product in Scheme 1 can inhibit its own enzymatic activity (k_3) or decrease the amount of the active enzyme ([E]) directly or indirectly, there is sufficient chance that the temperature dependence of individual elementary reactions will be compensated. The reaction rate (v) should increase transiently with increasing temperature, but the increased product is fed back negatively to the system until v becomes *apparently* temperature insensitive. Our recent observation suggested that $\mathrm{KaiC}^{\mathrm{pS/T}}$ carrying the expanded C2 ring is in a critical state that allows it to subordinate the assembly/disassembly processes by coupling them to the negatively fed-back ATPase activity of KaiC [39].

ATPase activity of KaiC

Terauchi and colleagues reported three outstanding characteristics of the ATPase activity of *S. elongatus* KaiC [59]. First, the ATPase activity of KaiC alone without both KaiA and KaiB is linearly correlated with the frequency (reciprocal of the period) of the phosphorylation cycle in the presence of both KaiA and KaiB. Second, the full-length *S. elongatus* KaiC hydrolyzes only a limited amount of ATP daily ($\sim$15 ATP molecules/day). Third, the ATPase activity of the full-length KaiC remains constant across a wide range of temperatures. On the basis of these characteristics, they proposed that KaiC ATPase is controlled by negative feedback and acts as a basic timing system underlying the circadian periodicity of the Kai oscillator: energy released upon hydrolysis is somehow stored in the KaiC hexamer as structural/state changes that in turn down-regulate its own ATPase activity.

Roughly speaking, ATPase and kinase/phosphotase activities occur in the C1 and C2 rings in the KaiC steady state, respectively. Although the C1 domains of *S. elongatus* KaiC do not incorporate inorganic phosphate groups as the C2 domains do, a recombinant *S. elongatus* KaiC having only the C1 domains (KaiC-C1) consumes roughly 70% of total ATP hydrolyzed by the full-length *S. elongatus* KaiC [59]. A similar observation was also reported for *T. elongatus* KaiC [60], confirming that the C2 domain exhibits intrinsically lower ATPase activity than the C1 domain. However, KaiC ATPase is further activated or inactivated depending on both its phosphorylation state and the addition of KaiA and/or KaiB [59, 60]. Thus, the

overall view of the KaiC hexamer is that ATPase produces functional crosstalk with kinase/phosphotase through a structural coupling between the stacked C1 and C2 rings.

KaiC-R393C is a curious mutant showing loose coupling [39]. Interestingly, this mutation did not affect the temperature compensation of the ATPase of KaiC alone ($Q_{10} \sim 0.9$), although it did weaken the temperature compensation of the phosphorylation cycle in the presence of both KaiA and KaiB ($Q_{10} \sim 1.3$). R393 is located at the C2–C2 protomer interface [35] (Fig. 1c) and probably functions as a tether holding the C2 domains in an appropriate hexameric configuration on the scaffolding C1 ring. The R393C mutation consistently exhibited selective destabilization of $KaiC^{pS/T}$ in the expanded conformation of the C2 ring [39]. $KaiC^{pS/T}$ is a key intermediate in the assembly/disassembly processes because it preferentially forms the ternary Kai complex [13, 39]. The inherent temperature dependence of the assembly/disassembly reactions can be reduced by subordinating them to a robustly temperature-compensated slow ATPase through $KaiC^{pS/T}$ with its expanded C2 ring conformation.

Which is better for temperature compensation of the clock activity, balancing every biological reaction so as to realize overall temperature independence, or creating a compact, minimal balancing core unit to which others are subordinated? Whereas the former should be susceptible to every mutation, the latter could be sometimes sensitive and sometimes insensitive. Although no systematic studies have yet examined this important issue, KaiC-R393C can be interpreted as an example of the latter case.

Synchronization: inter- and intra-hexamer heterogeneities

Given that an individual molecule can cycle its structural or functional state, the molecules in ensemble do not necessarily reveal stable, robust oscillation unless their phases are synchronized. An earlier study proposed that KaiC phosphorylation is synchronized by physical shuffling of the KaiC protomer among two or more KaiC hexamers in a specific phase [43]. This proposal was further supported by subsequent studies. The in vitro Kai oscillator in the presence of both KaiA and KaiB is autonomously synchronized at a particular phase in which protomer–protomer shuffling is preferred [61] and can be entrained externally by a periodic train of temperature pulses [62].

Although there is no doubt about the *presence* of a synchronizing mechanism that minimizes the inter-hexamer heterogeneity of KaiC, the mechanism *itself* seems to be a matter of some argument. The evidence for physical shuffling of the KaiC protomers relies primarily on the results of pull-down assays [43, 61]. The concept of physical shuffling is also supported by a result from fluorescence resonance energy transfer analysis [45], although the effect of either KaiA or KaiB on the exchange efficiency remains controversial. Several of the mathematical models of the Kai oscillator encapsulate physical shuffling as the core synchronization mechanism [45, 63, 64]. A group led by Sasai demonstrated possible synchronization without strict discrimination between physical and indirect-state shuffling [65, 66]. Conversely, Clodong et al. [67] actively adopted both contributions (KaiA sequestration and monomer exchange) into their model. The remaining half of the mathematical models described the circadian fluctuation of KaiC phosphorylation in terms of indirect synchronization of the phosphorylation state via activation/inactivation of KaiA [14, 47, 57, 68–70] and/or via ADP/ATP ratio [71] instead of physical shuffling. An experimental strategy enabling direct discrimination between physical shuffling and state shuffling is desired.

Intra-hexamer heterogeneity is another important issue for further examination. In the X-ray crystal structure of KaiC [35], several protomers exhibit different phosphate modification patterns from the others. The difference in the stoichiometry of the complex between the steady and oscillatory states might be a sign of diverse KaiC hexamers, each of which incorporates an identical number of phosphate groups in a different intra-hexamer distribution [24]. Furthermore, tracking of the Trp fluorescence of KaiC suggested that binding of KaiA and/or KaiB imposes an asymmetric tension on the KaiC hexamer [39]. The intra-hexamer heterogeneity has been actively incorporated into some of the mathematical models of the Kai oscillator [45, 47, 63–67, 70].

Despite these suggestions, little direct experimental evidence exists for the involvement of asymmetric KaiC hexamers. For example, the time courses of R_g, Trp fluorescence, and the phosphorylation level of KaiC could be explained by considering only symmetric phosphorylation states [39]. Even with asymmetric binding of KaiA or KaiB, the KaiC hexamer within the binary complexes was modeled without considerable asymmetry or deformation [24, 25, 29]. However, these results are likely insufficient to exclude the involvement of asymmetric species, as discussed in detail elsewhere [39], considering the quality and spatio-temporal resolution of the present experimental data. A biochemical technique for rebuilding the AAA + pseudo hexamer has been successfully used to examine the structural and functional significance of asymmetric oligomeric species in ClpX [72]. A similar approach may also be applicable to KaiC. Single-molecule measurements may be useful for tackling this problem, but a special technique will be required to capture an individual single protomer within a single KaiC hexamer.

Conclusions and outlook

Which is the master oscillator, ATPase, kinase/phosphotase, or assembly/disassembly? The question makes practical sense if only one of them can operate successfully without significant interdependencies with the others. To my knowledge, no such reports exist to date. Therefore, each of these three factors likely affects the activities and behaviors of the others as inspected structurally in the stacked C1 and C2 rings. All the factors are likely to be important in maintaining the in vitro circadian oscillation.

Rather, the essential questions are: what determines the circadian period, and how can it be so slow and stable? The assembly/disassembly processes are intrinsically much faster than the circadian timescale and also are inherently dependent on both solvent viscosity and temperature (Eq. 1). A clock whose period is limited by the assembly/disassembly processes could be a risk factor, since the period of such a system would be considerably affected by the 3–13-fold higher viscosity of cytoplasm [73–75] and by stochastic noise coming from the surrounding dense macromolecules.

The KaiC phosphorylation cycle is considered to be a closed chain of four kinase/phosphotase activities [13, 14]. Thus, some intermediates of the four phosphorylation states could be temperature compensated, given an identical activation barrier for each defective (slow) reaction or a delicate balance among them. Note that the activation energy of particular reactions cannot be small as long as they are specific reactions. Without an energy barrier of a certain height, it will be difficult to realize a definite pathway for sequential interconversion of the four states.

At the same time, the slowness of ATPase activity even in KaiC-CI (without the C2 domain) must be kept in mind [59]. Negative feedback generally works well for oscillatory behaviors and may be useful for explaining both the slow oscillations and temperature compensation of the Kai oscillator. Given the negative feedback regulation of ATPase in KaiC, where and how is the released energy stored? The structural basis of the slow, temperature-compensated ATPase activity of KaiC is not well understood and should be crucial to revealing the slowness and specificity both hidden in KaiC.

Theoretical explicability and practical possibility are sometimes two different things. What do the extremely slow rates often used in mathematical models mean? It is time to revisit the biological and structural backgrounds for the slowness and specificity both experimentally and theoretically. For this purpose, we need to improve the spatio-temporal resolution enough to catch motions of and changes in the KaiC hexamer in relation to ATPase and kinase/phosphotase. If, as Aristotle suggests, we do not realize the existence of time when nothing seems to change, and we say time has elapsed when we perceive motion and change, evidently time is not independent of movement and change. Thus, we cannot observe the existence of molecular time unless we observe the changes by which KaiC counts time.

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