Circadian rhythms enable cells and organisms to coordinate their physiology with the cyclical environmental changes that come as a result of Earth’s light/dark cycles. Cyanobacteria make use of a post-translational oscillator to maintain circadian rhythms, and this elegant system has become an important model for circadian timekeeping mechanisms. Composed of three proteins, the KaiABC system undergoes an oscillatory biochemical cycle that provides timing cues to achieve a 24-h molecular clock. Together with the input/output proteins SasA, CikA, and RpaA, these six gene products account for the timekeeping, entrainment, and output signaling functions in cyanobacterial circadian rhythms. This Minireview summarizes the current structural, functional and mechanistic insights into the cyanobacterial circadian clock.

Form and function of circadian clocks

Circadian rhythms are processes through which cells and organisms predict and adapt to the repetitive environmental changes incident to the 24-h solar day. These rhythms originate intracellularly and persist in the absence of external cues to orchestrate temporal organization of physiology. To achieve this, circadian clocks must perform three distinct but necessarily intertwined functions: timekeeping, entrainment, and output signaling.

Timekeeping is achieved through a cycle of slow biochemical processes that together set the period of oscillation close to 24 h. For this temporal information to be relevant, biological clocks must appropriately synchronize with their environment through entrainment. Temporal information runs from the timekeeping apparatus to the rest of the cell through output signaling mechanisms that impart circadian changes in physiology.

Timekeeping mechanisms that have been observed in circadian clocks are broadly categorized as transcription-translation feedback loops (TTFLs) or post-translational oscillators (PTOs). TTFLs achieve oscillation via delayed negative feedback, where a gene product represses its own expression when its levels become high enough. This results in an oscillation in the expression level of the gene, with transitions between repression and derepression occurring at critical points in the oscillatory cycle. By contrast, PTOs utilize timekeeping mechanisms that are independent of transcription. Like TTFLs, PTOs involve a cycle of biochemical processes, but instead of being driven by changes in expression levels, their behavior is controlled by post-translational modifications, conformational changes, protein–protein interactions, and/or subcellular localization. It is important to note that although we make a distinction here between TTFLs and PTOs as general categories of negative feedback systems, many circadian clocks make use of both transcription/translation as well as post-translational timing steps to constitute a biochemical oscillator.

Post-translational steps in circadian clocks present unique biochemical challenges and opportunities because they link dynamic molecular processes to biological phenotypes through covalent and conformational changes in protein structure. PTOs can operate at constant protein concentrations and are therefore amenable to in vitro study. In the cyanobacterial Kai system, an entire PTO consisting of only three protein gene products (KaiA, KaiB, and KaiC or KaiABC) can reconstitute circadian biochemical oscillation in vitro. As a result, this system has become an important model for understanding how discrete biochemical steps are integrated to give rise to precise biological timing.

Circadian biology in cyanobacteria

Circadian rhythms are essentially ubiquitous in eukaryotes, but far fewer instances have been documented in prokaryotes. Cyanobacteria temporally segregate photosynthesis and nitrogen fixation, which require mutually exclusive oxidative environments. Evolutionary pressures such as this resulted in a cyanobacterial transcriptome in which at least one-third of transcripts undergo circadian variation in accumulation levels, and active transcription of nearly all-transcripts appears to be under circadian control. These changes in transcriptional
output are linked to global changes in chromosome ultrastructure (8, 9), but they are controlled basally by a core biochemical oscillator (3, 10).

The KaiABC gene cluster was originally identified in genetic screens aimed at finding TTFLs involved in cyanobacterial circadian regulation, with deletion resulting in an arrhythmic phenotype (10). It was subsequently shown that the products of this gene cluster can recapitulate circadian biochemical oscillation in vitro (3) and that this circadian oscillation persists in vivo even when transcription and translation are inhibited (11). Its ability to be reconstituted in vitro demonstrates that KaiABC can act as a self-sustained PTO. In vivo, this PTO also functions within a TTFL framework, i.e., circadian variation of clock protein expression results in a more robust rhythm (4, 12–14). Together with three other effector proteins, the KaiABC oscillator comprises a self-contained minimal system that is capable of integrating timekeeping, entrainment, and output signaling processes into the cyanobacterial cell.

Domain structures of the six core clock proteins in cyanobacteria are shown in Fig. 1. Genetic studies on cyanobacterial rhythms have made extensive use of the mesophilic cyanobacterium Synechococcus elongatus, from which these genes were originally identified (10). Biophysical studies have often taken advantage of proteins produced from homologous gene sequences in the thermophilic variant Thermosynechococcus elongatus for their improved solubility and in vitro behavior (15). The six proteins shown in Fig. 1 are conserved between S. elongatus and T. elongatus and appear to comprise a core unit capable of accounting for all of the required functions of a circadian clock. Several additional genes have been identified that play roles in the circadian rhythms of cyanobacteria (16–21); however, this Minireview will focus on the six best-characterized gene products and the mechanisms through which they mediate timekeeping, entrainment, and output signaling functions in cyanobacterial circadian rhythms. For a broader review on circadian biology in cyanobacteria, see Refs. 4, 22.

**Structure and function of KaiC**

The core cyanobacterial clock is centered around the hexameric ATPase KaiC, which achieves timekeeping through circadian oscillations in phosphorylation that are intertwined with oscillations between the quaternary structures depicted in Fig. 2. The primary sequence of KaiC is a concatenation of two P-loop ATPase domains (10), termed CI and CII. In solution, KaiC binds ATP to form a hexamer through interactions at the CI subunit interfaces (23–25). Homologous ATPase active sites...
Phosphorylation of Thr-432 and Ser-431 (31). Around dawn, CII phosphorylation is at a minimum, and affinity for KaiB is lost (30). The repressive complex is released, freeing KaiA to bind to the A-loops and begin the cycle anew.

Post-translational modification at residues Ser-431 and Thr-432 of KaiC occurs in a specific order, with Thr-432 acting as a substrate in the first reaction of both the phosphorylation and dephosphorylation arms of the cycle (29, 36). The order of the KaiC phosphorylation states is denoted in this Minireview using the shorthand: S/T → S/pT → pS/pT → pS/T. The S/T and S/pT states are dominant during the daytime phosphorylation arm of the oscillatory cycle, characterized by binding of KaiA at CII (26, 28). Accumulation of pS/pT initiates the nighttime state (28, 30, 35), characterized by the formation of the KaiABC complex, which persists through the pS/T state until phosphorylation reaches a minimum, and KaiA is recruited back to CII.

Sustained oscillation requires a power source, and KaiABC appears to achieve this through ATPase activity of the CI domain, whose enzymatic activity is correlated with a period both in vitro and in vivo (37, 38). CI ATPase activity oscillates as well, with the unphosphorylated forms of KaiC consuming ATP approximately twice as fast as the phosphorylated form (37). This enzymatic activity shows a relatively modest rate enhancement of ATP hydrolysis, with an apparent $k_{cat}/K_m$ on the order of $10^{-4} \text{M}^{-1} \text{s}^{-1}$, about 6 orders of magnitude slower than the structurally similar $F_1$-ATPase (38). High-resolution structures have been obtained for the isolated CI ring in the pre-ATP and post-ATP hydrolysis states (38) resulting in the three specific structural changes depicted in Fig. 3A. Together with studies showing that catalytically dead KaiC is unable to interact with KaiB (26), one emerging model is that CI ATPase functions in timing the recruitment of KaiB and resultant formation of the nighttime repressive complex, although no specific structural or biochemical information exists yet on how these two processes might be related.

A direct structural connection between CII phosphorylation and the interactions of KaiC with other clock proteins has also proven difficult to pin down. High-resolution crystal structures exist of different phosphorylated and phosphomimetic states (39), but these structures are nearly identical. This is unexpected given the well-established functional differences between the phosphostates of KaiC. One possible explanation for this structural convergence is that crystalization of KaiC variants in these structures was performed in the presence of a non-hydrolyzable ATP analogue (24, 39), and structural changes associated with CII phosphorylation could be masked in the pre-ATP hydrolysis state. Alternatively, phosphorylation may influence dynamic structural equilibria that are not observable within a crystal lattice.

The latter idea is supported by lower-resolution studies on the overall shape and dynamic structural properties of KaiC. Time-resolved small-angle X-ray scattering data show an increase in the apparent radius of gyration in the pS/T phosphate (40), providing evidence of a change in shape for KaiC in the night. Interactions between isolated CI and CII domains increase across the phosphorylation cycle with a minimum at the S/T state and a maximum in the pS/T state (41), and NMR
KaiA: Primary regulator of KaiC phosphorylation

KaiA acts as the primary regulator of enzymatic activity on the CII domain of KaiC and therefore the phosphorylation cycle in general. Throughout the day, KaiA promotes phosphorylation of CII via two distinct mechanisms: 1) structural rearrangement of the CII catalytic core (32, 45), and 2) exchange of active-site ADP molecules for ATP (46). The enzymatic equilibrium of CII favors phosphatase activity when KaiC is in isolation (31), making KaiA occupancy the primary determinant of enzymatic equilibrium on CII.

Without KaiA bound, the A-loop residues project into the central cavity of KaiC (32). KaiA competes away interactions in this position, binding the loops and causing them to take on an α-helical conformation (47). When these loops are deleted, CII is in constitutive phosphorylation mode (32), indicating that the interaction of the A-loops with the cavity of KaiC abrogates CII phosphorylation and that KaiA disrupts those interactions. Additionally, KaiA has been shown to act as a nucleotide exchange factor, controlling the equilibrium of phosphorylation on CII at the level of substrate availability (46). KaiA increases the rate of ATP/ADP exchange on KaiC as well as the ratio of bound ATP at equilibrium (46). Hence, KaiA promotes CII phosphorylation by increasing the local concentration of ATP, as well as by direct perturbation of the active site.

Inactivation of KaiA during the night occurs through a conformational change that abrogates its A-loop binding activity (Fig. 3B). In the daytime state, the A-loops of KaiC form a helical bundle with the KaiA dimerization interface, flanked on either side by its N-terminal pseudo-receiver (PsR) domains. In this conformation, each β-strand from the interdomain linker crosses close to the other to form an intermolecular β-sheet, followed by a helical linker connecting the PsR domain. A high-resolution crystal structure of KaiA in the nighttime state was recently obtained by crystallizing its C-terminal dimerization domain and interdomain linker as part of a complex with KaiB and an engineered monomeric form of the KaiC-CI domain (35). KaiA docks on an exposed β-sheet on KaiB in the KaiBC complex using the β-strand of its interdomain linker, which repositions the helical linker to dock into its own A-loop binding site. This intramolecular interaction between the α-helix of the interdomain linker and the dimerization interface precludes binding to an A-loop peptide from another KaiC hexamer, which demonstrates that binding to the KaiBC complex stabilizes an autoinhibited form of KaiA at night. The PsR domains of KaiA are not needed to bind KaiBC (48) and were omitted for this structure. However, this structure of an isolated CI–KaiB–KaiA complex agrees with a lower-resolution model obtained by cryo-electron microscopy using full-length wild-type KaiA, KaiB, and hexameric KaiC fully assembled in the nighttime state (49). Although the PsR domains were included here, they did not give sufficient density to be built into the model, suggesting that they are flexibly tethered to the complex.

KaiB: Attenuator of KaiC phosphorylation

The dephosphorylation arm of the KaiABC oscillatory cycle is initiated by the binding of KaiB at the CI domain of KaiC, followed by inactivation of KaiA. Historically, some discord has existed surrounding the location of KaiB binding on KaiC and how this leads to a shift of phosphorylation equilibrium that lasts through the night. Recently, a series of corroborative...
structural and biochemical studies (33–35, 49, 50) have put this controversy to rest. Our understanding of the role of KaiB in the clock has expanded immensely through the discovery of a major conformational rearrangement that occurs between the free and KaiC-bound states of KaiB. In the ground state, KaiB takes on the unique fold depicted in Fig. 3C and oligomerizes to form a dimer of dimers (51–53). When bound to the CI domain of KaiC, KaiB is in a monomeric thioredoxin fold (34), referred to as a “fold-switched” state because it comprises a major conformational shift, resulting in reassignment of secondary and tertiary structure in roughly 50 residues composing the C-terminal half of KaiB.

Binding of KaiB to KaiC occurs remarkably slowly, taking hours to go to completion in vitro (33, 53). This binding is quickened by mutations that favor the dimeric or thioredoxin-like monomeric states of KaiB (34, 53). Native mass spectrometry experiments have shown that binding occurs through a monomeric form of KaiB (54) and appears to be cooperative, as masses were obtained for KaiC bound to either one KaiB molecule or six, but none of the subsaturated stoichiometries in between (54). This is consistent with the observation of electrostatic complementarity between KaiB subunits in the recent structures of the KaiBC complex (35, 49).

The current biochemical data suggest that KaiB binds through a conformational selection mechanism, in which the ground state dimeric and tetrameric forms of KaiB act as a thermodynamic sink to decrease the concentration of the KaiC binding-competent KaiB monomer and limiting the rate of binding. Several different mutations have been identified that stabilize the KaiB monomer, each of which take on the fold-switched form (34, 35). In addition to increasing the rate of binding to KaiC in vitro, these mutations halt circadian oscillation in vitro and in vivo, resulting in a dominant arrhythmic phenotype that locks KaiC in the unphosphorylated state between (54). This is consistent with the observation of electrostatic complementarity between KaiB subunits in the recent structures of the KaiBC complex (35, 49).

The output signaling: SasA, CikA, and RpaA

Output signaling from the cyanobacterial PTO takes place through a two-component regulatory system (55). Two-component systems are pervasive in bacterial sensing and signal transduction (56). In the archetypal form, they consist of a sensor histidine kinase (HK) and a response regulator (RR) (57, 58). There is often overlap between cognate RRs and HKs; for example, an HK may activate multiple RRs, or an RR may be regulated by multiple HKs (59). Moreover, the relevant active domains of the components may reside within a single protein that has both kinase and substrate functions. Cross-talk between regulatory networks increases dramatically from prokaryotes to eukaryotes (60), and cyanobacteria appear to fall somewhere in the middle of this complexity. The circadian signaling network in cyanobacteria is not yet fully understood, and new components are still being identified (18, 19).

Despite the general complexity of regulatory networks in cyanobacteria, a two-component system consisting of two HKs and one RR stands out as being centrally involved in output signaling from the KaiABC oscillator (55, 61). RpaA is an RR that governs transcription from a locus encoding additional transcription factors that mediate the global changes in gene expression associated with circadian rhythms (62). This regulator includes the cotranscribed KaiBC genes, thus integrating the PTO into a TTFL (63).

RpaA is regulated by two cognate HKs, SasA and CikA (61). The sensor domain of SasA adopts a thioredoxin fold that is almost identical in structure to the fold-switched form of KaiB (34, 64) and binds to the pS/pT form of KaiC (34, 61, 65). Activation of SasA follows a canonical HK signaling mechanism. The sensor domains on SasA recognize and bind to phosphorylated KaiC, resulting in autophosphorylation of His-161 in the SasA kinase domain (61) and subsequent transfer of that phosphate to Asp-53 on RpaA, rendering the transcription factor competent for DNA binding (63). SasA has been shown by analytical ultracentrifugation to form a trimer in solution (65), and gel-filtration experiments support a stoichiometry of one SasA trimer per KaiC hexamer. HKs usually oligomerize as dimers, but in rare cases they have been found to function as higher order oligomers (66) or monomers (67). It is yet unknown whether this unusual oligomeric state for SasA is linked to its sensory function.

At the biochemical level, SasA competes with KaiB for the B-loops on the CI domain of phosphorylated KaiC (48). Binding of SasA is faster than that of KaiB, presumably due to the refolding requirement of KaiB for binding to KaiC (34, 65). This difference suggests an evolutionary selection for the metamorphic properties of KaiB in controlling the temporal window of SasA signaling, in addition to providing a timing cue for the oscillatory cycle, although more evidence is needed to support either of these models definitively. KaiA also plays a role in the competition between KaiB and SasA for the CI domain of KaiC. Although the presence of KaiA does not affect $k_{\text{obs}}$ for binding of KaiB to KaiC, it does make a difference in competitive KaiB binding when KaiC is pre-bound with SasA (48). This property implicates a role for KaiA in active eviction of SasA by KaiB, although no physical model for this currently exists.

The activating effect of SasA on RpaA is reversed by CikA, a multifunctional protein that, in addition to its role in dephosphorylating RpaA, ties the clock to subcellular localization and entrainment functions (68–71). CikA is incorporated into the Night Complex through the interactions of its PsR domain with fold-switched KaiB at an interface that overlaps with the KaiA-binding site (Fig. 2). Consistent with a potential competitive mechanism, inclusion of the CikA PsR domain in an in vitro oscillation reaction results in a shortened period and decreased amplitude (34). Combined with the observation that CikA knockout results in a relatively severe gene expression phenotype (72), these data suggest that CikA has a more integral role in the timekeeping functions in vivo than suggested by its known roles in entrainment and output signaling. The inte-
grated roles of CikA and the other core clock proteins are summarized in Fig. 4.

**Metabolic of entrainment of the clock**

The cyanobacterial circadian clock achieves entrainment by linking the oscillatory cycle to photosynthesis through metabolic processes (73), rather than direct entrainment by light via sensory photoreceptors, as is commonly found in eukaryotic organisms (74). This metabolic entrainment of the cyanobacterial clock occurs in two ways: through sensitivity of the phosphorylation cycle to ATP/ADP ratios within the cell (75) and through the presence of nighttime-associated photosynthetic metabolites (71, 72, 76–78). The former entrainment cue occurs as a result of enzymatic sensitivity of the core oscillator to ATP/ADP ratios (72), causing it to align with the maximal photosynthetic period at midday when this ratio is highest (75, 79).

Cyanobacteria utilize quinone pools as intramembrane electron shuttles, reducing them during the process of photosynthesis (80). As a result, the concentration of oxidized quinones rises rapidly but briefly when photosynthesis shuts down with the onset of darkness before other pathways restore the redox steady state (76). Ectopic introduction of oxidized quinones can entrain the clock both in vivo and in vitro, and both KaiA and CikA play roles related to entrainment through binding of their PsR domains to oxidized quinones (71, 72, 76–78). The aggregation state and/or stability of the proteins is affected by quinone binding in vivo (71, 77). Still, the precise mechanism by which quinone binding influences the PTO is not currently well-understood.

**Applications in synthetic biology**

There is considerable interest in synthetic biology to control temporally regulated gene expression (81, 82). However, even the most robust synthetic oscillators are far less effective than the fully integrated oscillation of gene expression in cyanobacteria (83, 84). Because of its ability to function at constant protein concentrations and provide relatively stable temporal regulation at different temperatures, it could be enticing to reverse engineer synthetic oscillators from the cyanobacterial PTO. This has been achieved, on a rudimentary level, in a transplantation study where the core oscillator was introduced into *E. coli*, resulting in weak but measurable in vivo circadian rhythms in KaiC phosphorylation in a natively non-circadian
organism (85). In this study, the clock was linked to transcriptional output signaling through a two-hybrid approach, although the researchers were unable to show circadian oscillation of gene expression originating from KaiABC. Thus, a great deal of opportunity still exists to integrate the cyanobacterial PTO more deeply into the physiology of the host organism and to achieve clock-driven oscillations in gene expression. Undeniably, these efforts benefit from the mechanistic advances in our understanding of the cyanobacterial PTO. Furthermore, a great deal of work has gone into computational modeling of the cyanobacterial PTO (36, 86 – 89). These studies can inform efforts to re-engineer the oscillatory period of the PTO, leading to future technologies in which the cyanobacterial PTO may be used as a starting point to introduce a flexible range of temporal routines of gene expression into target organisms.

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THEMATIC MINIREVIEW: Structural review of cyanobacterial clock


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