CHAPTER 13

PROTEIN-PROTEIN INTERACTIONS IN THE CYANOBACTERIAL KaiABC CIRCADIAN CLOCK

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Abstract: The discovery that the central oscillator of the cyanobacterial KaiABC circadian clock can be reconstituted in vitro by the protein components KaiA, KaiB and KaiC renders this biological timer a unique target for biochemical and structural studies. The oscillator can be monitored through changes in the KaiC phosphorylation status that is modulated by KaiA and KaiB. As the 24-h period of the recombinant clock remains unaltered as a result of modest variation of temperature, interactions between the three Kai proteins not only form the basis for rhythmic control of levels of KaiC phosphorylation but also provide temperature compensation. A profound understanding of how this biological timer works requires a dissection of the functions of, and interactions between, the three proteins. Three-dimensional structures of the individual Kai proteins have been determined, and the KaiA-KaiC complex has been studied using hybrid structural methods. This chapter provides an overview of progress in the characterization of the cyanobacterial circadian clock with an emphasis on structural aspects of individual Kai proteins and the binary KaiA-KaiC complex

INTRODUCTION

Circadian clocks are endogenous biological timers that rhythmically regulate numerous processes with a period of roughly 24 h and exhibit temperature compensation (Dunlap et al 2004). Circadian clocks exist in various eukaryotic systems including mammals, plants, fungi and insects, and have been found also in cyanobacteria (Johnson, 2004; Iwasaki and Kondo, 2004); the latter are the simplest organisms known to possess a clock. In the model organism *Synechococcus elongatus* PCC 7942, the *kaiA*, *kaiB* and *kaiC* genes that form a cluster on the chromosome

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were shown to be essential for proper circadian function (Ishiura et al 1998). The following basic properties of this biological timer have emerged: (i) circadian rhythm is lost when KaiC protein is overexpressed continuously due to shutdown of *kaiBC* expression, whereas transient increases of KaiC serve to set the phase of the rhythm (Ishiura et al 1998; Xu et al 2000); (ii) in continuous light conditions the proportions of *kaiBC* mRNA and KaiC protein oscillate in a circadian fashion and exhibit a phase shift (Xu et al 2000); (iii) KaiA and KaiC are positive and negative regulators, respectively, of *kaiBC* transcription (Ishiura et al 1998); (iv) because practically all promoter activities in cyanobacteria underlie circadian rhythm, the Kai clock system might appear not to work in a clock-gene specific fashion, but to control a process that governs genome-wide expression the mechanism of which is unknown (Liu et al 1995; Xu et al 2003; Johnson, 2004; Nakahira et al 2004); (v) the proteins encoded by the *kai* genes – KaiA, KaiB and KaiC – interact with each other in vitro and in vivo (Iwasaki et al 1999; Taniguchi et al 2001), and KaiC constitutes the central component of the protein complex (Kageyama et al 2003); (vi) KaiC is an auto-kinase and an auto-phosphatase in vitro and in vivo (Nishiwaki et al 2000; Iwasaki et al 2002; Xu et al 2003), and the clock speed is correlated with the level of phosphorylation (Xu et al 2003), and (vii) both in vitro and in vivo, KaiA enhances phosphorylation of KaiC, and KaiB antagonizes the action of KaiA (Iwasaki et al 2002; Williams et al 2002; Kitayama et al 2003; Xu et al 2003 Kageyama et al 2006) (Figure 13-1). The observation that Kai proteins (KaiA and KaiC) can positively and negatively regulate *kaiBC* transcription (Ishiura et al 1998) rendered the cyanobacterial clock consistent with an oscillatory (TTO) feedback model involving transcription and translation, believed to be at the core of all self-sustaining biological timers (Dunlap et al 2004).

Recent observations have provided clear evidence that in *S. elongatus* a TTO feedback model is not valid. One advance occurred when the behaviour of the cyanobacterial KaiABC clock was scrutinized under constant dark conditions. Originally such an experiment had disclosed that the phase of rhythm in *S. elongatus* was not affected significantly when bacteria were switched back to conditions of continuous light following a period of constant dark (Xu et al 2000). In the dark, the metabolism of *S. elongatus* including RNA and protein syntheses is normally suppressed, but Kondo and coworkers reported a robust circadian rhythm under a constant dark condition in the presence of transcription inhibitors in excess proportions that almost quantitatively block the synthesis of RNA and protein (Tomita et al 2005). Despite the absence of rhythmic accumulation of Kai proteins and the lack of kaiA and kaiBC mRNA, KaiC phosphorylation exhibited a robust circadian rhythm for more than two days. The cyanobacterial circadian clock is therefore able to function without synthesis *de novo* of clock gene mRNA and the proteins encoded by them, and the period is accurately determined without transcriptional feedback.

These findings define a minimal timing loop in vivo that functions without transcription and translation and is temperature-compensated (Figure 13-1). The three Kai proteins accordingly comprise the minimal components of the circadian

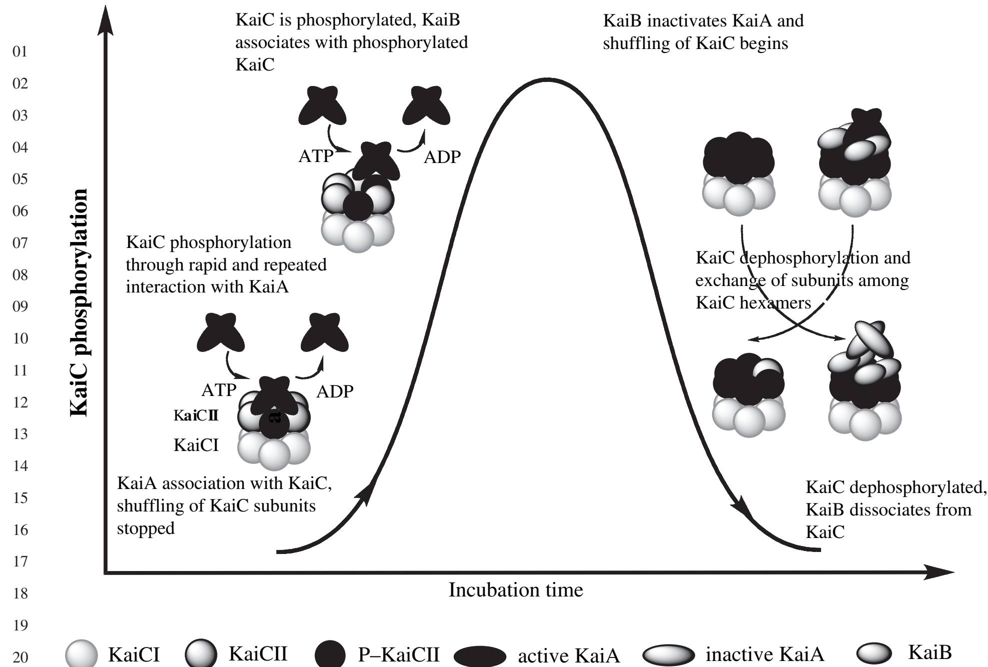

Figure 13-1. Model of the KaiC phosphorylation cycle. Schematic diagrams illustrate enhancement of KaiC phosphorylation (or inhibition of dephosphorylation) by KaiA dimer (left) and inactivation of KaiA by KaiB (right; adapted from Kageyama et al 2006). Only the KaiCII domains harbor phosphorylation sites (Xu et al 2004; see text)

oscillator and provide the output for the regulation of the general mechanism of transcription (Tomita et al 2005), perhaps using two associated histdine kinases – SasA and CikA (Schmitz et al 2000) – as signal mediators possibly to affect DNA superhelicality (Johnson, 2004). These observations raised also the spectre that KaiA, KaiB and KaiC might form a robust oscillator in vitro that exhibits rhythmic phosphorylation and dephosphorylation of KaiC and compensates for temperature changes (Figure 13-2). This condition was indeed demonstrated (Nakajima et al 2005), making the KaiABC system a unique target for a biochemical and structural dissection of the inner workings of a molecular timer.

STRUCTURAL STUDIES OF KAI PROTEINS

Three-dimensional structures based on crystallographic data and NMR data from solutions are available for all three Kai proteins from various cyanobacterial systems (Johnson & Egli, 2004; Golden , 2004) (Table 13-1). With regard to a structural characterization, the components of the cyanobacterial clock are the best studied, such that far more is known about them than the cogs of the eukaryotic circadian clocks

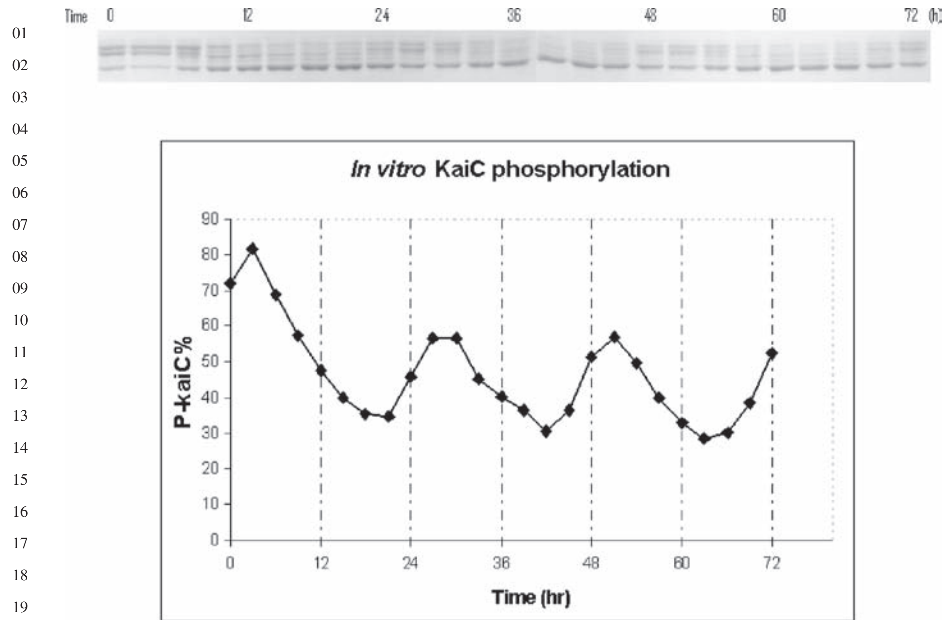

Figure 13-2. KaiC phosphorylation rhythm in vitro monitored over 72 h. Gel image courtesy of Ximing Qin and Tetsuya Mori (Johnson laboratory, Vanderbilt University)

for which only one partial structure has been reported (Yildiz et al 2005). Following the initial NMR determination of the structure of the N-terminal pseudo-receiver domain of KaiA from *S. elongatus* (Williams et al 2002) and EM investigations focusing on KaiC (Mori et al 2002; Hayashi et al 2003), high-resolution structural information for all Kai proteins emerged in 2004. The crystal structure of full-length KaiA was published for *S. elongatus* and revealed a domain-swapped arrangement with three dimer interfaces, one of which connects the N-terminal receiver domain with the C-terminal KaiC-interacting domain (Ye et al 2004) (Figure 13-3). The structures of the C-terminal dimerization and KaiC-interacting domain of KaiA from *Thermosynechococcus elongatus* BP-1 were solved separately by X-ray crystallography (Uzumaki et al 2004) and NMR (Vakonakis et al 2004a). The crystal structure and mutational data implicated grooves above the dimerization interface on opposite faces of the dimer as potential sites for interaction with KaiC.

A further crystal structure of the C-terminal domain of KaiA and a structure of full-length KaiB from the cyanobacterium *Anabaena* PPC7120 revealed a thioredoxin-like fold for the latter (Garces et al 2004) (Figure 13-4). This work also identified similarities in the dimensions and electrostatic potentials of particular regions in the KaiA and KaiB dimers as well as similar spacings between conserved arginine pairs on the surfaces of the respective Kai proteins. A crystal structure of

Table 13-1. Structures of cyanobacterial circadian clock proteins

Kai protein	Organism	Technique	Reference	PDB code[a]
KaiA N-terminal domain	PCC7942 *Synechococcus elongatus* (*S. elongatus*)	NMR	Williams et al 2002	1m2e
KaiA full-length	*S. elongatus*	X-ray	Ye et al 2004	1r8j
KaiA full-length	PCC7120 *Anabaena (Anabaena)*	X-ray	Garces et al 2004	1r5q
KaiA C-terminal domain	*Thermosynechococcus elongatus* BP-1 (*T. elongatus*)	X-ray	Uzumaki et al 2004	1v2z
KaiA C-terminal domain	*T. elongatus*	NMR	Vakonakis et al 2004a	1q6a
KaiB full-length	*Anabaena*	X-ray	Garces et al 2004	1r5p
KaiB full-length	PCC6803 *Synechocystis*	X-ray	Hitomi et al 2005	1wwj
KaiB full-length (T64C mutant)	*T. elongatus*	X-ray	Iwase et al 2005	1vgl
KaiB full-length (wild type)	*T. elongatus*	X-ray	Pattanayek et al unpubl. data	—
KaiC full-length	*S. elongatus*	X-ray	Pattanayek et al 2004	1tf7[b]
			Xu et al 2004	1u9i
KaiA - KaiC peptide complex	*T. elongatus*	NMR	Vakonakis & LiWang, et al 2004	1suy
KaiA - KaiC complex	*T. elongatus* / *S. elongatus*	X-ray/ EM	Pattanayek et al 2006	2gbl
SasA N-terminal domain	*S. elongatus*	NMR	Vakonakis et al 2004b	1t4y

[a] http://www.rcsb.org (Berman et al 2000).
[b] The 1tf7 and 1u9i entries are based on the same crystallographic data, but in 1u9i phosphate groups were added to T432 and S431 in six and four subunits, respectively.

KaiB from *Synechocystis* PCC6803 revealed formation of a tetramer with a positively charged perimeter, a negatively charged center and a zipper of aromatic rings important for oligomerization (Hitomi et al 2005). Additional evidence was based on mutational data that appeared to demonstrate the importance of the tetrameric state of KaiB for proper clock function. In the crystal structure of a *T. elongatus* mutant KaiB protein, a similar tetramer motif was found (Iwase et al 2005). The relevance of the tetrameric state of KaiB for its role in the control of the KaiC phosphorylation state has, however, been doubted as the protein appears to bind consistently to KaiC as a dimer, as judged from experiments using gel filtration chromatography (Kageyama et al 2006).

We determined the crystal structure of the full-length KaiC protein from *S. elongatus* (Pattanayek et al 2004). The structure of the central and largest protein from the cyanobacterial clock revealed the formation of a homo-hexamer in the

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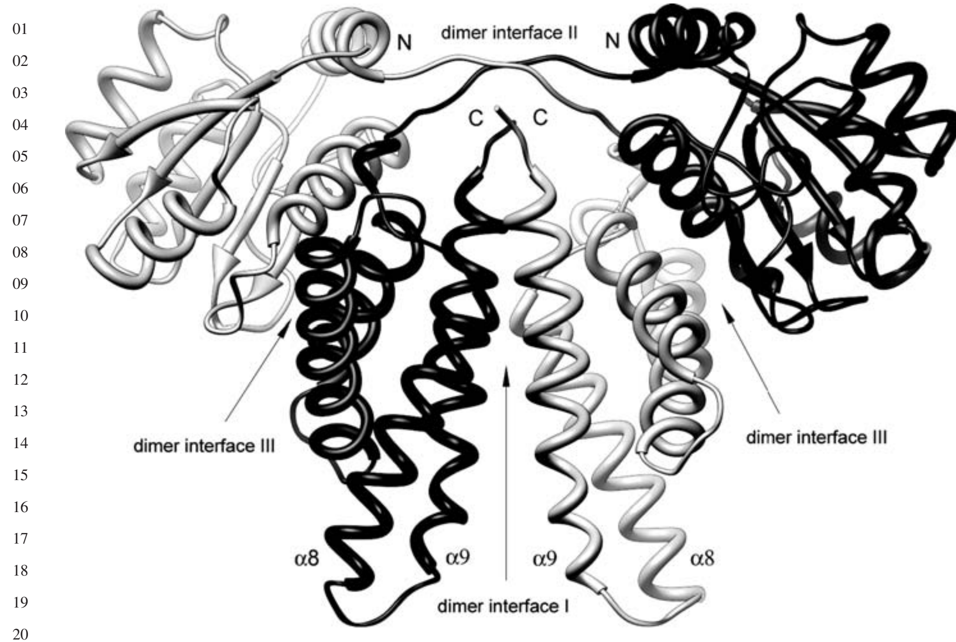

21 *Figure 13-3.* Crystal structure of the domain-swapped KaiA dimer from *S. elongatus* (Ye et al 2004).
22 Figures 13-3 – 13-7 were produced with Chimera (Huang et al 1996)

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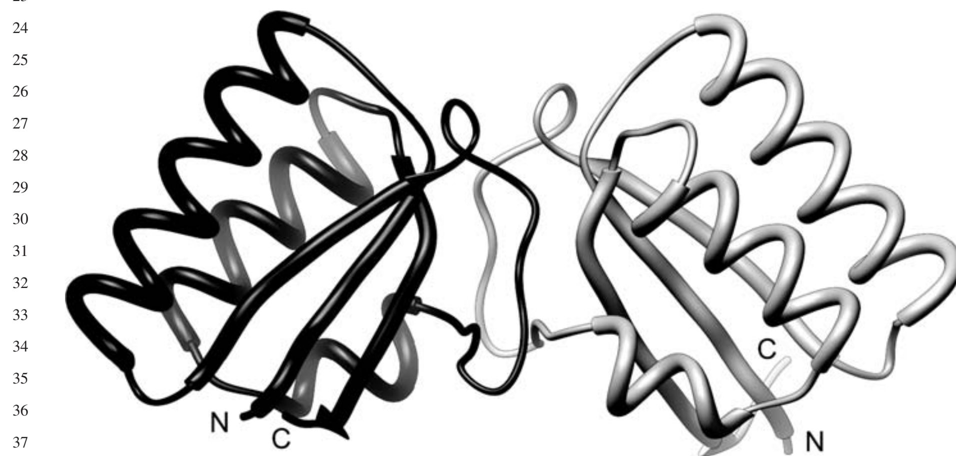

39 *Figure 13-4.* Crystal structure of the KaiB dimer from *Anabaena* (Garces et al 2004)

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41 shape of a double torus with a central pore and 12 ATP molecules bound between
42 the interfaces of monomers (Figure 13-5). The C-terminal 21 residues of KaiC
43 monomers were partly disordered in the original crystal structure, indicating great
44 conformational flexibility in this region for the unbound state of KaiC. Vakonakis

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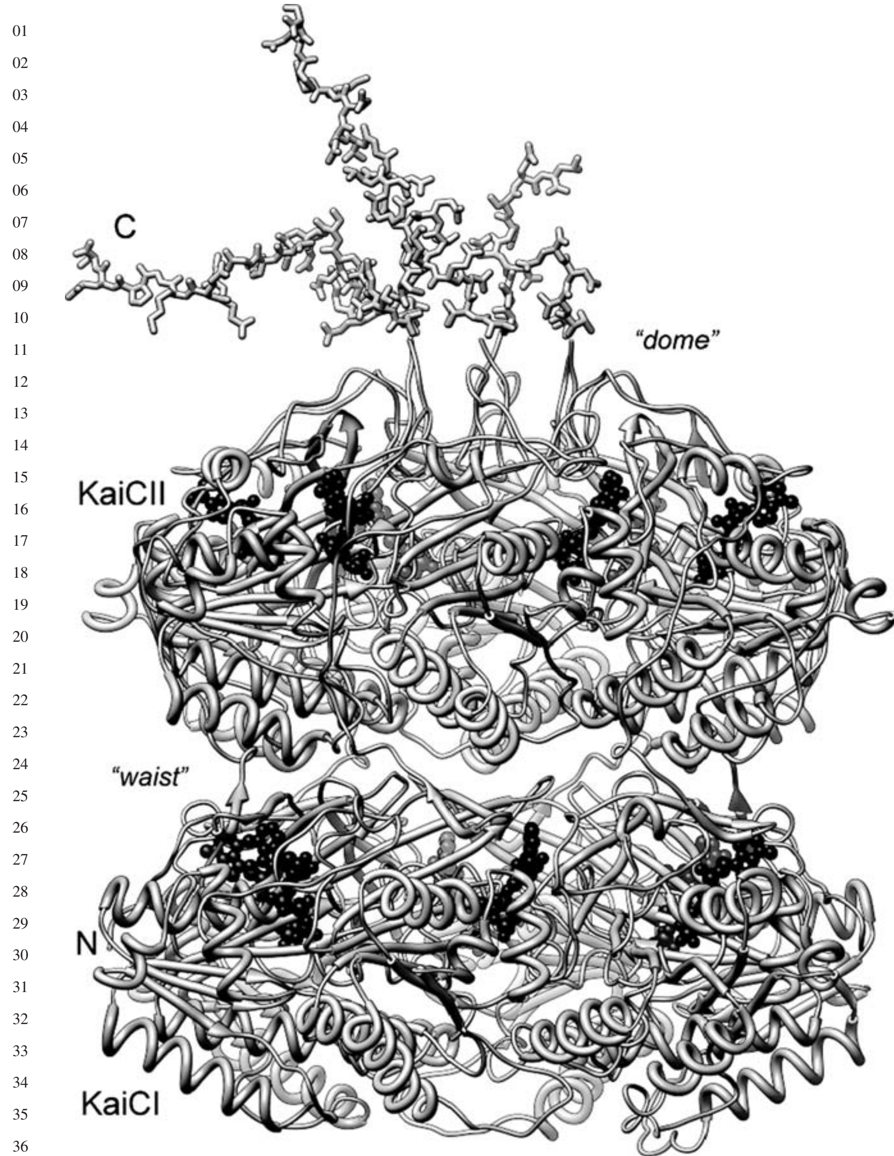

Figure 13-5. Crystal structure of the KaiC hexamer from *S. elongatus* (Pattanayek et al 2004). The model for full-length KaiC (519 amino acids) in the C-terminal region is complete for only two subunits (Pattanayek et al 2006). Atoms of the twelve ATP molecules bound between the KaiCI and KaiCII domains of individual subunits are shown as black spheres

and LiWang reported the NMR structure of a complex in solution between the dimeric C-terminal KaiA domain and 30mer peptides derived form the C-terminus of KaiC for the cyanobacterium *T. elongatus* BP-1 (Vakonakis & LiWang, et al 2004). Subsequent efforts to trace the C-terminal region of KaiC molecules in maps of electron density yielded a complete model for full-length KaiC from *S. elongatus* in the case of two subunits (Pattanayek et al 2006). The NMR structure of the monomeric N-terminal sensory domain of the SasA histidine kinase in solution has also been described (Vakonakis et al 2004b). Although KaiB shares with SasA and the thioredoxin family the initial beta-alpha-beta folding topology, the remaining structures and sequences diverge considerably (Hitomi et al 2005; Vakonakis et al 2004b).

DETERMINATION OF PHOSPHORYLATION SITES IN KAIC AND CONSEQUENCES OF THEIR MUTATION TO ALANINE FOR FUNCTION IN VITRO AND IN VIVO

The structure determined for *S. elongatus* KaiC was based on crystals grown from a mixture of proteins exhibiting various levels of phosphorylation as the protein had been purified as a hexamer and in the presence of Mg^{2+} and ATP (Pattanayek et al 2004). Following completion of the crystallographic model of the KaiC hexamer, inspection of difference electron-density maps allowed the identification of three sites, T432, S431 and T426 (Figure 13-6), of phosphorylation in the KaiCII domain; the KaiCI domain seems to contain no phosphorylation site (Xu et al 2004). Two residues, T432 and S431, were confirmed independently by mass spectrometry (Nishiwaki et al 2004).

The three serine and threonine residues, when mutated to alanine individually, render the clock arhythmic in vivo (Xu et al 2004). Individual T426A, S431A or T432A mutations as well as double mutations to alanine alter the phosphorylation patterns, and the triple mutant (T426/S431/T432$\rightarrow$A) is no longer phosphorylatable. Mutation of Ser and Thr residues does not affect hexamerization. All phosphorylation sites are located in the KaiCII half; phosphorylation proceeds across subunits, and the presence of phosphate groups is consistent with a more stable subunit interface (Xu et al 2004). Binding of ATP or ADP between the KaiCII domains of adjacent subunits is expected also to affect the stability of the complex. Lys and/or Arg residues can thus interact with the γ-phosphate group of ATP across the interface; such interactions are absent when ADP is bound (Hayashi et al 2006).

A STRUCTURAL MODEL OF THE COMPLEX BETWEEN KAIA AND KAIC

An intriguing feature of the cyanobacterial KaiABC circadian clock is that analysis of the structure and function of the central timer requires no concern with input and output. Beyond an understanding of how three proteins are able both to sustain a stable oscillation with a period of 24 h and to do

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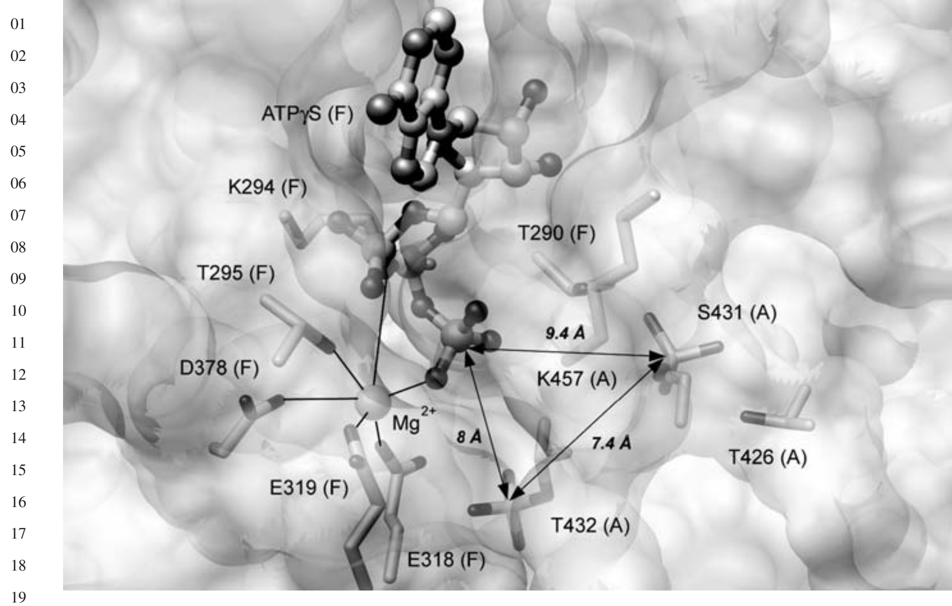

Figure 13-6. Location of phosphorylation sites in the KaiCII domain (T432, S431 and T426) at the interface between subunits A and F in the KaiC hexamer from *S. elongatus* (Xu et al 2004) The phosphoryl transfer occurs across subunits; selected distances in Å between the γ-phosphate and phosphorylated residues are shown

so in a temperature-compensated fashion, it is also important to acquire insight into how photoreceptors, and perhaps other sensors, are coupled with the clock (Schmitz et al 2000; Zhang et al 2006; Ivleva et al 2006). Similarly, how the ATP-dependent phosphorylation cycle driven by interactions between the three Kai proteins relates to global rhythmic control of gene expression (Nakahira et al 2004) remains to be worked out, although some players involved in output signaling have been identified (Katayama et al 1999; Iwasaki et al 2000; Ditty et al 2003; Takai et al 2006). In terms of an analysis of the output mechanism, the Kondo group has reported the identification of a protein, SasR, that interacts with SasA and has a leucine zipper DNA-binding motif (Kondo, 2005).

Based onsequence alignments, KaiC was shown to be a member of the RecA/DnaB superfamily of proteins (Leipe et al 2000), but, unlike classical helicases, KaiC is the result of a gene duplication and is composed of two hexameric rings (Figure 13-5). A 3D-structural alignment between the KaiCI or KaiCII hexameric rings and helicases revealed clear deviations in diameter, channel size and ATP position (Pattanayek et al 2004). Such alignments exhibited a fit that was somewhat inferior to superimpositions of the monomeric proteins. The best correspondence was found to exist between hexameric rings of KaiC halves and the F1-ATPase (Abrahams et al 1994), and was unanticipated from an alignment of the primary sequences. In light of these observations, KaiC is unlikely to act as

a helicase, consistent with the results of gel shift experiments that demonstrate the need for KaiC at picomole concentrations to cause a shift with poly-dT or forked oligodeoxynucleotides at femtomole concentrations (Mori et al 2002). As there is currently no experimental evidence that proves KaiC to be a helicase, it appears unlikely that clock-controlled regulation of genes involves a direct interaction between KaiC and DNA. The similarities at the structural level between F1-ATPase and the hexamers formed by the KaiCI and KaiCII halves are also unlikely to extend to the functional level. The molecular machine that produces ATP is anchored in the membrane, and features a central stalk that rotates inside the channel formed by the trimer of $\alpha\beta$-heterodimers. Neither the KaiA nor the KaiB dimer exhibit a conformation that indicates the possibility of insertion into the KaiC channel (Figures 13-3–13-5), and they have been shown to exert their functions as dimers, not monomers (Kageyama et al 2006).

Based on yeast two-hybrid screens, early attempts to map the binding sites of KaiA on KaiC resulted in the identification of two candidate regions in KaiC involving the C-terminal 60 and 100 amino acids of the KaiCI and KaiCII domains, respectively (Taniguchi et al 2001). In a model of the hexamer that had the KaiCI and KaiCII domains arranged tail to tail, the two regions were expected to lie close together. However, the arrangement head to tail of the two KaiC halves observed in the crystal structure places the putative KaiA-interacting sites at a significant distance from each other (Pattanayek et al 2004). One encompasses the dome-shaped surface formed by C-terminal regions of KaiCII domains, and the other is located in the constricted waist region between KaiCI and CII and includes the 15-amino acid peptide linking the two (Figure 13-5). Both deviating topologies of these sites – a concave surface in the waist and a convex dome surface on KaiCII – and the fact that KaiCI appears devoid of phosphorylation sites raise doubts about the need for an interaction between KaiA and KaiCI. The presumed function of KaiA is either to enhance phosphorylation of KaiC or to inhibit dephosphorylation (Figure 13-1), but the absence of phosphorylation sites, and hence kinase and phosphatase activity by KaiCI, renders unnecessary such a regulation.

Vakonakis and LiWang observed specific binding between a KaiCII C-terminal peptide and the C-terminal domains of the KaiA dimer from *T. elongatus* (Vakonakis & LiWang, et al 2004); the corresponding peptide at the C-terminus of KaiCI showed no binding. This observation is consistent with regulation by KaiA of the level of KaiC phosphorylation affecting only KaiCII. This finding prompted us to reexamine the electron density above the C-terminal dome in the KaiC hexamer crystal structure from *S. elongatus*, leading to complete models for full-length KaiC in two subunits and an addition of several residues to the remaining four (Figure 13-5) (Pattanayek et al 2006). Deletion of the C-terminal 25 residues in KaiC abolishes complex formation with KaiA in vitro and clock rhythmicity in vivo; the deletion does not affect hexamerization (Pattanayek et al 2006). Binding between a C-terminal peptide from a KaiC subunit and the KaiA dimer sheds no light on the mechanism according to which the latter enhances KaiC phosphorylation. A study focusing on the

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proteins from *T. elongatus* demonstrated that a single KaiA dimer is capable of upregulating KaiC phosphorylation to a virtually saturated level (Hayashi et al 2004a). The interaction between KaiA and KaiC is apparently dynamic in nature, involving rapid and repeated binding of KaiA to C-terminal peptides from KaiC subunits (Figure 13-1; Kageyama et al 2006).

Using a combination of X-ray crystallography, electron microscopy and assays in vitro and in vivo with native and mutant proteins from *S. elongatus* and *T. elongatus*, we have developed a model for the KaiA-KaiC 1:1 complex. This model leaves intact the binding interface between the KaiCII C-terminal peptide and the KaiA dimer worked out with solution NMR (Vakonakis & LiWang, et al 2004). The conformation of the peptide in the NMR structure and that of the C-terminal portion of one KaiC subunit in the crystal structure of full-length KaiC are similar (Pattanayek et al 2006). This discovery made possible replacement of that C-terminal peptide (from *S. elongatus*) by the NMR peptide with the C-terminal domains of KaiA dimer bound (from *T. elongatus*). With account taken of the EM-based envelope of the KaiA-KaiC 1:1 complex, the KaiA dimer based on the crystal structure of the full-length protein from *S. elongatus* (Ye et al 2004) was superimposed on the model of the KaiA dimer (C-terminal domains only) - KaiC complex. The resulting model of the complex has the α8-loop-α9 portion of the C-terminal domain of a KaiA monomer (Figure 13-3) in close proximity to the nucleobase portion of ATP bound between two KaiC subunits (Figure 13-7). The model discloses no detail of the interactions between KaiA and KaiC at this site, but main-chain atoms of residues in the apical KaiA helix-loop-helix region, of which mutation critically affects the period of the clock, lie as close as 12 Å from ATP.

There exists potentially a second binding site between KaiA and KaiC. The first involves the KaiA dimer and the flexible C-terminal peptide of a KaiC subunit, and the second a seemingly more transient interaction between a helix-loop-helix region of a KaiA monomer and the ATP-binding cleft formed between the KaiCII domains from two subunits. There are several scenarios for how this second interaction might affect the extent of phosphorylation at residues T432, S431 and T426. For example, sealing the cleft that harbors ATP might increase the residence period of the latter. Alternatively, the contact with KaiA might result in a conformational change of residues and facilitate the transfer of the ATP γ-phosphate group. In the crystal structure, the T432residues in all six subunits and S431 residues in four subunits are phosphorylated (Xu et al 2004). The side-chain oxygen atoms of T432 and S431 are more than 8 Å away from the γ-phosphate group of ATP, and the conformations of subunitinterfaces observed in the crystal are unconducive to phosphoryl transfer. A structure of non-phosphorylated KaiC hexamer with bound ATP is lacking, and no experimental data provide insight into the conformational changes that the subunit interface undergoes as a result of one or more of the above residues becoming phosphorylated. What appears clear is that this second interaction is not tight, consistent with a rapid and repeated association and dissociation of potentially just a single KaiA dimer on the dome-shaped surface of KaiCII (Figure 13-1). One is tempted to draw an analogy between this mode of interaction

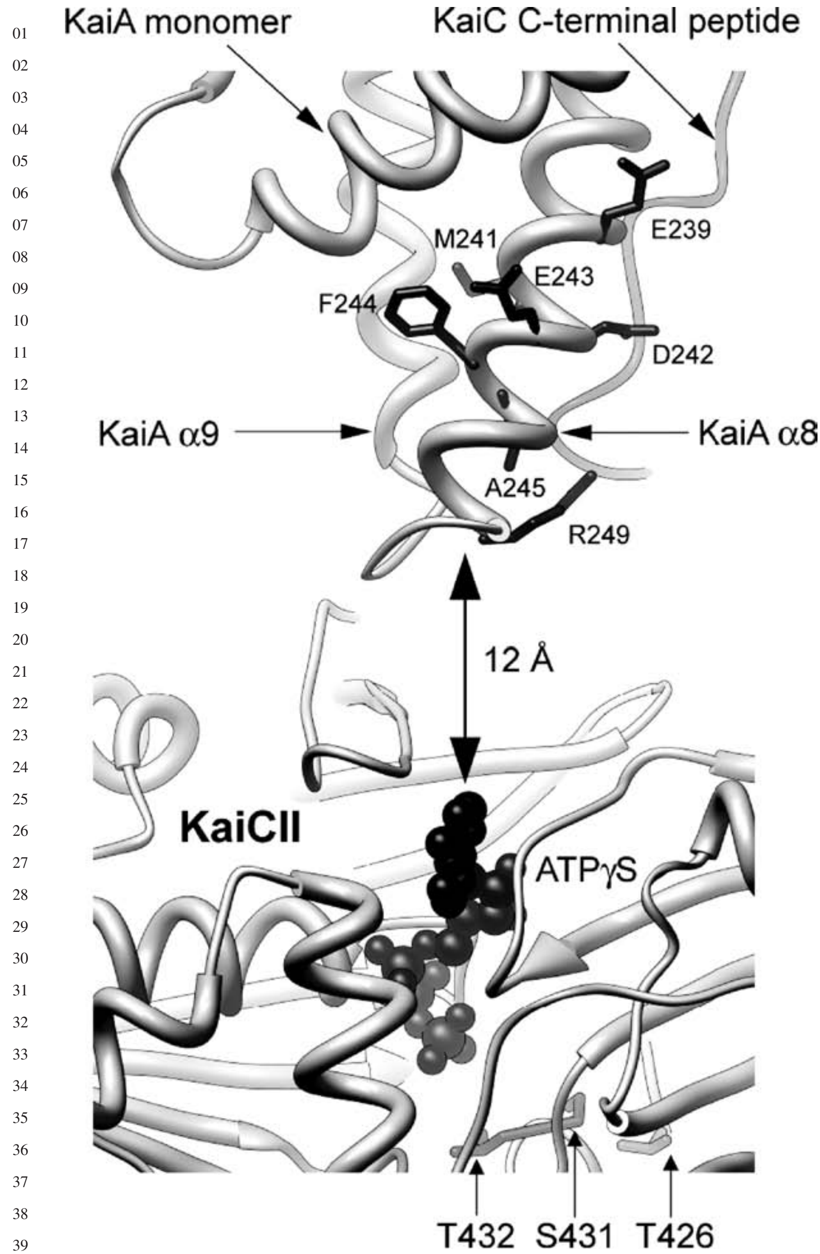

Figure 13-7. EM-based model of the 1:1 KaiA-KaiC complex from *S. elongatus* (Pattanayek et al 2006). Phosphorylation sites for a single KaiC subunit and selected residues in the α8-loop-α9 region of KaiA are highlighted

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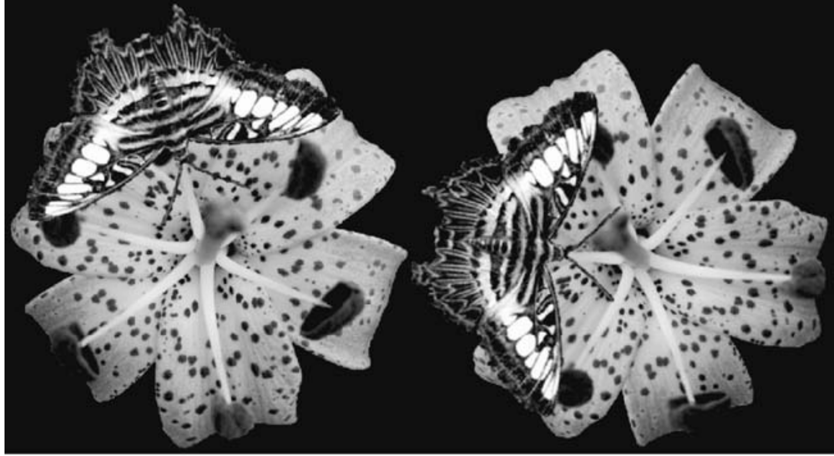

Figure 13-8. Artistic rendering of the interaction between KaiA dimer (*Parthenos sylvia subsp. lilacinus* – clipper butterfly) and KaiC hexamer (Tiger Lily)

for KaiA and KaiC and that of a butterfly drinking plant nectar and pollinating a flower. The butterfly (KaiA) hovers near a stamen (KaiC peptide) and eventually touches two petals (subunits; Figure 13-8, left), before moving to the next stamen or petals (Figure 13-8, right), thus pollinating the flower (phosphorylating KaiC).

DIVERGENT FUNCTIONS OF THE KAICI AND KAICII DOMAINS

There is mounting evidence for distinct roles of the two hexameric KaiC rings that comprise the central cog of the KaiABC clock in sustaining the phosphorylation rhythm. The crystal structure revealed formation of hydrogen bonds between P-loop residues and the nucleobase moiety of ATP molecules bound between KaiCI domains from adjacent subunits. Conversely, these hydrogen bonds are absent in the ATP binding pockets between subunits in the KaiCII ring. There is instead a tighter grip around the β- and γ-phosphates there (Pattanayek et al 2004, 2006). The structural data are consistent with distinct affinities for ATP by the KaiCI and KaiCII halves. The affinity for ATP in the CI half is accordingly greater than in the CII half (Hayashi et al 2004b). Work with proteins from *T. elongatus* demonstrated that the KaiCI domain expressed separately forms stable rings in the presence of ATP, but no hexamer formation was seen with KaiCII domains (Hayashi et al 2006). Beyond these differences in the recognition of and binding affinity for ATP, the two domains exhibit also topological (the C-terminal peptide tentacles protrude only from the KaiCII domains) and electrostatic differences

(the N-terminal dome is negatively and the C-terminal dome is positively polarized) (Pattanayek et al 2004). Most importantly, only KaiCII contains Thr and Ser residues that become phosphorylated, and KaiA seems to interact with only the KaiCII half. These observations together support a conclusion that the KaiCI hexamer serves as a structural platform whereas the KaiCII hexamer constitutes the business end of the homo-hexameric complex. Conformational changes as a result of KaiA-mediated phosphorylation might affect mostly the KaiCII half. Although no model of the interaction between KaiB and KaiC has been proposed, KaiB likely performs its role as a KaiA-antagonist also at the KaiCII end.

SUMMARY AND OUTLOOK

A dissection of the structure and function of the cyanobacterial KaiABC circadian clock offers the prospect of understanding a molecular timer – a nanoclock – in unprecedented detail. Whether key features of this clock, namely maintenance of a stable oscillation and temperature compensation decoupled from transcription and translation, are unique or will be established for other clocks in higher organisms remains to be seen. Significant progress has been made over the past two years in the analysis of the KaiABC clock. The availability of 3D-structures for proteins KaiA, KaiB and KaiC has enabled an examination of the interactions between them. X-ray, NMR and EM data with the results of assays in vitro and in vivo were thus compiled into a model of the 1 : 1 KaiA-KaiC complex. The model features two binding sites between the proteins that are both located on the outer surface of KaiC. There is no evidence for the central KaiC channel being used by either KaiA or KaiB for regulation of the level of phosphorylation of KaiC. Only the KaiCII hexameric ring that harbors all phosphorylation sites is likely contacted by KaiA and KaiB. The KaiCI and KaiCII domains that are the result of a gene duplication have divergent functions: the CI hexamer serves as a structural platform and is conformationally more rigid, whereas the CII hexamer is the functional center, and conformational changes in KaiCII domains triggered by phosphorylation and dephosphorylation are key to the generation of the rhythm with a ca. 24 h period. Application of hybrid structural methods will likely provide insight into the conformational properties of the binary KaiB-KaiC and the ternary KaiABC complexes, but only X-ray crystallography in combination with modeling of the dynamic processes underlying the interactions between the three clock components will disclose the atomic details required to understand the mechanism of this molecular timer. A central problem that remains to be solved is the origin of the temperature compensation – the independence of the clock period of temperature within a limited range – seen with the KaiABC clock reconstituted in vitro. Isolation of mutant proteins that lack temperature compensation and insight into potentially altered interactions between Kai proteins as a consequence of specific mutations might yield an improved understanding of this fascinating property exhibited by a complex of three proteins with bound ATP.

Acknowledgements

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