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Review

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Author for correspondence:
Martin Egli
e-mail: martin.egli@vanderbilt.edu

Architecture and mechanism of the central gear in an ancient molecular timer

Martin Egli

Department of Biochemistry, Vanderbilt University, Nashville, TN 37232, USA

ME, 0000-0003-4145-356X

Molecular clocks are the product of natural selection in organisms from bacteria to human and their appearance early in evolution such as in the prokaryotic cyanobacterium *Synechococcus elongatus* suggests that these timers served a crucial role in genetic fitness. Thus, a clock allows cyanobacteria relying on photosynthesis and nitrogen fixation to temporally space the two processes and avoid exposure of nitrogenase carrying out fixation to high levels of oxygen produced during photosynthesis. Fascinating properties of molecular clocks are the long time constant, their precision and temperature compensation. Although these are hallmarks of all circadian oscillators, the actual cogs and gears that control clocks vary widely between organisms, indicating that circadian timers evolved convergently multiple times, owing to the selective pressure of an environment with a daily light/dark cycle. In *S. elongatus*, the three proteins KaiA, KaiB and KaiC in the presence of ATP constitute a so-called post-translational oscillator (PTO). The KaiABC PTO can be reconstituted in an Eppendorf tube and keeps time in a temperature-compensated manner. The ease by which the KaiABC clock can be studied *in vitro* has made it the best-investigated molecular clock system. Over the last decade, structures of all three Kai proteins and some of their complexes have emerged and mechanistic aspects have been analysed in considerable detail. This review focuses on the central gear of the *S. elongatus* clock and only enzyme among the three proteins: KaiC. Our determination of the three-dimensional structure of KaiC early in the quest for a better understanding of the inner workings of the cyanobacterial timer revealed its unusual architecture and conformational differences and unique features of the two RecA-like domains constituting KaiC. The structure also pinpointed phosphorylation sites and differential interactions with ATP molecules at subunit interfaces, and helped guide experiments to ferret out mechanistic aspects of the ATPase, auto-phosphorylation and auto-dephosphorylation reactions catalysed by the homo-hexamer. Comparisons between the structure of KaiC and those of nanomachines such as F1-ATPase and CaMKII also exposed shared architectural features (KaiC/ATPase), mechanistic principles (KaiC/CaMKII) and phenomena, such as subunit exchange between hexameric particles critical for function (clock synchronization, KaiABC; memory-storage, CaMKII).

1. Introduction

'Each thing is of like form from everlasting and comes round again in its cycle' (Marcus Aurelius, second century A.D.). The emperor's words spoken almost 2 millennia ago attest to the timeless fascination with the nature of time, and recurring patterns such as day and night or the seasons. Keeping track of time and correctly anticipating daybreak and nightfall is of such crucial importance that organisms from simple cyanobacteria to humans all rely on precise molecular timers. Circadian clocks set the pace for a multitude of processes that include gene expression, cell division, development, metabolism and behaviour [1]. Circadian rhythms are characterized by three hallmarks independent of organism [2]: (i) endogenous oscillations with a period of approximately 24 h that persist under constant conditions, i.e. light or dark and temperature,

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(ii) temperature compensation, i.e. the period is only minimally affected by changes in temperature, and (iii) entrainment of the endogenous timer by exogenous, environmental cues, i.e. the light/dark cycle. The adaptive importance of circadian programmes was rigorously tested in cyanobacteria: competition experiments between *Synechococcus elongatus* strains showed that those with clocks that resonated with their light/dark environment outcompeted other strains, even if not set at 24 h [3]. Further, strains with any type of functional clock outcompeted clock-disrupted strains, although not under constant conditions [4]. Overall, such experiments demonstrated that a precise clock system enhances reproductive fitness.

Although all circadian oscillators share the above hallmarks, the molecules that make up the timers vary greatly among the lower and higher organisms. The KaiABC clock in *S. elongatus* is composed of the KaiA, KaiB and KaiC proteins and emerged some 3.5 billion years ago [5,6], but other organisms whose clocks have been studied extensively, from *Neurospora crassa* and *Arabidopsis thaliana* to *Drosophila melanogaster* and *Homo sapiens* differ from the cyanobacterial oscillator in their molecular make-up. For recent reviews of the molecular clock components in diverse organisms, mechanistic, functional and structural aspects as well as emerging shared features and unique attributes, see [7–14]. Research into the inner workings of the KaiABC cyanobacterial clock shattered the assumption that a transcription/translation-based feedback loop (TTFL) is at the heart of all biological timers [7,15]. Initially, it was discovered that circadian rhythms of KaiC phosphorylation persisted in the absence of transcription and translation [16]. Subsequent investigations established that mixing the KaiA, KaiB and KaiC proteins in an Eppendorf tube in the presence of ATP constitutes a temperature-compensated circadian oscillator, a so-called post-translational oscillator (PTO) [17]. The cyanobacterial timer thus comprises a PTO—the master clock—coupled to a TTFL that is required for robust oscillations [1,18,19]. The discovery of the KaiABC PTO in *S. elongatus* has led to a renewed interest in examining the experimental evidence for the existence of PTOs or non-transcriptional oscillators in eukaryotic clocks ([7] and references therein). A PTO may have proved more resilient in maintaining robust rhythms despite noise arising from cell division, changes in metabolic rate and environmental stress. The reduced susceptibility to external factors may have served as a powerful evolutionary driving force for convergent clock mechanisms in diverse organisms [20]. In cyanobacteria, the clock orchestrates gene expression and, although it is not clear whether metabolism is regulated in a similar fashion, changes in metabolism apparently serve the entrainment of the clock [1]. In that context it is fascinating to contemplate the possibility that temperature compensation, a shared property of all biological timers, merely constitutes a subset of metabolic compensation.

Among the three Kai proteins that constitute the *S. elongatus* PTO, only KaiC has enzymatic activity [21,22]. Actually, KaiC exhibits multiple activities—kinase [17], ATPase [23] and phosphotransferase/ATP synthase [24,25]—and its (auto-) phosphorylation and (auto-) dephosphorylation proceed with a period of approximately 24 h [16,17], thus setting the daily cycle. KaiC features N- and C-terminal domains (KaiCI and KaiCII, respectively) that display high sequence similarity (gene duplication) [26] (figure 1), and create a double ring upon formation of KaiC homo-hexamer [27–29]. Importantly, function, three-dimensional structure and dynamics of the CI and CII domains and rings differ [30]. Thus, both act as ATPases, but only CII possesses kinase activity [31–34], whereby phosphates from phosphorylated threonine and serine residues are returned to ADP to produce ATP, therefore rendering KaiC an ATP synthase [24]. The CI ring acts as a platform or hub and tighter inter-domain contacts compared with CII are at the origin of the higher stability of the CI ring [35]. KaiA binding to the CII C-termini stimulates KaiC phosphorylation [36–40] and KaiB sequesters KaiA in a KaiABC ternary complex during the dephosphorylation phase [41]. Binding by KaiB coincides with KaiC subunit shuffling, a process that is crucial for the robustness of the oscillation [42–44]. Multiple activities make this enzyme a fascinating target for mechanistic studies and the observation that KaiC together with KaiA and KaiB constitute a fully functional and temperature-compensated circadian clock *in vitro* have advanced the KaiABC nano-machine to the best studied molecular timer [1,7,45].

Circadian rhythms in cyanobacteria are pervasive from molecules to cells. In addition to the aforementioned rhythmic oscillation between the KaiC hypo- and hyperphosphorylated states and the above circadian assembly and disassembly of binary and ternary Kai protein complexes, the concentrations of the three Kai proteins as well as those of the KaiAC, KaiBC and KaiABC complexes wax and wane in a circadian fashion [44]. Bioluminescence assays using a bacterial luciferase reporter demonstrated genome-wide rhythmic promoter activity that is not disrupted by cell division and persists in daughter cells without a change in phase (reviewed in [29]). Additional cellular phenomena that exhibit a circadian rhythm are chromosomal compaction and expansion, and chromosomal topology as evidenced by assaying supercoiling of an endogenous plasmid [29].

This review focuses on KaiC, the central cog of the cyanobacterial clock, and some of its fascinating properties such as multiple enzymatic activities. Functionally, structurally and mechanistically, KaiC is the most interesting component of the three-protein PTO of *S. elongatus*, a recognition that prompted us to target it for initial three-dimensional structure determination by X-ray crystallography some 15 years ago. Structures of the full-length enzyme indeed provided insight into the order of phosphorylation events at the interfaces between KaiCII subunits [28,31,33,34], alternative phosphorylation sites besides the primary threonine 432 (T432) and secondary serine 431 (S431), and the absence of kinase activity in the KaiCI half [46]. Recent crystal structures of the KaiCI ring provided a better understanding of the slow ATPase activity in the N-terminal half [47]. Structural data also confirmed that KaiC exhibits not only sequence similarities to other proteins such as helicases (DnaB) and recombinases (RecA) [26], but that the architectures of these proteins show close resemblance, at least at the subunit level of single-ring hexameric assemblies. By comparison, the similar CI/CII and F1-ATPase active-site organizations revealed by structural overlays [28], are not evident from sequence alignments, but are consistent with the mechanism of KaiC dephosphorylation, i.e. the transfer of phosphates on threonine and serine back to ADP with concomitant synthesis of ATP in CII [24]. Other unexpected structural and mechanistic similarities (double ring structure, kinase and

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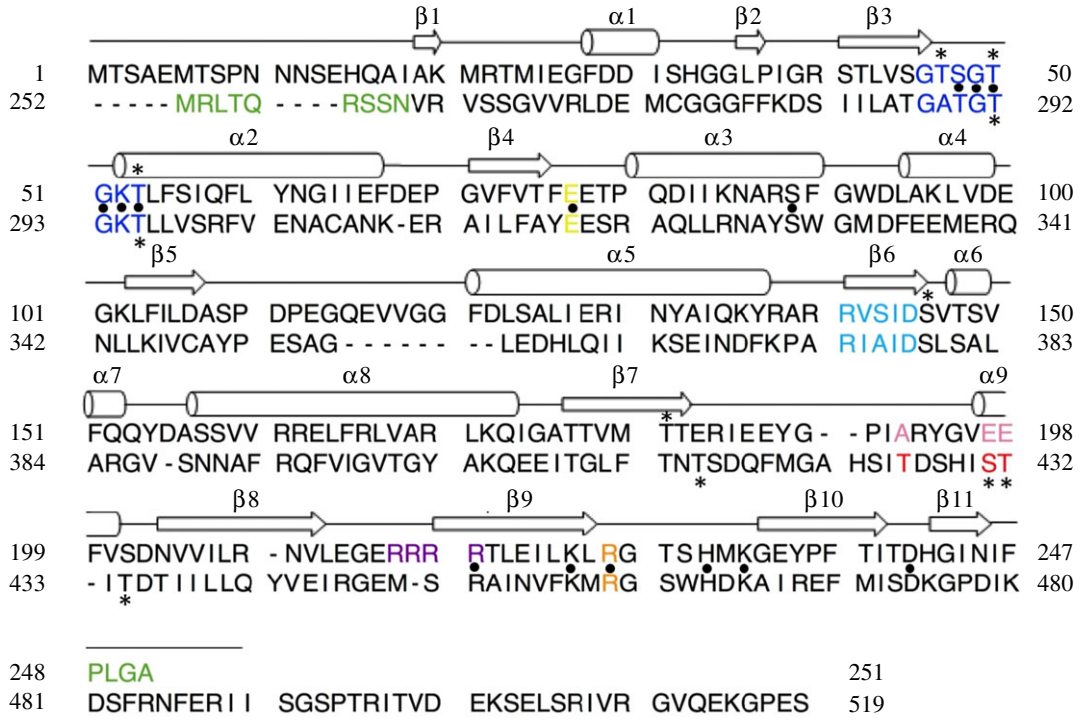

Figure 1. Sequence alignment between the KaiCI (upper line) and KaiCII (lower line) halves. Secondary structure elements observed in the crystal structure are depicted above the CI and CII sequences. Selected motifs and residues are highlighted in colour: Walker motif A ('P-loop', GXXXXGKT, blue), imperfect, truncated, Walker motif B (RXXXD, cyan), catalytic carboxylate glutamic acid (yellow), phosphorylation sites (CII, red) and equivalent residues in CI (pink), arginine finger (orange) and arginine linker (CI, purple), and CI–CII linker (green). Residues contacting ATPs are marked by a black dot and threonines and serines within 8 Å from γP are starred. Residues E487 to I497 in the longer C-terminal region of CII are referred to as A-loop (binding of KaiA), and residues Q115 to D122 in CI are referred to as B-loop (binding of KaiB).

phosphotransferase activity, and subunit exchange) also exist between the KaiC clock protein and the Ca^{2+}/calmodulin-dependent kinase II holoenzyme (CaMKII) that is involved in memory formation [48].

2. Differences in the sequences and architectures of the KaiCI and CII domains

Amino acid sequence comparisons established an evolutionary relationship between the RecA/Rad51/DCMI family of ATP-dependent recombinases in archaea, bacteria and eukaryota, the DnaB replication fork helicase in bacteria and the Sms and KaiC families [26]. KaiC arose as a result of the duplication of a gene that codes for approximately 250-amino acid ATP- and DNA-binding domain. However, although this typically more than 500 amino acids long protein composed of two RecA-like domains and joined in a head-to-tail fashion is present in cyanobacteria (e.g. *S. elongatus*), the *Archeoglobus* and *Pyrococcus* archaea, and *Methanobacterium*, it is not found in *Aeropyrum* and *Methanococcus*. Besides the double-domain KaiCs, there are many archaeal single-domain homologues and the KaiC protein in the bacterium *Thermotoga maritima* (hyperthermophile) is also of the single-domain type. Thus, it was suggested that the single-domain protein was laterally transferred from the bacteria to the archaea, followed by duplication and fusion [26]. Sequence alignment of the KaiCI and KaiCII domains from *S. elongatus* reveals similarities and clear differences (figure 1). The two halves display some 20% identity and 30% similarity, and these numbers are similar for the proteins from *Thermosynechococcus elongatus*, *Synechocystis* and *Anabaena*. As expected, the KaiC domains feature a number of sequence motifs and residues that are hallmarks of ATPases, such as the Walker A and B motifs, catalytic glutamate and the Arg finger that commonly contacts the ATP γ-phosphate at the active site. The N- and C-terminal regions in the two domains differ significantly: at the N-terminus, the CII domain exceeds in length the CI one by a short stretch; as well the former domain features a more than 30-residue long C-terminal tail. KaiC C-terminal tails exhibit relatively high sequence conservation among cyanobacterial strains and a noteworthy characteristic is the presence of four or more acidic residues [40].

The likely absence of an N-terminal helix like those present in DnaB or RecA was thought to preclude hexamerization by KaiC and the head-to-tail fusion of two RecA-like domains expected to perhaps promote a functional dimer [26]. However, our crystal structure revealed that KaiC does form homo-hexamers [28]. In fact, prior to the determination of the structure, negative-stain electron micrographs provided unequivocal proof that KaiC molecules assemble into hexamers (figure 2*a*). Once crystals of *S. elongatus* KaiC became available [49], the self-rotation function calculated based on diffraction data to better than 3 Å resolution was consistent with the presence of non-crystallographic sixfold symmetry (figure 2*b*). Indeed, once the structure was phased using a single $Ta_6Br_{12}^{2+}$ site, the experimental electron density map showed two hexameric rings composed of KaiC subunit domains that were slightly twisted relative to each other (figure 2*c*). The initial structural model contained about 490 amino acids per subunit, from *ca* residue 10–500, whereby the two domains are nearly linearly arranged (figure 3*a*). Translating KaiCI by 42 Å and rotating it

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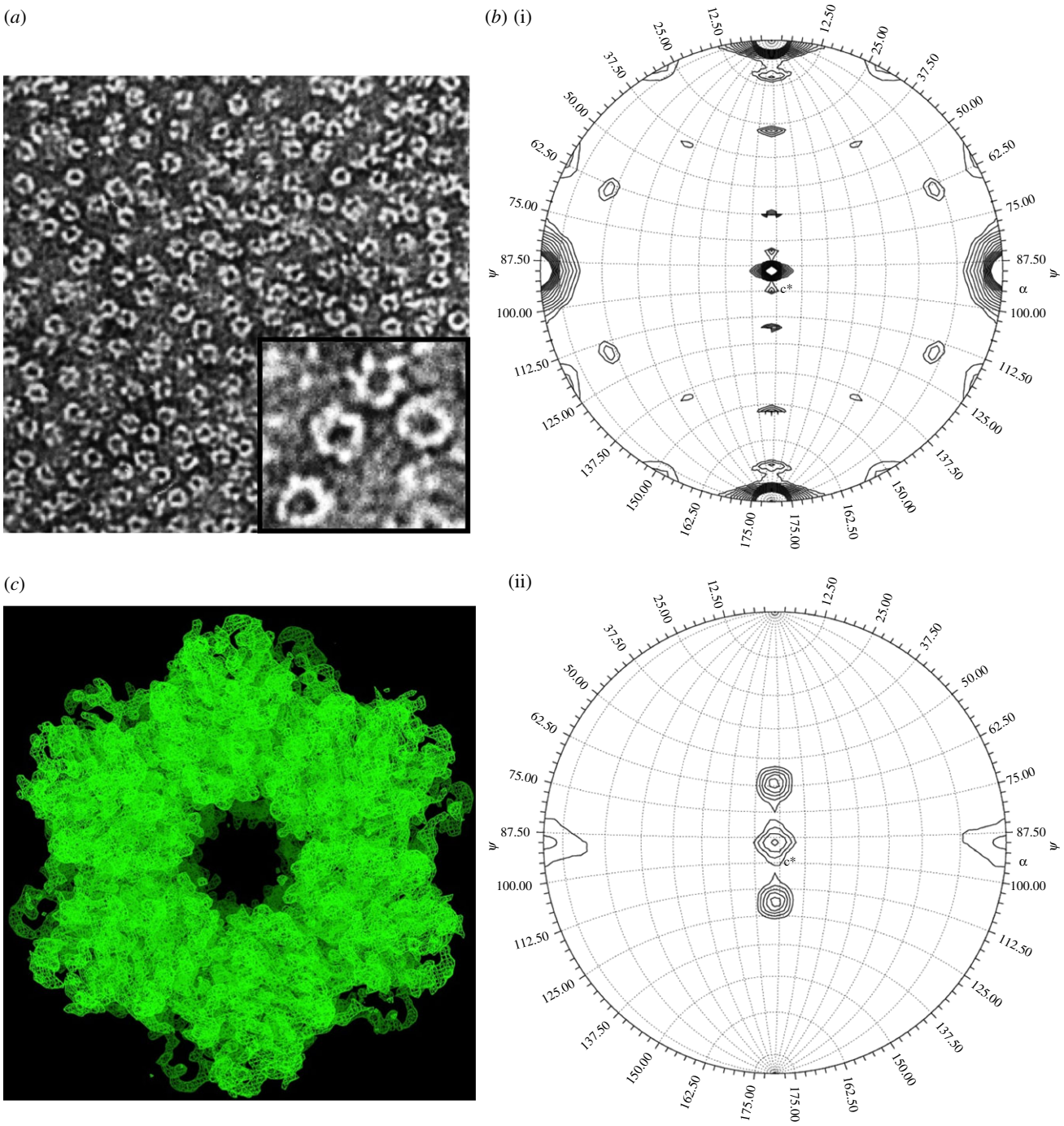

Figure 2. *Synechococcus elongatus* KaiC forms a hexamer. (*a*) Negative-stain EM micrograph [27]. The magnified image in the inset shows hexameric rings. (*b*) Self-rotation function in space group $P2_12_12_1$: $\kappa = 180^\circ$ section, revealing non-crystallographic dyads every 30° (i); $\kappa = 60^\circ$ section, revealing non-crystallographic sixfold rotation axes that are slightly tilted relative to the crystallographic *z*-axis (ii). (*c*) Solvent flattened experimental electron density map at 2.8 Å resolution.

clockwise by 15° around the axis of translation superimposes it roughly onto KaiCII. The structural overlay of the two domains results in an RMSD of approximately 2.5 Å, whereby loop regions exhibit a somewhat less optimal fit than α helices and β strands (figure 3*b*). The 13-residue linker region connects the β11(CI) and β1(CII) strands and the most striking deviation concerns the C-terminal ends of the two domains. Unlike the KaiCI domain that ends in two short antiparallel β strands (β10/11), KaiCII continues in an S-shaped turn and the last 20 residues then project away from the surface. In the original structure, these tails were not traced or traceable in the electron density [28], but in a subsequent paper we reported a more complete KaiC model with complete C-terminal tails in two subunits [37] (figure 4*a*). Not surprisingly the tails exhibit considerable flexibility, but as a result of packing interactions in the KaiC crystal lattice that bring CI and CII surfaces of opposite electrostatic polarizations into close vicinity [28,37], some of the C-terminal regions become more ordered. Thus, in the KaiC crystals, these tails adopt a random coil conformation or are intrinsically disordered. However, in the structure of a complex between full-length KaiA dimer and C-terminal KaiCII peptides determined more recently, KaiC tails adopt an α-helical conformation and form a coiled-coil motif with KaiA helices [40].

ATP molecules are wedged between subunits of KaiC hexamers (the crystal structure contains the slowly hydrolysing ATPγS analogue [49]), and the structure disclosed some

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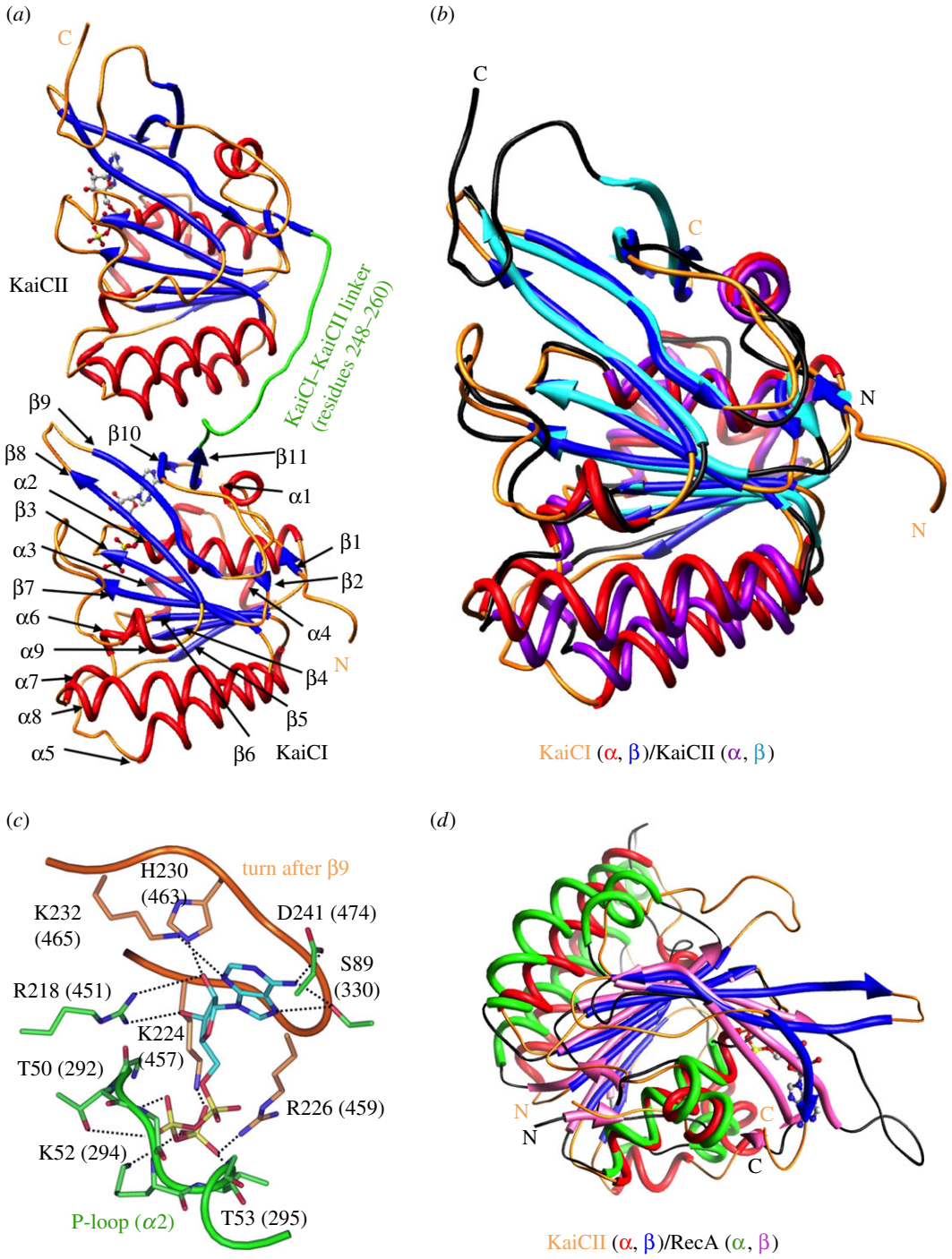

Figure 3. Three-dimensional structure of *S. elongatus* KaiC (PDB code 1TF7) [28]. (*a*) Structure of a KaiC monomeric subunit composed of CI and CII domains that adopt similar folds. Individual α helices (red) and β strands (blue) are labelled and the central linker is highlighted in green. (*b*) Overlay of the CI (residues 14–247; colour code identical to panel *a*) and KaiCII domains (residues 261–497; α helices, β strands and loops coloured in purple, cyan and black, respectively) with an RMSD of 2.45 Å based on 208 matching Cα pairs. (*c*) ATP-binding site in the CI domain. Amino acids from subunits A and B are coloured in green and orange, respectively, and are labelled. Selected H-bonds are shown as dashed lines and corresponding residues in the CII domain engaged in similar H-bonds are shown in parentheses. (*d*) Overlay of the KaiCII domain (residues 262–477; α helices, β strands and loops are coloured in red, blue and orange, respectively) and the central domain of *E. coli* RecA (PDB code 1G19; residues 39–269; α helices, β strands and loops are coloured in green, pink and black, respectively) [26,50]. The RMSD for Cα pairs amounts to 1.3 Å.

important differences between the ATP-binding modes in the CI and CII halves. Thus, the adenine nucleobase is only involved in contacts to protein in CI (figure 3*c*); no interactions occur at CII active sites. This is consistent with the observation that CI displays a strong preference for ATP relative to GTP, whereas the discrimination between these two nucleoside triphosphates is diminished in CII [51]. A further difference is found between corresponding residues K52 and K294 from the P-loop in CI and CII, respectively. K52 forms a salt bridge with the γ-phosphate but K294 does not. It is, therefore, not surprising that the K52H mutation causes loss of rhythm [51]. Conversely, the K294H mutation only results in a longer period. At both domain interfaces a lysine and an arginine (R finger), K224 and R226 in CI, and K457 and R459 in CII, hold the γ-phosphate group in a tight grip (figure 3*c*). Overall, the specificity for ATP in CI is in-line with the ATPase activity there (CII also displays ATPase activity). However, multiple amino acids are

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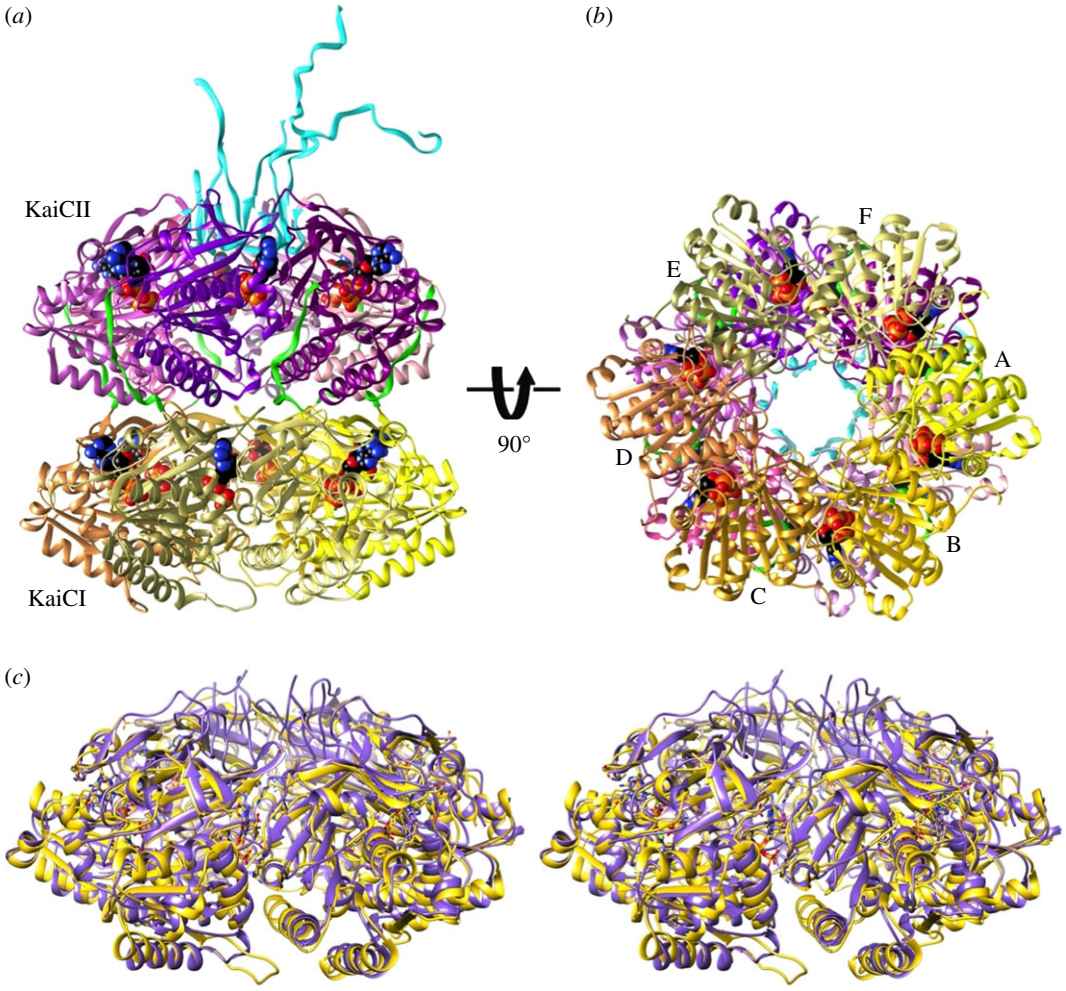

Figure 4. Structure of the KaiC homo-hexamer (PDB code 3DVL) [28,37]. (*a*) View from the side with the N-terminal CI and C-terminal CII domains from individual subunits coloured differently, and assuming the shape of a double doughnut. CI–CII linkers across the constricted waist are light green, C-terminal peptide tails are cyan and ATP molecules are shown in space filling mode, with carbon, oxygen, nitrogen and phosphorus atoms coloured in black, red, blue and orange, respectively. (*b*) The KaiC hexamer rotated by 90° around the horizontal axis and viewed from the CI side, exposing the central channel. Subunits are labelled A–F in a clockwise manner. (*c*) Stereo diagram depicting the superimposed CI (residues 19–247; coloured in gold) and CII rings (residues 261–497; coloured in lilac).

contacting the γ-phosphate group both at CI and CII subunit interfaces, and subtle differences between the interactions do not allow a rationalization as to why CI is exclusively an ATPase, whereas CII exhibits both ATPase and kinase activities. Both core domains, as predicted by sequence comparisons, adopt a fold that exhibits close similarity to the structure of *Escherichia coli* RecA [50] (figure 3*d*).

3. Three-dimensional structure and dynamics of the KaiCI and CII rings

The KaiC hexamer resembles a double doughnut with a diameter and height of approximately 100 Å. The view along the non-crystallographic sixfold rotation axis shows a central channel that is somewhat wider at the CI end (figure 4). The CI–CII linker that comprises residues 248–260 runs on the outer surface and across the constricted waist region, and C-terminal tails (residues 497–519) protrude from the dome-shaped surface of the CII ring. Whereas CI and CII core domains of about 245 residues adopt similar folds, the C-terminal extension breaks the more or less symmetrical stack formed by the hexameric rings. Binding sites of ATP molecules between subunits are located closer to the upper (C-terminal) surface of rings, i.e. ATPs bound in the CI half lie close to the central waist and those bound in the CII half lie close to the dome-shaped surface that is marked by clefts harbouring the ATPs [28]. The lower (N-terminal) and upper (C-terminal) surfaces of rings exhibit opposing electrostatic surface potentials (ESPs). Thus, the N-terminal surface of the double doughnut is strongly negatively polarized, but the C-terminal surface (bottom and top, respectively, in figure 4*a*) displays a largely positive ESP [28,30]. Analysis of the ESPs at subunit interfaces reveals the strongly hydrophilic nature of the interaction between individual KaiC molecules. Similarly, the central pore is rich in arginine, histidine and glutamate. However, KaiC shows only weak affinity for DNA [27] and no helicase activity has been established to date for the central cog of the KaiABC clock. Consistent with these observations, structural overlays of KaiC and helicase hexamers reveal only a suboptimal fit and misaligned ATP-binding sites at interfaces [28]. This is in contrast with superimpositions of monomeric domains, e.g. KaiCII and RecA [50] (figure 3*d*), that reveal an excellent match.

Several motifs in the CI and CII domains mediate contacts among subunits besides the extensive interactions at subunit interfaces that harbour ATP molecules. Among them is the arginine linker (residues R215, R216, R217 and R218, figure 1) in CI that does not just mediate contacts between

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CI domains from adjacent subunits but also reaches across the CI–CII waist region [52], and the A-loop (residues E487–I497, figures 1 and 3*a*) near the CII C-terminal end [38,39]. Another motif in the CII half is constituted by D417(A) and H429(B) and D427(B) from neighbouring subunits (A, B, C, etc.) that form a circle around the central channel, whereby the histidine side chain is sandwiched between the D417 carboxylate moiety and the D427 main chain keto group [24]. Importantly, mutation of some of the residues that are part of the above motifs results in drastically distorted clock periods or arrhythmic behaviour. Examples of the latter are the R216A and R217A [50], D427A [24] and E487A [38] mutants. Proper functioning of the central cog of the cyanobacterial clock is dependent on allosteric effects among subunits and distorting the latter by mutation of key residues severely impairs or destroys the oscillator. In addition to non-covalent lateral, inter-subunit CI–CI and CII–CII and intra-subunit CI–CII interactions, the CI–CII linker that joins the two domains is crucial for clock function. Severing the linker abolishes rhythmicity and therefore the clock cannot be reconstituted from separate CI and CII rings [35]. The CI domain from *S. elongatus* KaiC expressed separately forms a stable hexamer but the CII domain does not [53]. The crystal structure of the KaiCI hexamer has recently been published and the ATPase mechanism and activity of the isolated CI half can be assayed [47]. However, this has not been possible for the CII half, although the CII domain expressed separately still exhibits auto-kinase activity [53].

A superimposition of the KaiCI and CII hexamers reveals two regions where their conformations obviously differ from each other (figure 4*c*). The A-loops surround the channel at the C-terminal end of the double doughnut in a crown-like fashion and the 20-residue CII tails then protrude from the dome-shaped surface. The tails serve the interaction with KaiA and the activation of the kinase in the CII half [36,37,40], and this entire 35 amino acids long stretch is missing in the CI half. At the other end, the loop between β5 and α5 in CII is much tighter than in CI as a consequence of a deletion (figure 1). The more extended loop in CI (B-loop) is readily recognizable at the bottom of figure 4*c* and has been implicated in KaiB binding [30,54,55]. Molecular dynamics simulations of KaiC hexamers (including residues 14–497 in all subunits) demonstrated the drastically enhanced mobility of the A- and B-loop motifs located at the top and bottom, respectively, of the double doughnut [39]. The crystal structure thus unveiled unique conformational properties of the two regions that obviously deviate also in terms of the KaiCI and CII primary sequences. These regions turn out to be of critical importance for mediating Kai protein–protein interactions and clock mechanism and also exhibit distinct dynamic properties, such that altering the latter via mutation affects clock period and/or temperature compensation.

4. Functional divergence between KaiCI and CII and why CII acts as a kinase but not CI

The observation that the KaiCI half has ATPase activity and that KaiCII is both an ATPase and a kinase adds to the fascination with the cyanobacterial PTO and prompted us to take a closer look at the reasons for water being the only nucleophile in one case, whereas threonine and serine act as nucleophiles in the other [56]. KaiCII is a Ser/Thr kinase and it turns out that the identity of the phosphorylation sites is very important for proper working of the oscillator, but more about that in the next chapter. The initial structure of KaiC found that T432 in all six subunits was phosphorylated and that S431 was phosphorylated in four of them [28]. Although no order of the phosphorylation events could be deduced based on this observation, it was apparent that Oγ of T432 was situated closer to the γ-phosphate group of ATP than Oγ of S431 on average, and it seemed therefore reasonable to view T432 as the primary phosphorylation site [31]. A mass-spectrometric analysis of proteolytic fragments of KaiC confirmed the T432 and S431 phosphorylation sites [32]. Phosphorylation occurs across the subunit interface such that the γ-phosphate of ATP bound to subunit A is transferred to threonine and serine from subunit B (figure 5*b*). Subsequent work revealed a strict order of phosphorylation and dephosphorylation: TS → pTS → pTpS → TpS → TS [32,33]. An interesting difference in the environments of the T432 and S431 residues is that the latter is positioned in the vicinity of T426 across a tight turn. When S431 is phosphorylated, the γ-hydroxyl group of T426 engages in an H-bond to the phosphate group. An initial round of mutations showed that the T432A, S431A and T426A mutants were all arrhythmic *in vitro* [31]. A more in-depth investigation using the T426A, T426N and T426E mutants along with others demonstrated that alterations at residue 426 abolished circadian rhythms of luminescence *in vivo* [57]. The finding that the T426N mutant was arrhythmic supports the conclusion that it is not sufficient for the side chain of residue 426 to be able to engage in an H-bond (as seen in the crystal structure of T426N-KaiC [46]). Rather, it has to be phosphorylatable in order for 426 to fulfil its proper regulatory role. Indeed, we discovered that T426 carried a phosphate group in a single subunit and in four subunits in the crystal structures of the S431A and T432E/S431A KaiC mutants, respectively [46]. For an overview of phosphorylation sites and number of phosphates in CII based on crystal structures of mutant enzymes, see [30]. What is clear is that a full understanding of the complex mechanistic aspects of the KaiC auto-kinase activity and its regulation requires one to look beyond the two main sites and the strict order, starting from the hypo- to the hyper- and back to the hypo-phosphorylated state, over the daily cycle.

Alignment of the CI and CII sequences shows that the CI residues that correspond to T432, S431 and T426 in CII are E198, E197 and A192 (figure 1). Neither glutamate nor alanine can be phosphorylated; examination of the separate CI half by polyacrylamide gel electrophoresis (SDS-PAGE) and inspection of the electron density in the CI region did not reveal phosphorylation. It appears that nature deliberately precluded kinase activity in KaiCI by mimicking pT432 and pS431 with glutamates in CI and T426 with a non-phosphorylatable alanine (figure 5). We investigated the possibility of phosphorylation in a KaiCI triple mutant E198T/E197S/A192T (TST-KaiCI) using the *T. elongatus* system, but did not detect kinase activity [52]. Closer examination of the superimposed CI and CII active sites reveals subtle deviations in the loop region leading up to the α9 helix that harbours the phosphorylation sites in CII. Thus, a slight kink at G195 changes the register of the following V196, E197, E198 and F199 in CI relative to I430, S431, T432 and I433 in CII. Instead of the correspondence between E197/E198 and S431/T432, as suggested by the sequence alignment, the three-dimensional structural alignment shows that E198 maps to S431 and F199 maps to T432. As a result,

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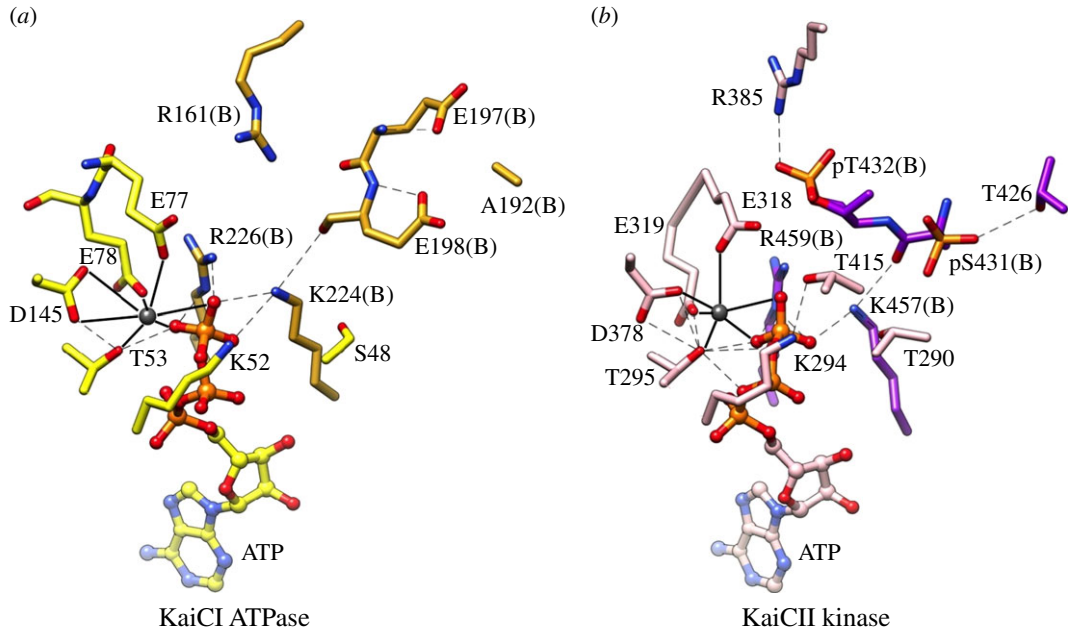

Figure 5. Active sites at subunit interfaces in the (*a*) KaiCI and (*b*) KaiCII halves. Carbon atoms of residues from adjacent subunits are coloured in yellow and gold (CI subunits A and B, respectively), and pink and purple (CII subunits A and B, respectively), Mg^{2+} ions are depicted as grey spheres with coordination geometries indicated by solid lines, and H-bonds are dashed lines.

E197 is turned away from the γ-phosphate (figure 5*a*) and F199 is inserted between E198 and the γ-phosphate. This would explain the absence of phosphorylation in the KaiCI-TST mutant. We subsequently also examined whether the ΔF199 deletion mutant combined with the TST mutations sparks kinase activity but this turned out not to be the case [52]. Subtle changes in sequence and length of the loop regions preceding residues 197 and 198 in CI and 431 and 432 in CII (figure 1) combined with the identity of these residue pairs themselves (EE and ST, respectively) seem to determine the fate in terms of enzymatic activity in CI (ATPase) and CII (kinase). However, altered dynamics in the CII phosphorylation loop relative to the corresponding loop in CI as well as differential stabilities of the CI (stable hexamer) and CII (no hexamer formation by isolated CII domains observed) rings cannot be ignored when considering the origins of kinase activity in CII and the absence thereof in CI.

Structural alignments disclosed a close correspondence between the F1-ATPase trimer of α−β heterodimers [58,59] and the hexameric KaiCI and CII rings [24,28] (figure 6*a*). The structural fits are significantly closer than those comparing KaiC and helicase hexamers and extend to the subunit interfaces and active sites (figure 6*b*,*c*). The structural similarities are in line with the transfer of phosphate groups on T432 and S431 in KaiCII back to ADP and formation of ATP during the dephosphorylation phase of the daily clock cycle [24]. However, F1-ATPase is not known to possess kinase activity and the question in regard to the absence of auto-phosphorylation in the KaiCI ring explored above also applies to F1-ATPase. An inspection of the ATPase active site at the α/β interface shows that T340 and S344 are positioned at distances from the γ-phosphate (figure 6*b*) that are not too different from the corresponding distances for T432 and S431 at the KaiCII inter-subunit active site (figure 6*c*). However, unlike the latter, the F1-ATPase active-site features three aromatic residues F259, F299 and Y311 that likely alter the polarity of the environment in the vicinity of the potentially phosphorylatable T340 and S344 amino acids, provide space limitations and thus restrict the conformational freedom. It is interesting that the insertion of an aromatic residue, F199, into the KaiCI active site seems to also be implicated in limiting the activity there to hydrolysis of ATP.

5. KaiC is a Ser/Thr (position 432) and a Ser kinase (position 431)

Given the transfer of phosphate to T432 and S431 KaiCII can be considered a serine–theronine (Ser/Thr) kinase. But in most aspects—fold, domain organization, active site make-up, activation loop and specificity—KaiC, as a member of the RecA/DnaB superfamily of proteins, differs from the classical kinases. Most Ser/Thr kinases display a phosphoacceptor preference, Ser or Thr, and some show promiscuous behaviour. The majority of kinases in the human and yeast proteomes appear to prefer Ser [60,61] and for several kinases, the amino acid at the Asp-Phe-Gly+1 (DFG+1) position in the activation loop has been identified as a major determinant of phosphoacceptor preference [62]. Thus, among the yeast kinases examined, those selective for Ser had larger hydrophobic residues such as Leu, Phe or Met at the DFG+1 position. Those mostly selective for Thr had the β-branched aliphatic residue Ile at the +1 position, and non-selective kinases featured either Leu or Ser there. This analysis was extended to human kinases with similar results, i.e. Thr-/Ser-specific kinases had Ser, Val or Ile at the DFG+1 position and Ser-selective ones featured mostly Phe at that site, such that the F187 V mutation (F187 is the DFG+1 residue) in protein kinase A inverted its preference from Ser- to Thr-specific. Human death-associated protein kinase 1 (DAPK1) has a Leu at position DFG+1 (L164) in the activation loop [63] and is known to phosphorylate both Ser and Thr sites in target proteins [64–66].

Although phosphorylation sites (T432/S431) and order (T432 before S431) of phosphorylation in KaiCII are known,

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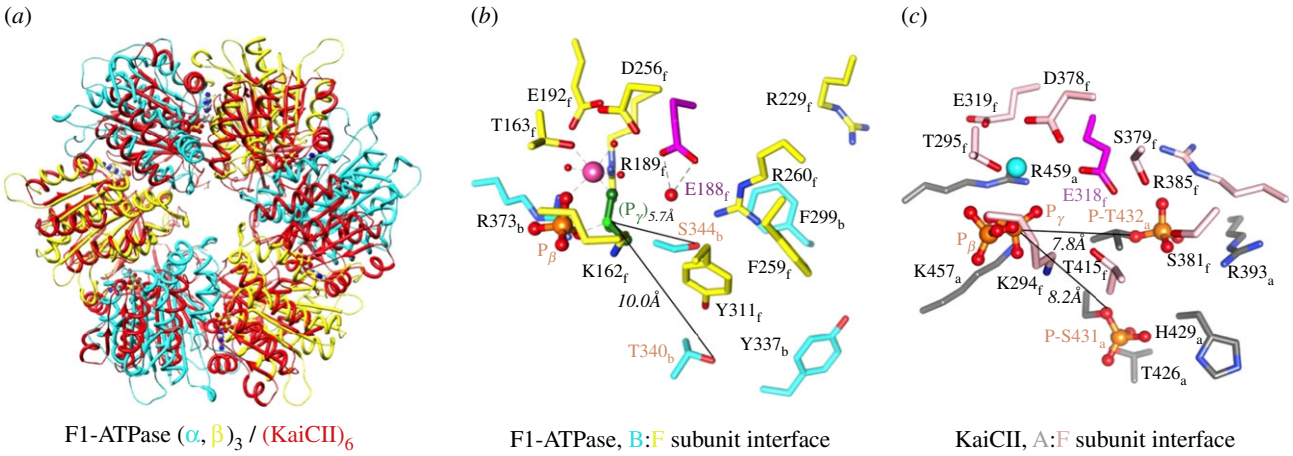

Figure 6. KaiC and F1-ATPase form similar ring-shaped complexes and active sites at subunit interfaces. (*a*) Overlay of the KaiC hexamer (red; CII ring only) and the α3β3 ring from F1-ATPase (PDB code 1BMF) [58]. The α and β subunits of F1-ATPase are coloured cyan and yellow, respectively. (*b*) ATP-binding site at the α − β heterodimer interface in F1-ATPase, taken from the crystal structure of the bovine enzyme inhibited by ADP and beryllium fluoride (PDB code 1W0 J) [59]. Carbon atoms of amino acid side chains in α chain B of F1-ATPase are cyan, and carbon atoms of side chains in β chain F of F1-ATPase are coloured yellow and Mg^{2+} is a pink sphere with coordinated water molecules depicted as smaller red spheres. (*c*) ATP-binding site at the subunit interface in KaiCII, taken from the crystal structure of the full-length enzyme from *S. elongatus* (PDB code 3DVL) [28,37]. Carbon atoms of CII subunit A are grey and carbon atoms of CII subunit F are pink, and Mg^{2+} is a cyan sphere. Only β- and γ-phosphates of ATP are depicted, whereby the latter is mimicked by BF_3 (green) in the structure of F1-ATPase. Carbon atoms of catalytic glutamates (E188 in F1-ATPase and E318 in KaiCII) are highlighted with magenta carbon atoms, and the water nucleophile H-bonded to E188 (F1-ATPase) is shown as a red sphere of slightly larger radius.

the phosphoacceptor preference of the auto-kinase remained unknown. We found in crystal structures that mutation of either T432 (T432S/S431), S431 (T432/S431T), or both (T432S/S431T), resulted in changes in the phosphorylation levels compared with wild-type KaiC (table 1). All three mutants were arrhythmic (T Mori, Y Xu, CH Johnson, 2015), indicating that tampering with phosphorylation sites results in an identity crisis and abolishes clock function, despite the fact that KaiC can phosphorylate Thr or Ser at position 432 (but apparently only Ser and not Thr at position 431; table 1). Altering the identity of the secondary phosphorylation site T426 to Ser while retaining T432 and S431 abolishes phosphorylation of the latter according to the crystallographic analysis of this KaiC mutant (table 1) and triggers an extremely long period. Once again, these data suggest a crucial role of residue 426 in KaiC conformation and clock regulation.

6. Origins of the 'slowness' of the KaiCI ATPase

One of the most fascinating and difficult to explain properties of molecular clocks is the long-time constant of the period. Recent crystal structures of the KaiCI hexamer alone led to the identification of two possible origins of the slowness of the ATPase [47]. One was a suboptimal (not in-line with respect to the scissile Pγ-O3β bond) orientation of the water molecule initiating hydrolysis of ATP. The other relates to the *cis*/*trans* isomerization of the peptide bond between D145 and S146, i.e. between a predominantly *cis* conformation in the pre-hydrolysis state and a *trans* conformation in the post-hydrolysis state (see figure 5*a* for the location of D145). The switch from *cis* to *trans* is accompanied by a repositioning of the α6-α7/α8 helices (see figure 3*a* for the location of these helices in KaiCI), and modified interactions with ATP at the adjacent subunit interface. Interestingly, the conformational changes in the helix–loop–helix region seen in KaiCI as a result of ATP hydrolysis are very similar to the deviating positions between α6-α7/α8 helices of KaiCI and KaiCII in the superimposed rings. In figure 4*c*, the different orientations of α8 helices in one subunit are visible near the bottom exit of the central channel, adjacent to the B-loop. Thus, it is feasible that the ATPases in CI and CII were trapped in different states in the crystal structure of full-length KaiC. The actions of the two enzymes in the upper and lower rings are coupled and ATPase activity in CII is required for auto-dephosphorylation [47]. Crystals of full-length KaiC were grown in the presence of ATPγS [28,49], a slowly hydrolysing analogue of ATP, and ATPase activity is therefore not prevented. However, the structures of the separate CI half of KaiC and mutants thereof in the presence of ADP or ATP were determined at resolutions of around or better than 2 Å [47]. Conversely, the resolutions of crystal structures of full-length KaiC are typically closer to 3 Å. The highest resolution for a KaiC structure we obtained so far was for the A422S mutant protein (2.6 Å resolution; Egli lab, 2013). However, even at this resolution, the quality of the electron density does not permit one to distinguish between the *cis* and *trans* conformations of a peptide bond and both isomers of the D145-S146 dimer can be built into the density. Moreover, a careful inspection of the MD simulation data generated for KaiC (with subunits comprising 14–497 or 14–487 residues [39]), did not reveal any transition from the preferred *trans* to the *cis* conformation. This is perhaps not surprising given the steep energy barrier of 14–16 kcal mol^{-1} that needs to be overcome for *cis*/*trans* isomerization to occur [47].

7. Comparison between CaMKII and KaiC

Looking beyond one's own field of investigation or particular subject of interest can be challenging and confusing, but ever so often an initially strange territory presents familiar views and reveals common principles. The structural similarity between KaiC and F1-ATPase despite very different sequences, distinct functions and cellular locations offers a case in point. The comparison between KaiABC, a

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Table 1. Phosphorylation sites and levels in KaiC mutant enzymes.

residue	T432	S431	T432	T431	S432	S431	S432	T431	T432	S431	S426
phosphorylated	6	4	6	—	6	6	6	—	6	—	—
non-phosphorylated	—	2	—	6	—	—	—	6	—	6	6

nano-machine in cyanobacteria that keeps track of time (figure 7*a*), and human Ca^{2+}/calmodulin-dependent kinase II (CaMKII, figure 7*b*), a dodecameric complex of multi-domain subunits that is involved in synaptic and behavioural memory [67] also shows that the two share quite a few traits. For one, both use atypical folds for kinase function—oligomeric assemblies that allow cooperative effects, fluctuations in the interactions at subunit interfaces and *trans*-phosphorylation. Both KaiC and CaMKII catalyse auto-phosphorylation and the dephosphorylation reactions proceed through a reversal of auto-phosphorylation with synthesis of ATP [24,68]. The use of ATPγS for structural and functional assays demonstrated in both cases that the analogue does not affect the forward reaction but that phosphorothioate-threonine and -serine can no longer be dephosphorylated. CaMKII forms hexamers of dimers, with the hub domains on the inside, establishing the core, and the kinase domains on the outside (figure 7*b*). In the double ring composed of hub domains, the vertical dimer is more stable than the equatorial one. This is not unlike the architecture of the KaiC hexamer, although CI and CII in the clock enzyme form a covalently linked and serially arranged 'dimer' that arises from a gene duplication. However, the KaiCI ring can be considered a hub because separate CI domains form a stable hexamer; conversely separate CII kinase domains do not hexamerize. *Trans*-phosphorylation in KaiC involves the two primary sites, T432 and S431, and a secondary site, T426, and KaiA acts as an activator. In CaMKII, the initial phosphorylation concerns T286 in the regulatory domain and requires activation by Ca^{2+}/calmodulin; subsequently, two further threonines, T305 and T306, undergo phosphorylation [47] (figure 7*b*). The activated form of CaMKII shows spontaneous subunit exchange as binding of a peptide portion to the hub as a result of phosphorylation causes subunits of the hub ring to fall apart. Also the deletion of the kinase domain results in subunit exchange [48]. Eventually, fluctuations in the lateral interfaces between vertical hub dimers open up a gap and this allows capture of a free dimer unit and expansion from dodecameric CaMKII to a tetradecameric form (figure 7*b*). KaiC subunit exchange is also a key feature of the daily cycle in the KaiABC circadian clock. KaiC molecules alone swap subunits as well and as in the case of CaMKII, experiments with fluorescently labelled complexes were used to establish this principle [44]. However, in the full *S. elongatus* PTO, subunit exchange sets in during the dephosphorylation phase when KaiB is associated with KaiC (figure 7*a*). Unlike in the case of CaMKII, the KaiC hexamer does not expand to a heptameric particle upon subunit exchange; it seems that monomeric units join pentameric complexes that have released a subunit. That both CaMKII and KaiABC rely on subunit exchange is possibly the most remarkable shared property of the two systems. Mathematical models demonstrated that prevention of subunit exchange in the clock system leads to rapid attenuation of the amplitude of the oscillation and loss of function [44,45]. Phase-dependent exchange of subunits allows individual KaiC hexamers to maintain similar levels of phosphorylation and thus provides a mechanism of clock synchronization. In CaMKII that is a likely candidate for a memory-storage molecule, subunit exchange allows passing on an activated state from an activated complex to an unactivated one. In this fashion, a level of activation can be maintained in a CaMKII holoenzyme even when some subunits are degraded and certainly long after the initial activation signal has disappeared [48].

8. Conclusion

One of the key points, I tried to convey in this review is the crucial role structural insights have played for a better understanding of the KaiABC clock. Apart from obvious observations such as fold, active-site configuration (i.e. ATP binding) and phosphorylation sites, the KaiC structure revealed upfront important differences between the KaiCI and KaiCII rings that would, however, not necessarily have been obvious to the observer in terms of their meaning. Thus, a simple superimposition between the two hexamers exposes three regions of notable conformational and/or dynamic variance: the KaiCII A-loop and tail, the KaiCI B-loop, and misaligned α8 helices in CI and CII (figure 4). These three regions turn out to play key roles in clock protein–protein interactions (A-/B-loop), regulation (A-/B-loop) and ATP processing (α8 and preceding loop and helix). An examination of the differences in the primary sequences of the CI and CII halves would also have led one to the CII-terminal A-loop and tail as well as the CI B-loop regions, but there is no arguing as to which approach—sequence alignment versus three-dimensional structure and dynamics—is more informative.

Although we have gained a much better understanding of the KaiA–KaiC and KaiB–KaiC interactions over the last decade, much more remains to be learnt in terms of the activation of KaiC phosphorylation by KaiA (single molecule pulling experiments could tell us more about the forces involved and cooperative effects among subunits) and the precise role(s) played by KaiB in the PTO and in linking the PTO to the TTFL. There also exists a void in terms of getting a better grip on the molecular underpinnings of and specific involvement of individual amino acids and regulatory motifs in temperature compensation. Not only are the reactions catalysed by KaiC subjects to temperature compensation, but the entire PTO and, therefore, the various protein–protein interactions that take place over the daily cycle also have to be temperature-compensated. As far as KaiC is concerned, a detailed mutational analysis combined with *in vitro* or

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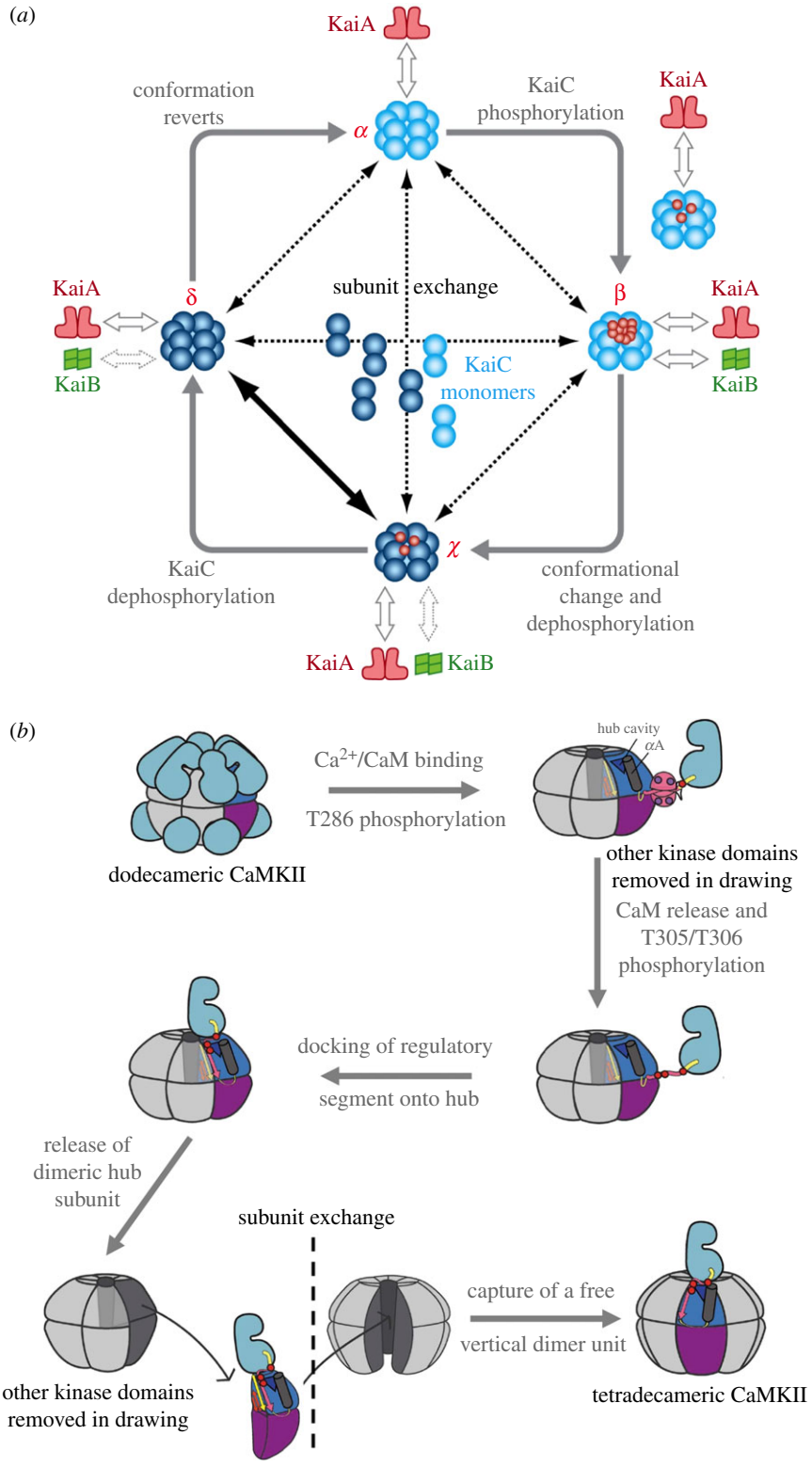

Figure 7. Subunit exchange in a hexameric molecular complex that measures time, the cyanobacterial KaiABC circadian clock, and in a dodecameric molecular complex that is critical for memory formation, human CaMKII. (*a*) Diagram depicting the daily clock cycle controlled by KaiA, KaiB and KaiC and featuring phosphorylation and dephosphorylation of the KaiC homo-hexamer [44,45]. Cyan and blue dumbbell shapes represent two conformational states of KaiC subunits/monomers in the phosphorylated and non-phosphorylated forms, respectively. Phosphate groups at the T432 and S431 sites of KaiC are red dots. A subpopulation of KaiC hexamers associates with and dissociates from KaiA and KaiB during the 24-h cycle. Double-headed arrows in the centre indicate subunit exchange between KaiC hexamers. Rates of subunit exchange vary among KaiC states: a solid line indicates a high rate and a dashed line indicates a low rate. (*b*) Diagram depicting a potential mechanism of subunit exchange in CaMKII [48]. The sequence starts with an auto-inhibited dodecamer CaMKII holoenzyme in a compact conformation. The hub domain is grey, the kinase domain is blue, the regulatory segment is yellow, the CaM footprint is red, and upper and lower hub domains of one vertical dimer are coloured blue and purple, respectively. In the subsequent drawings, only the kinase of a single subunit is shown. CaMKII is auto-phosphorylated at T286 in the regulatory element. After CaM dissociates, T305 and T306 are phosphorylated if T286 is already phosphorylated. The regulatory segment is then released and can bind the available β-sheet of its own hub domain. As a result of binding by the regulatory domain, the lateral association between hub domain dimers is weakened, opening a crack between adjacent hub domains and causing release of a domain dimer from the dodecameric holoenzyme. Owing to fluctuations in the conformation of the auto-inhibited form of the CaMKII holoenzyme lateral interactions are weakened, thus allowing re-entry of the domain dimer into a dodecamer with formation of a CaMKII tetradecamer. The panels are adapted versions of illustrations published in [45] (KaiABC) and [48] (CaMKII).

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in vivo measurements of temperature-dependent changes in the period as a consequence of mutation could pinpoint key residues and key regions in this regard.

Finally, the emergence of common principles in molecular machines that keep track of time and play a role in memory formation is particularly exciting. Subunit exchange has been studied to some degree in the KaiABC clock and in greater structural and mechanistic detail in CaMKII. In the former, more remains to be learnt about how KaiB affects subunit exchange. There is some evidence from cryo-EM and negative-stain EM in combination with gold labelling that KaiB monomers congregate at some point in the daily cycle (KaiB–KaiC complex) along the KaiCII rim [69,70], thereby potentially covering ATP-binding clefts and serving KaiA sequestration and initiation of the dephosphorylation stage. However, in CaMKII subunit exchange is triggered by destabilization of lateral interactions between hub domains. The KaiCI ring constitutes the hub in KaiC and destabilizing interactions among CI domains would be expected to be the best way to induce subunit exchange and degradation of the KaiABC particle. There is good support for KaiB binding to KaiCI and interactions between KaiB and KaiCI in the monomeric state [55]. However, it is worth mentioning that the above data in support of the CII binding mode by KaiB were obtained using wild-type proteins, whereas the studies supporting a CI binding mode used B and CI proteins that both contained mutations (reviewed in [30]). Neither the structures of complexes composed of monomeric species nor those of KaiCI hexamers in different states (e.g. with ADP or ATP bound) can enlighten us as to the changes at the CI/CII interface in the KaiC homo-hexamer. Moreover, crystal structures of KaiC have all trapped the hexamer in the hyper- or near hyper-phosphorylated state, thereby limiting our understanding of the detailed conformational changes and cooperative effects that a hexamer undergoes as it transitions over the daily cycle from the hypo- to the hyper- and back to the hypo-phosphorylated state before coming apart. Thus, many more questions exist regarding the detailed mechanism of the KaiABC cyanobacterial circadian clock.

Competing interests. The author declares that he has no competing interests.

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