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Protein−Protein Interactions in the Cyanobacterial Circadian Clock: Structure of KaiA Dimer in Complex with C-Terminal KaiC Peptides at 2.8 Å Resolution

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Supporting Information

ABSTRACT: In the cyanobacterial circadian clock, the KaiA, -B, and -C proteins with ATP constitute a post-translational oscillator. KaiA stimulates the KaiC autokinase, and KaiB antagonizes KaiA action. KaiA contacts the intrinsically disordered C-terminal regions of KaiC hexamer to promote phosphorylation across subunit interfaces. The crystal structure of KaiA dimer from *Synechococcus elongatus* with two KaiC C-terminal 20mer peptides bound reveals that the latter adopt an α-helical conformation and contact KaiA α-helical bundles via mostly hydrophobic interactions. This complex and the crystal structure of KaiC hexamer with truncated C-terminal tails can be fit into the electron microscopy (EM) density of the KaiA:KaiC complex. The hybrid model helps rationalize clock phenotypes of KaiA and KaiC mutants.

The discoveries that the circadian rhythm of KaiC phosphorylation in the cyanobacterium *Synechococcus elongatus* proceeds in the absence of transcription−translation feedback[1] and that the clock can be reconstituted *in vitro* from the KaiA, KaiB, and KaiC proteins in the presence of ATP[2] allowed detailed biochemical and biophysical investigations of this molecular timer.[3−6] Post-transcriptional and -translational regulatory mechanisms have also been identified in clocks of mammals and other eukaryotes (ref 7 and references cited therein).

KaiC undergoes phosphorylation and dephosphorylation at two sites, Thr-432 and Ser-431,[8,9] in strict order[10,11] and with a period of ∼24 h. In addition to phosphorylation [autokinase in the C-terminal hexameric CII ring (Figure S1)] and autodephosphorylation (CII ATP synthase[12,13]), KaiC also displays ATPase activity in the CI and CII rings.[14] The KaiC phosphorylation level is a marker for clock phase and regulates its activities and nanocomplex formation with KaiA and KaiB.[6] Three-dimensional (3D) structures for all three proteins have been determined and revealed a KaiC hexamer, a domain-swapped KaiA dimer, and a dimer of dimers for KaiB.[15]

Kai hexamer displays the shape of a double doughnut with N-terminal CI and C-terminal CII rings formed by gene-duplicated domains, whereby the N-terminal peptides of CII subunits link domains on the outer surface of the barrel (Figure S1). Six ATP molecules are bound at subunit interfaces in both CI and CII, and C-terminal peptides protruding from the dome-shaped CII surface constitute an intrinsically disordered region (IDR). These peptides are contacted by KaiA dimer,[16,17] and this interaction is key to stimulation of KaiC phosphorylation by KaiA.[18,19] However, the lack of high-resolution structures of full-length KaiA:KaiC[17] and KaiB:KaiC[20] complexes has precluded a full understanding of the mechanisms of KaiC kinase promotion by KaiA[21,22] and KaiB's antagonistic role,[23] respectively.[24]

To visualize the interaction between C-terminal KaiC peptide and KaiA, we determined the crystal structure of full-length *S. elongatus* KaiA homodimer (amino acids V1−T284/His_6) in complex with two KaiC 20mer peptides (D500−S519) NH_3^+-DEKSELSRIVRGVQEKGPES-[K-5-FITC]-COO^- (Figure 1).

Figure 1. Sequence alignment of C-terminal portions of KaiC proteins from selected cyanobacterial strains. The sequence alignment was generated with CLUSTAL Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/)[25] and modified manually in UCSF Chimera.[26]

The KaiC peptide carried a C-terminal lysine that was modified with fluorescein 5-isothiocyanate (5-FITC), i.e., the primary amino group of the lysine side chain reacts with the isothiocyanate group, N═C═S, of 5-FITC under formation of a thiourea moiety. The labeled peptide has a yellow tint, which allows for facile monitoring of the formation of complex crystals with the KaiA protein (Figure S2). Crystals of the complex were obtained

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by soaking the peptide into KaiA crystals or by cocrystallization of KaiA with KaiC peptide. The latter crystals were of better quality and were used for the determination of the structure described here. The structure was phased by molecular replacement using the full-length KaiA dimer as the search model and refined to 2.8 Å resolution (see the Supporting Information for details and Table 1 for crystal and refinement parameters).

Table 1. KaiA:KaiC Complex Crystal Structure Statistics

Crystal Data	
space group	$P4_32_12$
unit cell constants ($a = b$, c) (Å)	97.43, 124.52
resolution (Å) (last shell)	46.2−2.82 (2.89−2.82)
no. of unique reflections (last shell)	15075 (1082)
completeness (%) (last shell)	100 (100)
R_{merge} [a] (last shell)	0.110 (0.805)
$I/\sigma(I)$ (last shell)	37.1 (4.7)
Refinement	
no. of working set reflections	15075
no. of test set reflections	732 (4.9%)
no. of protein non-H atoms	5139
no. of water molecules	108
R_{work}, [b] R_{free} [c]	0.239, 0.298
average B factor (Å^2)	
protein (all residues)	57.3
solvent	54.3
root-mean-square deviation	
bond lengths (Å)	0.01
bond angles (deg)	1.2
Ramachandran analysis (%)	
favored	91
allowed	6
outliers	2

[a] $R_{merge} = \sum_{hkl}\sum_{j=1,N}|\langle I_{hkl}\rangle - I_{hkl,j}|/\sum_{hkl}\sum_{j=1,N}|I_{hkl,j}|$, where the outer sum ($hkl$) is taken over the unique reflections. [b] $R_{work} = \sum_{hkl}||F_{ohkl}| - k|F_{chkl}||/\sum_{hkl}|F_{ohkl}|$, where $|F_{ohkl}|$ and $|F_{chkl}|$ are the observed and calculated structure factor amplitudes, respectively. [c] $R_{free} = R_{work}$ for the set of reflections omitted from the refinement process.

The electron density permits visualization of amino acids D500−K515 from one KaiC peptide (G chain) and amino acids D500−S519 from the other (H chain) (Figure 2; KaiA subunit chains are termed A and B). The five C-terminal residues of peptide G, including the added lysine with 5-FITC, are disordered. For peptide H, only that C-terminal lysine is invisible.

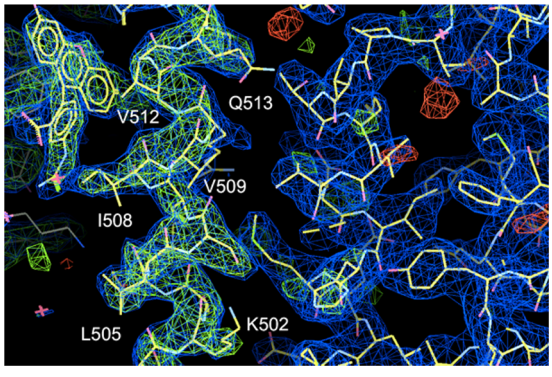

Figure 2. Omit ($2F_o - F_c$) Fourier electron density drawn at the 1σ level around the KaiC peptide (H chain; K502 is at the bottom and Q513 at the top) and one of the 5-FITC molecules (top left).

The electron density also showed two 5-FITC molecules, and their locations [adjacent to V512.G/H and thus at some distance from the C-termini (Figure 2)] suggest that the fluorescent probe had undergone partial hydrolysis (during crystallization, as matrix-assisted laser desorption ionization time of flight showed the stored peptide to be intact). However, FITCs do not mediate interactions between KaiC peptides and KaiA dimer and can be ignored in terms of the analysis of the KaiC peptide conformation and the KaiA:KaiC binding interface.

KaiC peptides bind on opposite sides of the KaiA dimer, and adjacent to the dimer interface formed by two helices from C-terminal α-helical bundle domains of KaiA subunits (Figure 3).

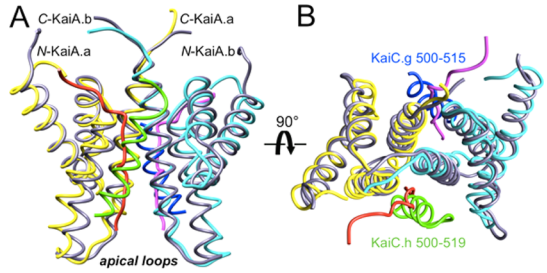

Figure 3. KaiA dimer (C-terminal domains of subunits in the crystal structure colored yellow and cyan) in complex with KaiC peptides (green, H chain; blue, G chain), viewed (A) perpendicular to the noncrystallographic dyad and (B) rotated by 90° around the horizontal. The NMR solution structure of C-KaiA dimer (gray) in complex with the corresponding portions of KaiC peptides (red and magenta; the complex exhibits precise 2-fold rotational symmetry)[16] is superimposed.

The stretch comprising D500−K515 in both peptides adopts a regular α-helical conformation, and the four C-terminal residues of the H chain end in a curl (Figure 3A). The distance between Cα atoms of D500 and S519 is just above 28 Å, and the length of the KaiC peptide thus matches the height of the KaiA C-terminal α-helical bundles minus the apical loop. Thus, C-terminal KaiC peptides contact almost exclusively C-terminal domains of KaiA dimer. The closest contacts between KaiC peptide (H chain) and a residue from the KaiA N-terminal domain (R127.A) are formed Oγ of S519 (4.4 Å) and the C═O group of P517 [3.2 Å; the primary contact of R127 is to E124 (Figure S3)].

The ordered α-helical KaiC peptides in the complex differ drastically from the dynamic and partially disordered random coil conformations of C-terminal tentacles in the crystal structure of free KaiC hexamer (Figure S1).[17] The isolated KaiC peptide forms a random coil (Figure S4). The switch to the α-helix seems to be induced by binding to KaiA and allows for a facile binding mode with KaiA α-helical bundle domains via a coiled coil motif. Indeed, each peptide forms a parallel, right-handed coiled coil with a portion of the long KaiA helix comprising residues E255−S279 from one subunit (Figure 3B, KaiC.H:KaiA.B). Further interactions are established with residues V230−R249 of the antiparallel helix from the other KaiA subunit (KaiC.H:KaiA.A).

Perhaps not surprisingly, the KaiC peptide does not feature the heptad repeat sequence motif hpphcpc (a-f; h = hydrophobic, p = polar, c = charged amino acid) characteristic of coiled coils,[27,28] as the same stretch in free KaiC forms an IDR. KaiC C-terminal peptides evidently fluctuate through a range of conformations, but docking to KaiA may organize the region from at least D500 to K515 into an α-helix. The KaiC sequence in this region displays a fairly high degree of conservation across proteins from different cyanobacterial strains (Figure 1). No fewer than 13 of the 20 C-

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terminal residues are charged or polar in the protein from *S. elongatus*, and just four are hydrophobic: L505, I508, V509, and V512. Beyond residue 515, the sequence variations increase, and KaiC proteins exhibit a range of lengths. This is consistent with the KaiA:KaiC peptide interface in the crystal structure of the complex: among amino acids 515−519, only P517 (via C═O) interacts with KaiA [R127.A NH2, 3.2 Å (Figure S3)]. The relatively low content of bulky hydrophobic amino acids is not untypical for a disordered region, especially considering the solvent-exposed C-terminal location.[29−31]

In the α-helical segment encompassing residues 500−515, the only charged amino acid to engage in an interaction with KaiA is R510 [to E235 (Figures 4 and 5)]. However, R510 is exposed to

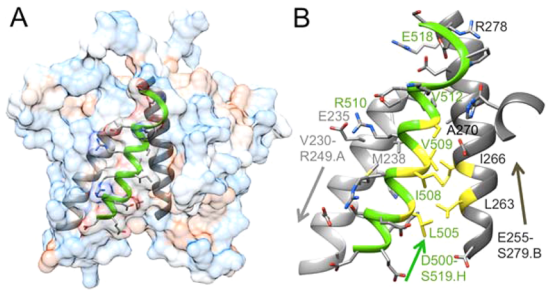

Figure 4. Coiled coil interaction between KaiC peptide (H chain, green) and KaiA dimer (only C-terminal domains are shown). (A) Surface model of $KaiA_2$ colored according to hydrophobicity (pink, most hydrophobic, light blue, least hydrophobic). (B) Three-stranded coiled coil between KaiA (gray) and KaiC α-helices (green).

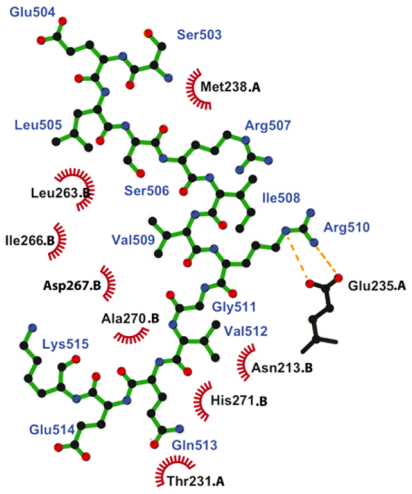

Figure 5. Ligplot[32] analysis of interactions of KaiC peptide (H chain, residues S503−E514) with $KaiA_2$. Peptide bonds are colored green and KaiA bonds black. H-Bonds are shown as dashed orange lines, and hydrophobic contacts are indicated with spoked red arcs.

solvent, as are D500, E501, S503, E504, R507, E514, and K515. In addition, the side chains of K502 and S506 establish a somewhat long H-bond (3.5 Å) in the core but appear not to form any electrostatic interactions with KaiA. This leaves only hydrophobic KaiC amino acids L505, I508, V509, V512, and Q513, the Cβ and Cγ methylene groups of the latter, as mediators of stabilizing interactions with KaiA residues. Indeed, all of these take part in the KaiA:KaiC interface (Figure 4, side chains colored yellow, and Figure 5), consistent with the nature of stabilizing contributions in coiled coils and basic principles of protein−protein interactions (i.e., the hydrophobic effect dominates).[33,34] The experimentally determined K_D for the KaiA:KaiC complex (full-length proteins) was as low as 0.2 μM (native PAGE, AMPPnP) and as high as 1.3 μM (IAsys biosensor, ATP).[35] There is thus no need to expect an extensive buried surface, and the structure of the complex between KaiC peptides and the KaiA dimer is in line with the transient nature of the interaction in the clock cycle.[4,5,15]

No high-resolution structure of the complex between the full-length KaiA and KaiC proteins is currently available. However, the crystal structures of KaiC hexamer[15,17] and the KaiA dimer in complex with KaiC C-terminal peptides can be fused readily (Figure 6A). The distances between Cα atoms from correspond-

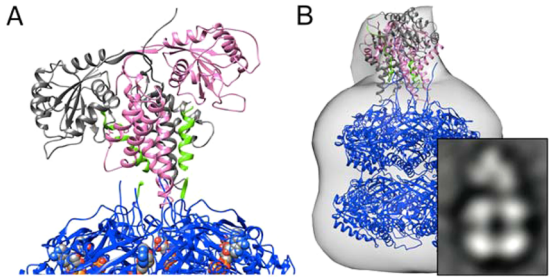

Figure 6. Model of the 3D structure of the KaiA:KaiC complex. (A) Crystal structure of the complex between *S. elongatus* KaiA dimer (subunits colored gray and pink) and KaiC C-terminal peptides (green), described here, combined with the crystal structure of KaiC hexamer[15,17] (Protein Data Bank entry 3DVL, blue ribbon). C-Termini of KaiC in the model were truncated at V499, and a slight gap was left between valines from A and C subunits and D500 residues from G and H peptide chains in the KaiA:KaiC-peptide complex. (B) Combined complex from panel A, fit into the electron density based on the negative-stain EM structure of the *S. elongatus* KaiA:KaiC complex.[17] The inset shows an example of an EM class sum image of the $KaiA_2$:$KaiC_6$ complex.

ing residues in KaiC subunits at the locations where C-terminal tails protrude from the hexameric barrel (I497) range from ~10 to 22 Å (adjacent, i.e., A/B, B/C, etc., and opposing, i.e., A/D, B/E, etc., subunits, respectively). The distance between Cα atoms of D500 residues in the G and H chains of the KaiA:KaiC-peptide complex is 28 Å. Because KaiC peptides contact opposing sides of the KaiA dimer (Figure 2), it is unlikely that the peptides capturing KaiA are from adjacent KaiC subunits. We joined the two structures by roughly aligning the N-termini (D500) of the G and H chains in the peptide complex and the C-termini (V499) of the A and C chains of a truncated KaiC hexamer (Figure 6A). Although some adjustments are necessary to fuse the chains, these appear to be rather minor and no large-scale conformational changes need to be envisioned. The combined model of the complex fits nicely into the low-resolution density (<20 Å) derived from negative stain electron microscopy (EM)[17] (Figure 6B). The 3D model of the KaiA:KaiC complex based on the two fused crystal structures is consistent with the appearance of the complex particle in EM class sum images (Figure 6). Further, the 1:1 $KaiA_2$:$KaiC_6$ complex is supported by biochemical experiments, and a single KaiA dimer was found to be sufficient for converting KaiC to the hyperphosphorylated form.[35]

The conformation of KaiC peptides in the crystal structure of the complex with full-length KaiA dimer described here is completely different from the conformation of the corresponding portion in a longer C-terminal peptide that also contains the adjacent A-loop residues E487−V497 (Figure 1), in complex with the C-terminal domain of KaiA from *Thermosynechococcus elongatus* BP-1[16] (Figure 2). The structure was derived by solution NMR, and KaiC peptides adopt a random coil

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conformation with a sharp turn between the stretched E487−I497 region and the portion corresponding to D500−E518 [Figure S5; KaiC from *T. elongatus* is one residue shorter than the protein from *S. elongatus* (see Figure 1)]. In fact, the conformation of the *T. elongatus* I497−E518 peptide in the solution structure of the complex closely resembles that of the corresponding region in subunit A in the crystal structure of free KaiC hexamer that was tracked to full length[17] (Figure S6). It is unlikely that both models of the KaiA:KaiC-peptide complex are correct. On one hand, there are differences in the constructs used and the methods applied: NMR, solution, *T. elongatus* C-terminal KaiA domain, and KaiC 35mer peptide versus X-ray, crystal, *S. elongatus* full-length KaiA, and KaiC 20mer peptide. On the other hand, the KaiABC clocks from *S. elongatus*[2] and *T. elongatus*[36] can both be reconstituted *in vitro*, and they form a PTO. One would expect matching structures to underlie the same function in closely related strains. However, deviating KaiC conformations result in different interactions with KaiA dimer (Figure 5, Figure S7, and Table S1). Further, the NMR KaiA:KaiC peptide complex cannot be fused with a single KaiC hexamer as N-termini of KaiC peptides point in opposite directions (Figure S5).

In summary, the crystal structure of the KaiA:KaiC peptide complex reveals that C-terminal IDRs of KaiC bound to KaiA dimer adopt an α-helical conformation. The interaction is predominantly stabilized by hydrophobic contributions, and this complex and the structure of KaiC hexamer can be almost seamlessly combined to generate a model of the full-length KaiA:KaiC complex. Docking of KaiA to KaiC peptides will likely destabilize the underlying A-loops (E487−I497 region), but not necessarily require a complete unravelling of the loops,[16,18] to boost KaiC phosphorylation via a concerted allosteric mechanism.[19] The model of the KaiA:KaiC complex derived here is fully consistent with the consequences of site-directed mutagenesis. Thus, deletion of the KaiC C-terminal tails (Δ25 mutant) abolishes clock function,[17] and mutations in the KaiA apical loop region (located close to the KaiCII dome surface) result in severely distorted clock periods (Figure S8).[17]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00694.

Methods and materials, Figures S1−S8, Table S1 (PDF).

Accession Codes

The Protein Data Bank entry for the complex is 5C5E.

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Notes

The authors declare no competing financial interest.

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