Crystal Structure of Circadian Clock Protein KaiA from *Synechococcus elongatus*

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The circadian clock found in *Synechococcus elongatus*, the most ancient circadian clock, is regulated by the interaction of three proteins, KaiA, KaiB, and KaiC. While the precise function of these proteins remains unclear, KaiA has been shown to be a positive regulator of the expression of KaiB and KaiC. The 2.0-Å structure of KaiA of *S. elongatus* reported here shows that the protein is composed of two independently folded domains connected by a linker. The NH2-terminal pseudo-receiver domain has a similar fold with that of bacterial response regulators, whereas the COOH-terminal four-helix bundle domain is novel and forms the interface of the 2-fold-related homodimer. The COOH-terminal four-helix bundle domain has been shown to contain the KaiC binding site. The structure suggests that the KaiB binding site is covered in the dimer interface of the KaiA “closed” conformation, observed in the crystal structure, which suggests an allosteric regulation mechanism.

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Circadian timekeeping systems are present in virtually all eukaryotic organisms and modulate diverse physiological processes ranging from leaf movement to transcription regulation (1, 2) with a ~24-h periodicity. Important mechanistic similarities, such as positive and negative transcription-translation feedback loops, are shared between all circadian clocks studied thus far (3). The evolutionary importance of an endogenous circadian oscillator is further emphasized by its presence in ancient organisms such as cyanobacteria (4), the subject of this work. Indeed, it has been shown that a circadian oscillator tuned to the light-dark cycle period increases the fitness of the organism (5). The cyanobacterial clock is the simplest and the oldest (6) identified thus far, composed of at least three interacting clock proteins, KaiA, KaiB and KaiC (4, 7), that form heteromultimeric complexes in vivo of sizes that oscillate in a circadian manner (8).

Genomic analysis indicates that KaiC is a member of the bacterial RecA/DnaB family (9). The KaiC phosphorylation state has been shown to change in *vivo* with a circadian pattern (10, 11), and it is known that ATP binding by KaiC promotes formation of a hexameric KaiC particle (12, 13), which is presumed to be the functional form of the protein. Experimental evidence suggests that KaiC binds forked DNA when in the ATP-dependent hexameric form (13). KaiA, the positive element of the cyanobacterial circadian oscillator, activates KaiC autophosphorylation and/or inhibits KaiC dephosphorylation, which is believed to be important for its circadian clock function (10, 14). KaiB alone does not affect KaiC autophosphorylation *in vitro*, but it antagonizes the action of KaiA both *in vivo* and *in vitro* (11, 14, 15).

Iwasaki et al. (7) demonstrated that Kai proteins directly associate in all possible combinations with data from a two-hybrid system, *in vitro*, and in cyanobacterial cells. The association between KaiA and KaiB is weak but can be dramatically enhanced by KaiC (7). A KaiA long period mutation allele (E103K, kaiA1) was reported to dramatically enhance the KaiA-KaiB interaction *in vitro* but without any effect on KaiA-KaiC interaction (7). More interestingly, KaiC failed to enhance the kaiA1-KaiB interaction (7).

KaiA is composed of two domains, both solution structures of which were recently solved (14, 16). The structure of the NH2-terminal domain of KaiA is that of a pseudo-receiver domain (14), similar to those found in bacterial response regulators (17). Although the fold is that of a canonical receiver domain, the primary sequence is dissimilar, and it lacks the conserved aspartate residue necessary for phosphorylation. The solution structure of KaiA COOH-terminal domain showed a novel all α-helical homodimeric fold (16). Our earlier results show that the COOH-terminal domain of KaiA possesses a groove, which likely binds to the linker region between the CI and CII domains of KaiC. This interaction between KaiA and KaiC enhances the autokinase activity of KaiC.

Here, we present the structure of full-length KaiA, which was determined by x-ray crystallography to a resolution of 2.0 Å. The full-length structure provides insight into the mechanism of KaiA regulation.

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**EXPERIMENTAL PROCEDURES**

Expression, Purification, and Crystallization—The gene corresponding to *Synechococcus elongatus* KaiA was subcloned in a pET32a+ (Novagen) expression vector, and protein was overexpressed in *Escherichia coli* BL21(DE3) by making the solution 1 mM in isopropyl-β-d-thiogalactopyranoside. Cell lysates were prepared by French press in a buffer containing 200 mM potassium chloride, 20 mM Tris-HCl, pH 7.0. The protein was purified by metal affinity chromatography, cleaved by...
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enterokinase (Novagen), and further purified by metal affinity chromatography, two steps of ion exchange chromatography and gel filtration. During the last gel filtration step KaiA was exchanged to the final buffer of 20 mM sodium chloride, 20 mM HEPES, pH 7.0. The protein was concentrated to 10–15 mg/ml by stirred ultrafiltration. Protein concentration was determined by absorbance at 280 nm, using a corrected extinction coefficient as suggested by Pace et al. (18). KaiA, as prepared, has the following leading NH2-terminal sequence AMADIV followed by the wild-type sequence starting on residue Leu2 from our overexpression system.

The protein was crystallized in Micro Batch plates at 20 °C under AFs oil (Hampton Research) with a 4-μl sitting droplet consisting of a 1:1 mixture of the stock protein solution and a solution containing 130–180 mM ammonium sulfate, 85 mM sodium cacodylate, pH 6.5, 19–22% polyethylene glycol 8000, and 15% v/v glycerol. Thick plate shaped crystals appeared in about 2 days and grew to about 0.5 mm in the longest dimension and varying width and thickness. Our attempts to obtain crystals of Se-Met incorporated KaiA were unsuccessful.

X-ray Data Collection and Structure Determination—For x-ray data collection, crystals were flash-frozen at 100 K using paratone as cryoprotectant. KaiA crystals belong to space group P21 with unit cell dimensions of a = 47.1 Å, b = 125.8 Å, c = 56.8 Å and β = 114.9°. Two monomers per asymmetric unit, and a solvent content of 45.6%. Data were collected at Advanced Photon Source (APS) beamlines and processed with HKL2000 programs (19). Several native data sets were collected from crystals that were diffracted to 2.0 Å. For heavy atom derivatization, the native crystals were soaked in varying concentrations of heavy atom solutions made in synthetic mother liquid. Extensive screening of a large number of heavy atom soaked crystals resulted in one useful 2.8-Å Nd derivative. This derivative was obtained by 24-h soaking of crystals grown under the aforementioned conditions in a 1:1 mixture of stock solution (20 mM sodium chloride, 20 mM HEPES, pH 7.0) and a solution containing 170 mM ammonium acetate, 85 mM sodium cacodylate, pH 6.5, 25% polyethylene glycol 8000, 15% v/v glycerol, and 10 mM neodymium acetate.

Molecular replacement trial with the KaiA135N model (14) using the program AMoRe (20) yielded a weak solution for the first molecule with correlation coefficient of 28.8%, but failed in searching the second KaiA135N molecule. A highly redundant data set to 2.8 Å collected at an in-house x-ray generator lacked isomorphism with the existing native data sets. Experimental phases were derived by SAD1 methods using only the Nd derivative. The substructure of Nd in the asymmetric unit was solved with the program SHELXD (21) with only one Nd site. The experimental phases were further improved by solvent flattening using the program DM (20). The resulting phases were too poor to be used for chain tracing and model building. Real space molecular replacement search was performed with the KaiA135N model on the molecular replacement server of the New York Structural Genomics Research Consortium (NYSGRC) (russel.bioc.acem.yu.edu/cgi-bin/nhouse/rotptf/rotptf.cgi). Encouragingly, the top solution from the real space molecular replacement search matched the solution from AMoRe. Therefore, the top two solutions were combined to be a dimer model. The dimer model looked reasonable judged by their packing in the lattice. A molecular replacement search with the dimer model yielded a solution with a correlation coefficient of 33.8%. The experimental phases were further improved by solvent flattening and 2-fold non-crystallographic symmetry averaging averaging using the program DM (20) by assuming the model was correct. Since CD spectra of the KaiA COOH-terminal domain revealed primarily α-helical structures (14), a real space molecular replacement search was performed with several α-helices with different α-helix lengths. The results were confirmed upon electron density map, and six α-helices were added to the model (two of the six α-helices, corresponding to c9 of the two molecules, turned out to be correct).

Model Building and Refinement—Iterative cycles of model building with XiaView (23) using the ARPwARP improved phases figure of merit, Fobs − Fcalc, maps (24) and refinement with the program SHELXL (25) on the 2.0 Å resolution data set were performed for improving the quality of the model. Water molecules were added in the difference electron density maps at positions corresponding to peaks (>3.0 σ) and with appropriate hydrogen bonding geometry. The details of the final refinement parameter are presented in Table I. The figures were prepared using Molscript (25), Bobscript (26), and GLR and rendered by POV-Ray (www povray.org). The atomic coordinates of KaiA have been deposited in the Protein Data Bank with the ID code 1R8J.

RESULTS AND DISCUSSION

Structure Determination and Overall Structure—KaiA with NH2-terminal sequence AMADIV followed by the wild-type sequence starting from residue Leu2 was overexpressed in E. coli BL21 (DE3). The crystals belong to space group P21 with unit cell dimensions of a = 47.1 Å, b = 125.8 Å, c = 56.8 Å, β = 114.9°. The asymmetric unit consists of two chains forming a homodimer. The structure was determined by a combination of SAD phasing, real space molecular replacement, ARP/wARP phase improvement and non-crystallographic symmetry averaging. Five NH2-terminal non-KaiA residues, two COOH-terminal residues, and two loop regions (residues 85–92 and 137–146) are highly flexible and were omitted from the refinement (Table I). The current model, refined at 2.0-Å resolution, has a crystallographic R value of 21.0% and an R free of 27.1%. The model has good geometry (Table I), and 100% of the non-glycine backbone dihedral angles is in the most favored or allowed regions.

KaiA exists as a dimer in the crystal (Fig. 1A), in agreement with the data from gel filtration and ultracentrifugation equilibrium (16) and previous reports that KaiA exists as a homodimer or heteromultimeric protein complexes with other clock components in vivo in a circadian fashion (8). One subunit of KaiA contains two independently folded domains connected by a canonical linker (NH2-terminal pseudo-receiver domain, residues 1–164, canonical linker, residues 165–173, COOH-terminal four-helix bundle domain, residues 174–284). One unique feature of the KaiA dimer is domain swapping (27), in that the domains are swapped so that the NH2-terminal domain of one chain pairs with the COOH-terminal domain of the other. There are no interdomain interactions within a subunit, unlike most other multidomain proteins. The two subunits twist around each other forming a dimer, related by a non-crystallographic 2-fold axis. The dimer interface between the two monomers buries a total of 3440 Å2 accessible surface areas per subunit, about 21% of the total surface of a single subunit, which is on the high side of the range of buried surface area observed in other dimeric proteins (28).

The NH2-terminal portion (residues 1–135) of this domain is structurally similar to the response regulator receiver family, which is involved in a wide range of regulatory processes (29), discussed in detail below. This domain consists of a central five-stranded (β1 to β5) parallel β-sheet flanked by two groups of α-helices (α1, α4 and α2, α3) packed on either side of the β-sheet and an additional α-helix (α5) lying near the amino terminus of the central β-strand (Fig. 1A). The COOH-terminal domain contains two parallel helix-hairpin-helix motifs that form a four-helix bundle, which represents a new protein folding motif (16).

Structure and Function of the NH2-terminal Pseudo-receiver Domain—The portion of the NH2-terminal domain that is structurally similar to the response regulator receiver family was previously studied by NMR (14). The backbone root mean square deviation (r.m.s.d.) between the KaiA crystal structure and KaiA135N solution structure (average minimized structure, Protein Data Bank ID code 1M2E) is 1.31 ± 0.04 Å for residues 4–83 and 98–135. The sequence alignment (Fig. 1B) shows gaps/insertions in two loop regions of the NH2-terminal domain among the four known homologs. Most of the gaps/insertions are in disordered regions of the protein except for those that happen to fall in crystal lattice contacts. The first

1 The abbreviations used are: SAD, single wavelength anomalous dispersion; r.m.s.d., root mean square deviation.

2 L. Esser, personal communication.
disordered loop (residues 84–97) was also observed to be highly dynamic in the solution structure (14). In addition, a cis-peptide (Ala$^{129}$Pro$^{130}$) was observed in the structure and this proline is well conserved in KaiA sequences (Fig. 1B).

The structural similarity between the KaiA NH$_2$-terminal domain and the response regulator receiver family was unanticipated since no amino acid sequence homology was detected prior to solving the structure. All receiver domains contain four highly invariant residues necessary for a common Mg$^{2+}$-dependent phosphoryl-transfer mechanism in the carboxy-terminal crevice of the central $\beta$-strand, which correspond to Asp$^{12}$, Asp$^{52}$, Asp$^{57}$ (phosphate-accepting residue) and Lys$^{109}$ in E. coli CheY (17). We aligned the amino acid sequence of KaiA NH$_2$-terminal domain against the response regulator receiver family (Fig. 2).

Thirty-four KaiA alleles involving mutations to 28 different residues have been reported to alter the periodicity of clock-regulated gene expression rhythms in S. elongatus (4, 31). The alleles are mapped onto the KaiA structure (Fig. 1, B and C) and are distributed throughout the two domains and the canonical linker, with 44.1% of the alleles mapping to the pseudo-receiver domain, 52.9% to the four-helix bundle domain and 3.0% to the canonical linker. The alleles map to residues with structural roles, to residues with roles in dimer interactions, or to residues with potential roles in KaiC interactions, indicating that both domains and the linker are important for the circadian clock function of KaiA.

The alleles mapping to the NH$_2$-terminal pseudo-receiver domain involve mutations of twelve residues. Eight of the twelve residues (Ile$^{9}$, Ile$^{16}$, Leu$^{31}$, Ser$^{36}$, Cys$^{53}$, Val$^{76}$, Glu$^{103}$, and Val$^{121}$) are buried residues. Mutation of these residues resulted in unfolded domains or aggregation (14), reflecting their structural roles. Three residues (Gln$^{113}$, Gln$^{117}$, and Asp$^{119}$) mapped to the $\alpha4$ helix are involved in the dimer interface, as discussed later. The role of Asp$^{136}$ is unclear, since it is in a flexible surface loop.

**Table I**

| Data set       | Nd           | Native         |
|----------------|--------------|----------------|
| Wavelength (Å) | 1.5418       | 0.9792         |
| Resolution (Å) | 2.8          | 2.0            |
| Measured reflections | 79,217    | 140,389        |
| Unique reflections | 15,128     | 38,494         |
| Redundancy      | 5.4          | 3.6            |
| Completeness (%, highest shell) | 99.2 (99.1) | 95.2 (54.3) |
| Mean I/Î of (highest shell) | 6.6 (2.7) | 30.6 (2.7) |
| $R_{sym}$ (%), highest shell | 9.2 (30.0) | 4.0 (36.2) |
| SAD analysis    |              |                |
| Resolution (Å) | 2.8          |                |
| Number of Nd sites | 1          |                |
| Phasing power   | 0.575        |                |
| $R_{sym}$ (anomalous) | 0.930     |                |
| Refinement      |              |                |
| Resolution (Å) |              | 20.0–2.0       |
| No. of reflections |             | 36,552         |
| $R$-factor$R_{calc}$, (%) | 21.0–27.1 |                |
| Missing residues |              | Molecule A: -6 to -5, 87–92, 142–145, 283–284 |
| No. of non-hydrogen atoms |       | 4577           |
| No. of solvent atoms |            | 246            |
| Cis-peptide     |              | Ala$^{129}$–Pro$^{130}$ |
| r.m.s.d. bond lengths (Å) |         | 0.009          |
| r.m.s.d. bond angles (°) |         | 1.1            |
| Ramachandran plot (%) |        | 93.1           |
| Most favored regions |            | 6.9            |
| Additional allowed regions |       | 6.9            |
| Generously allowed regions |       | 0.0            |
| Disallowed regions |              | 0.0            |

TABLE I Statistics from the crystallographic analysis

Details of the crystallization and structure determination see text. $R_{calc} = \Sigma i|F_{o} - F_{c}| / \Sigma i|F_{o}|$ for the intensity (I) of h observations of reflection $h$. Phasing power = ($F_{o} - F_{c}$)/$E$, where ($F_{o}$) is the root mean square heavy atom structure factor, and $E$ is the residual lack of closure error. $R_{calab}$ is the mean residual lack of closure error divided by the dispersive or anomalous difference. $R$-factor = $\Sigma F_{o} - F_{calc}$/$\Sigma F_{o}$, where $F_{o}$ and $F_{calc}$ are the observed and calculated structure factors, respectively. $R_{calc} = R$-factor calculated by using a subset (~5%) of reflection data chosen randomly and omitted throughout refinement. r.m.s.d., root mean square deviations from ideal geometry.
Three alleles were mapped to Phe\textsuperscript{178} and Arg\textsuperscript{180} in this loop, which are well conserved (Fig. 1, B and C). Arg\textsuperscript{180} forms a salt bridge with conserved residue Asp\textsuperscript{227}, and the side chain of Phe\textsuperscript{178} stacks with Pro\textsuperscript{175} and forms hydrophobic interactions with the C\(^\beta\) of Asp\textsuperscript{227} and C\(^\beta\) of Arg\textsuperscript{173} (Fig. 3B). Hence, the role of Phe\textsuperscript{178} and Arg\textsuperscript{180} might be structural.
The two subunits within a KaiA dimer in the crystal interact with each other throughout the dimer interface. We divide the dimer interactions to three regions (Fig. 1A). The first region (dimer interface I) is between the four-helix bundle domains of the two monomers. The two domains pack head-to-head in a 2-fold symmetric manner (Fig. 3A). The interactions are dominated by the coiled-coil interactions along the long helices (a9) involving residues 260–280 from each chain. Additional interactions involve the connecting loop between a7 and a8 from 227–230. The interface is primarily hydrophobic, containing hydrophobic clusters and aromatic stacking interactions including residue Pro229, Val230, Leu263, Leu265, Ile266, Ile269, Ala270, Leu272, Cys273, Tyr276 and Ile280 from both molecules. The hydrophobic interactions appear to be the main force driving dimer formation in this interface. The packing is further stabilized by three salt bridges, including AspA260–ArgB262, AspA227–ArgB277 and AspB227–ArgA277, and four hydrogen bonds, including that between Oa2 of the carboxylate side chain of E274 from one molecule and the backbone amide of Val230 from another molecule, and the carbonyl of Asp227 from one molecule and the NH1 of Arg277 from another molecule. The hydrophobic interactions appear to be the main force driving dimer formation in this interface. The packing is further stabilized by three salt bridges, including AspA260–ArgB262, AspA227–ArgB277 and AspB227–ArgA277, and four hydrogen bonds, including that between Oe2 of the carboxylic acid side chain of E274 from one molecule and the backbone amide of Val230 from another molecule, and the carbonyl of Asp227 from one molecule and the NH1 of Arg277 from another molecule. Interestingly, a water molecule (Wat168) forms a mediate contact between AspA260 and ArgA262, asymmetric to the salt bridge between AspA260 and ArgA262 (Fig. 3A). The dimer interface I buries a total of about 990 Å² surface areas per subunit. Three alleles were mapped to Ile266, Cys273, and Glu274 that are involved in this dimer interface (Figs. 1, B and C, and 3A).

The second interface (dimer interface II) forms between the strictly conserved (Fig. 1B) linkers of the two subunits (Fig. 1A). The two linkers adopt an atypical anti-parallel β-strand conformation, zipping with each other through eight main chain hydrogen bonds and two additional hydrogen bonds formed between the Nζ of Lys172 from one molecule and the carbonyl of Arg163 from another molecule. Two hydrogen bonds also form between the Nζ of Lys172 from one molecule and the carbonyl of Val169 from another molecule, which is the last NH2-terminal non-KaiA residue replacing Met1 position (Fig. 3B). Four hydrophobic residues (LeuA167, LeuB167, ValA169, and ValB169) cluster together and are exposed to solvent, forming a hydrophobic surface patch. The COOH terminus conformation is stabilized by three hydrogen bonds forming in same molecule, including two between the side chain hydroxyl of Tyr166 and the carbonyl of Pro281, and one between the side chain hydroxyl of TyrB170 and the Oγ of Ser279. Asymmetrically, the side chain of TyrA170 stacks between ProA281 and ProB281, occupying the non-crystallographic 2-fold axis. Only one allele was mapped to Tyr166 in the canonical linker (Fig. 1B and C). Mutation of this residue (Y166C) causes arrhythmia (31).
The interface between the NH$_2$-terminal pseudo-receiver domain of one molecule and the COOH-terminal four-helix bundle domain of the other molecule in the dimer forms the third interface (Fig. 1). Each interface buries a total of about 860 Å$^2$ of surface area per monomer. β-Strand 5 (β5) and α-helix 4 (α4) from the pseudo-receiver domain, along with α-helix 7 (α7) and the NH$_2$-terminal loop region linking the canonical linker and α-helix 6 (α6) from the four-helix bundle domain, are involved in forming this interface. The interactions in this interface are a mixture of hydrophobic, hydrogen bonding, and salt bridge interactions. Mutations in several residues involved in this dimer interface extend the period of the circadian rhythm (31). These include Gln$^{113}$, Gln$^{117}$, and Asp$^{119}$ from α4 and Phe$^{225}$ from α7. The interactions formed by Gln$^{113}$ in the third interface are asymmetric. Only Gln$^{113}$ forms two hydrogen bonds with Asn$^{214}$. Gln$^{117}$ forms two hydrogen bonds, one with Asn$^{221}$, another with the carbonyl of Asn$^{214}$. Asp$^{119}$ forms a salt bridge with Arg$^{177}$ and an ion-dipole with the side chain hy-
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KaiA allele (R249H, kaiA2) with a mutation at the KaiA COOH-terminal domain was reported with reduced effect of KaiA stimulated KaiC autokinase activity, and this effect can be restored by a KaiC allele (A422T, kaiC15) (10), which has been mapped to one of two KaiA binding domains (C_{KAI2} of KaiC (33). This suggests that Arg^{249} is involved in the direct interaction with KaiC. Residue Arg^{249}, together with the other six residues (Glu^{241}, Met^{241}, Asp^{242}, Glu^{243}, Phe^{244}, and Ala^{245}), cluster on the apical portion of α8 in the four-helix bundle domain. Mutations of these residues exhibit only long period phenotypes (31). Multiple sequence alignment shows that they are either conserved or chemically similar (Fig. 1B).

Structural analysis shows that Met^{241} and Phe^{244} are buried residues, whereas Glu^{239}, Asp^{242}, Glu^{243}, Ala^{245}, and Arg^{249} are well exposed to the solvent (Fig. 1C). The surface formed by these residues is likely involved in the direct association with KaiC.

Although the function of KaiC in the clock apparatus is still unclear, it appears to involve interaction with DNA. Its forked-DNA substrate binding activity was detected by electrophoretic mobility-shift assay (13). The ATP-induced hexameric KaiC ring structure was revealed by electron microscopy (12, 13, 16). Each KaiC subunit in the electron micrographs showed a dumbbell-shaped structure with two domains corresponding to duplicated CI (the first half) and CII (the second half) domains and connected by an ~10-Å narrow linker. Based on electron microscopy data, a KaiA-KaiC interaction model has been proposed (16), in which KaiA binds to the KaiC linker region through the groove formed between the KaiA COOH-terminal domains. Our speculation of the KaiC binding site mapping to this model is consistent with results of previous studies that show that both CI and CII domains of KaiC interact with KaiA.

Potential KaiB Binding Site and an Allosteric Regulation Mechanism—How KaiB antagonizes the action of KaiA is still unclear. It might affect KaiA through direct interaction, or it might interact with KaiC, thus indirectly attenuating the KaiA function. KaiA influence on KaiA-stimulated KaiC phosphorylation was mapped to its COOH-terminal domain, and KaiA COOH-terminal domain (KaiA180C) has a similar effect (2:1:2:6) on KaiC autophosphorylation, compared with that of full-length KaiA (14). KaiB causes a 50% reduction in the KaiA-stimulated KaiC autophosphorylation when the KaiB:KaiA molecular ratio is 40:1 (15) and a 30% reduction when the ratio is 1:1 (14). Interestingly, KaiB caused almost 100% reduction in the KaiA COOH-terminal domain (KaiA180C)-stimulated KaiC autophosphorylation when the KaiB:KaiA180C molecular ratio is 1:1 (14). These data suggest that KaiB directly interacts with KaiA and that the NH_{2}-terminal pseudo-receiver domain of KaiA antagonizes the KaiB effect.

Analysis of the three dimer interfaces reveals distinctive features. First, the dimer interface I is primarily hydrophobic and the interactions are strong. Without the NH_{2}-terminal pseudo-receiver domain and the canonical linker, the COOH-terminal four-helix bundle domains still strongly associate to form a dimer, as observed in the ThKaiA180C solution structure (16). Second, the main driving force in the dimer interface II is eight main chain hydrogen bonds, which are also strong. Third, the dimer interface III is a mixture of hydrophobic, hydrogen bonding, and salt bridge interactions but is primarily hydrophilic and weak, indicating that the NH_{2}-terminal pseudo-receiver domain in one monomer and the COOH-terminal four-helix bundle domain in another monomer might be able to dissociate, exposing the surface between the domains.

We therefore propose an allosteric regulation mechanism, in which there exist two alternative conformations of KaiA: an open and a closed form. What we observed in the crystal structure represents the "closed" conformation. We propose that the...
KaiB binding site is in the KaiA COOH-terminal domain and is covered by the NH2-terminal pseudo-receiver domain from the other KaiA monomer in the closed conformation, forming part of the dimer interface III. The NH2-terminal pseudo-receiver domain allosterically regulates the KaiB binding activity. This would be similar to how the receptor binding site on the insulin surface is covered by the extended B-chain COOH-terminal peptides, which have great mobility (34). Also, an allosteric regulation mechanism has been reported for integrin (35). Furthermore, domain-swapped dimer has been indicated to confer some advantages to allosteric properties, as in bovine seminal RNase (36).

The proposed allosteric regulation mechanism may explain several previous experimental results. KaiA exists as closed conformation in regular physical condition, in which the KaiB binding site is buried in the dimer interface III. This explains the weak KaiA-KaiB interaction. KaiA activates KaiC autophosphorylation through KaiA-KaiC interaction. We propose that the KaiC-phosphorylated form might allosterically induce phosphorylation through KaiA-KaiC interaction. We propose the weak KaiA-KaiB interaction. KaiA activates KaiC auto-

Conclusions—In conclusion, the KaiA structure reported here provides the first complete three-dimensional view of this circadian clock protein. The KaiA monomer is composed of two domains. The NH2-terminal pseudo-receiver domain is structurally similar to the bacterial response regulator receiver domain, whereas the COOH-terminal four-helix bundle domain is a novel fold. It further shows the formation of a homodimer through extensive dimer interface I and of the canonical linker. Analysis of the dimer interface suggests that there exist two alternative conformations. The structure in the crystal structure represents the closed conformation. We have proposed an allosteric regulation mechanism for KaiA-KaiB interaction and time-specific clock protein complex formation. The structure of KaiA reported here is the first step in efforts to use structural studies for elucidation of circadian clock function. It serves as a framework for further studies of the KaiABC system and other clock-related proteins.

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