Tetrameric Architecture of the Circadian Clock Protein KaiB

A NOVEL INTERFACE FOR INTERMOLECULAR INTERACTIONS AND ITS IMPACT ON THE CIRCADIAN RHYTHM* [S]

Kenichi Hitomi‡, Tokitaka Oyama§§, Seungil Han‡, Andrew S. Arvai‡, and Elizabeth D. Getzoff‡

From the Department of Molecular Biology and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, California 92037 and the Department of Biological Science, Graduate School of Science, Nagoya University and Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan

Received for publication, October 4, 2004, and in revised form, January 24, 2005
Published, JBC Papers in Press, February 16, 2005, DOI 10.1074/jbc.M411284200

Cyanobacteria are among the simplest organisms that show daily rhythmicity. Their circadian rhythms consist of the localization, interaction, and accumulation of various proteins, including KaiA, KaiB, KaiC, and SasA. We have determined the 1.9-Å resolution crystallographic structure of the cyanobacterial KaiB clock protein from Synechocystis sp. PCC6803. This homotetrameric structure reveals a novel KaiB interface for protein-protein interaction: the protruding hydrophobic helix-turn-helix motif of one subunit fits into a groove between two β-strands of the adjacent subunit. A cyanobacterial mutant, in which the Asp-Lys salt bridge mediating this tetramer-forming interaction is disrupted by mutation of Asp to Gly, exhibits severely impaired rhythmicity (a short free-running period; ~19 h). The KaiB tetramer forms an open square, with positively charged residues around the perimeter. KaiB is localized on the phospholipid-rich membrane and translocates to the cytosol to interact with the other Kai components, KaiA and KaiC. KaiB antagonizes the action of KaiA on KaiC, and shares a sequence-homologous domain with the SasA kinase. Based on our structure, we discuss functional roles for KaiB in the circadian clock.

In bacteria to humans, daily rhythms endogenously consist of ever-changing protein localization, accumulation, and interactions (for reviews of cyanobacterial clocks, see Refs. 1–5). In cells, proteins involved in the circadian clock interact with other clock components, sometimes self-assemble, and take part in a complex protein network with the right timing, placement, and organization to create and maintain a robust rhythm. Meanwhile, the clock core is adjusted by exogenous factors, e.g. light. In any organism, biological phenomena defined as a circadian clock have three characteristic features: a free running periodicity of about 24 h, phase resetting by environmental cues, and temperature compensation of the period. The proper associations among individual clock proteins are essential, but their interplay with each other can be subtle and difficult to decipher.

Cyanobacteria, the evolutionary predecessors of chloroplasts, are both the simplest photosynthetic cells and the most primitive organisms that maintain a circadian clock. The circadian clock gene cluster, kaiABC, was originally discovered and cloned from the cyanobacterium Synechococcus elongatus PCC7942 (6). Interestingly, kaiB and kaiC homologues of unknown function have been discovered in Archaea and Proteobacteria, but the three kai genes have very different evolutionary histories (7). In S. elongatus PCC7942, however, inactivation of any single kai gene abolishes the circadian rhythms (6). The KaiA, KaiB, and KaiC proteins can associate in all possible combinations, including self-assembly in vitro and in vivo (8). Crystal and solution structures of cyanobacterial KaiA domains reveal a dimer with tight interactions (9–13). In the structure of the full-length KaiA dimer, the two independently folded domains are connected by a canonical linker with domain swapping (11). The two domains of the KaiC protein, which belongs to the family of ATPase/GTPase homologues, stack into a dumbbell shape and assemble into a symmetric double-layered hexameric ring in an ATP-dependent manner (13–15). The histidine kinase designated SasA closely associates with the cyanobacterial clock machinery, and is necessary for maintaining robust circadian rhythms, although the SasA gene lies outside the kaiABC cluster (16). Interestingly, SasA contains a KaiB-like sensory domain that mediates binding of SasA to KaiC.

The Kai and SasA proteins dynamically associate into heteromultimeric protein complexes in a circadian fashion (19). KaiA enhances KaiC phosphorylation and KaiB attenuates KaiA-enhanced KaiC autokinase activity (17, 18). The KaiB function as an attenuator of KaiC phosphorylation requires the presence of KaiA, suggesting that a heteromultimeric KaiABC protein complex would be formed following the KaiA-KaiC interaction. In in vitro studies, the KaiA dimer interaction with the KaiC hexamer was suggested to vary from 1:1 (two molecules of KaiA against six molecules of KaiC) to up to 2:1, depending on ATP hydrolysis by KaiC. It is still unknown how KaiB stoichiometrically interacts with the KaiA-KaiC complex.

* This work was supported in part by grants-in-aid from the Japanese Ministry of Education, Culture, Sports, Science, and Technology 15GS0308 and 15770025 (to T. O.) and National Institutes of Health Grant R01 GM37684 (to E. D. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence may be addressed: Division of Biological Science, Graduate School of Science, Nagoya University, Furu-cho, Chikusa-ku, Nagoya 464-8602, Japan. Tel.: 81-52-789-2507; Fax: 81-52-789-2963; E-mail: oyama@bio.nagoya-u.ac.jp.

‡ To whom correspondence may be addressed: Dept. of Molecular Biology, The Scripps Research Institute, MB4, 10550 North Torrey Pines Rd., La Jolla, CA 92037. Tel.: 858-784-2878; Fax: 858-784-2288; E-mail: edg@scripps.edu.

The atomic coordinates and structure factors (code 1WWJ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

The Journal of Biological Chemistry Vol. 280, No. 19, Issue of May 13, pp. 19127–19135, 2005
Published by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
Recently, a crystal structure of the *Anabaena* KaiB homologue was determined, showing that the KaiB homologue is a dimer in the crystal as are the KaiA structures (12). On the other hand, it was shown *in vivo* that the KaiC-based complex changes in size in response to day and night, accompanied by the night-specific interaction with KaiA and KaiB (19). In the cyanobacterial cell, although the amount of KaiA is constant, KaiB and KaiC exhibit robust circadian rhythms, being the most abundant at the early circadian night phase (circadian time 15–16). The amount of KaiB and KaiC changes rhythmically with the KaiB:KaiC ratio up to 2:1 (17), even though these two genes form an operon (6). Kitayama et al. (17) showed that KaiB protein is localized in both the cytosol and the membrane, suggesting that a KaiB regulatory link between subcellular localization and protein-protein interactions is important in the cyanobacterial clock system.

The KaiB protein is essential to the circadian rhythm of cyanobacteria: rhythmicity disappearing in the *kaiB* null mutant (6). Because most *kaiB* mutants showed a short period phenotype (6), hypomorph mutations are likely to shorten the period. Although genetic evidence shows a key role for KaiB in pacemaking, the biochemistry of KaiB function is poorly understood. Here we report the crystallographic structure of the KaiB tetramer from *Synechocystis* sp. PCC6803, identify interesting structural features, including the (i) positively charged perimeter, (ii) negatively charged center, and (iii) zipper of aromatic rings, and discuss their implications for KaiB function. Furthermore, we demonstrate that a mutation at the tetrameric interface affects the clock, suggesting that the inability to form this newly identified tetrameric complex may cause abnormality in the circadian rhythm.

### Experimental Procedures

#### Expression and Purification of *Synechocystis* sp. PCC6803 KaiB—The *Synechocystis* sp. PCC6803 *kaiB* gene was introduced into a pET11a (Novagen) vector. *Escherichia coli* BL21(DE3) cells transformed by this plasmid were grown at 30 °C and protein expression was induced with 0.2 mM isopropyl-D-thiogalactopyranoside. The cells were disrupted by sonication, and the supernatant was applied to an ion exchange (Poros 20 HQ), cation exchange (Poros HS), and gel filtration (3-200 26/60) columns. The anion and cation exchange columns did not bind the cyanobacterial protein, whereas the other *E. coli*-derived proteins were efficiently removed. The protein yield was 30 mg/liter. Purified KaiB protein was concentrated by Amicon Centriprep (Millipore) to 10 mM in a solution containing 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 0.5 mM EDTA, and stored at −80 °C.

#### Dynamic Light Scattering—Dynamic light scattering studies were performed at 20 °C by using a DynaPro 99 instrument (Protein Solutions, Inc.). Scattering of *Synechocystis* sp. PCC6803 KaiB was analyzed at 10 mW in the storage buffer (20 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 0.5 mM EDTA). The DynaPro 99 instrument measures fluctuations in the intensity of scattered laser light caused by the Brownian motion of molecules in solution. Reported scattering values are the averages of 30 scans, each including measurements at 20 different time points between 3 and 3000 µs. Data were analyzed with DYNAMICS version 5.5.56.38 software (Protein Solutions, Inc.). The scattering data are fitted with an exponential autocorrelation function, which is used to determine the molecular translational diffusion coefficient, \(D_p\), and to assess the polydispersity of the sample (Table I). The radius of hydration, \(R_H\), is then calculated by using the equation \(D_p = kT\eta/(6\pi\eta R_H)\), where \(k\) is the Boltzmann constant, \(T\) is temperature in Kelvin, and \(\eta\) is the solvent viscosity. \(R_H\) is defined as the radius of a hypothetical hard sphere that diffuses with the same speed as the particle under examination. But macromolecules are non-spherical and solvated. Therefore, the molecular weight (\(M_r\)) of a macromolecule is estimated by using \(M_r \) versus \(R_H\) calibration curves developed from standards of known molecular weight and size. Thus, the \(M_r\) estimate of a given particle is subject to error if it deviates from the shape and solvation of the molecules used as standards. The molecular weight for protein macromolecules is estimated from a curve that fits the following \(M_r = [1.6800R_H^{2.3398} + 0.249]^{0.283}\), as implemented in the software. Statistics for dynamic light scattering are summarized in Table I.

#### Crystallization and Diffraction Data Collection—Crystals of the *Synechocystis* sp. PCC6803 KaiB protein were obtained at both 4 °C and room temperature, by the hanging drop method. The 2-microliter drop, containing a 1:1 ratio (\(v/v\)) of protein (10 mM in storage buffer) to reservoir solution (below) was equilibrated against 500 ml of a reservoir solution containing 60–80% saturated betaine and 100 mM imidazole/maleate buffer (pH 6.0). Crystals grown at 4 °C diffraction to higher resolution than those grown at room temperature, giving diffraction data to 1.9-Å resolution data at SSRL Beam Line 7-1. The data were processed by DENTZ/SCALEPACK (20). KaiB crystals belong to space group \(P2_12_12_2\), with unit cell dimensions: \(a = 52.8\) Å, \(b = 66.0\) Å, \(c = 114.8\) Å. Four monomers per asymmetric unit give a 41.3% solvent content.

| TABLE I  | Dynamic light scattering study of *Synechocystis* sp. PCC6803 KaiB |
|----------|---------------------------------------------------------------|
| \(D_p^a\) | \(R_H^b\) | Mass | Polydispersity |
| 646.3 | 3.251 | 53.2 | 9.2 |

\(a\) \(D_p\), translational diffusion coefficient.  
\(b\) \(R_H\), hydrodynamic radius.

| TABLE II | *Synechocystis* sp. KaiB |
|----------|-----------------------------|
| Diffraction data |
| Beamline | SSRL-7-1 |
| Resolution (Å) | 30–1.9 (1.97-1.90)\(^{a}\) |
| Wavelength (Å) | 1.08 |
| Observations | 96,330 |
| Unique reflection | 31,143 (2,882) |
| Completeness (%) | 95.9 (88.9) |
| \(R_{sym}\) | 3.7 (25.1) |
| \(I/\sigma(I)^c\) | 23.9 (3.9) |
| Space group | \(P2_12_12_2\) |
| \(a = 52.8\) Å | \(b = 66.0\) Å |
| \(c = 114.8\) Å | Molecular in asymmetric units | 4 |

\(a\) Values for the highest resolution shell are given in parentheses.  
\(b\) \(R_{sym}\) = the unweighted \(R\) value on \(I\) between symmetry mates.  
\(c\) \(I/\sigma(I)\) is the observed intensity and \(\sigma(I)\) is the standard deviation of \(I\).  
\(d\) \(R = \Sigma_i [F_{\text{calc}} - F_{\text{obs}}]/\Sigma_i F_{\text{calc}}\), the cross-validation \(R\) factor for 5% of reflections against which the model was not refined.  
\(e\) r.m.s.d., root mean square deviation.

*FIG. 1.* Dynamic light scattering study of *Synechocystis* sp. PCC6803 KaiB. The single peak centered at the hydrodynamic radius of 3.25 nm (see also Table I) corresponds to the tetrameric state.
Model Building and Refinement—Phases were obtained by molecular replacement with the AMoRE program package (21). Results with the Anabaena KaiB dimer (Protein Data Bank code 1R5P) search model gave a correlation factor of 54.3% and an R factor of 41.1% in resolution range from 10 to 4 Å. Structure refinement was carried out with CNS (22), with iterations of positional refinement, simulated annealing, and torsion angle dynamics followed by temperature factor (B factor) refinement. The model fitting was done with Xfit (23). Statistics for crystallographic data processing and refinement are summarized in Table II. The coordinates for Synechocystis KaiB are deposited in the RCSB Protein Data Bank under the Protein Data Bank code 1WWJ.

Mutational Analysis: Measurement of Circadian Rhythms of Bioluminescence in S. elongatus PCC7942—Bioluminescence rhythm assays used cyanobacterial strains, culture conditions, and methods described previously (24). NUC42 and NUC43 were used as wild type and ΔkaiABC deletion strains, respectively. The S. elongatus PCC7942 kaiBS

FIG. 2. Overall structure of tetrameric Synechocystis sp. PCC6803 KaiB. A, Synechocystis KaiB, shown as a ribbon diagram, forms flattened (side view) and Kai-like (top view) structure: α-helices, green; β-strands, yellow; coil, pink. B, tetrameric Synechocystis KaiB assembles from two symmetric homodimers. We define the dimer interface as Interface I, and the interface between dimers as Interface II. C, electrostatic potential surface for tetrameric KaiB complex reveals characteristic features: a positively charged rim (side view) and a negatively charged cluster at the center of one Kai face (top and bottom views). Electrostatic potential color code is from red, −2.0 kT/e, to blue, +2.0 kT/e.
A (D90G) mutant was originally isolated from a PCR-based random mutagenesis library at the kaiB locus. The library was constructed by using the strategy described for kaiA mutagenesis by Nishimura et al. (24), except for the restriction enzymes (AflII and HpaI) to target the kaiB locus. Because the original mutant strain was gone, we recreated the mutant construct by PCR-based site-directed mutagenesis. The PCR product was amplified by using the primer set (5′/H11032-AACGACAGTAGAAGTCGTCGGAATCTTGAAGTTCGCCGTAGAGTAAACCAAGGC-3′/H11032(55-mer) and 5′/H11032-GAGGACATTTTGCTGGATTA-3′/H11032(20-mer)), digested with AflII and HpaI and introduced into the pCkaiABC vector from which the kaiB locus had been excised using AflII and HpaI (24).

RESULTS
Overall Tetrameric KaiB Structure—We obtained our best diffraction quality crystals of Synechocystis KaiB by using trimethylglycine, also known as betaine, as a precipitant. Synechocystis KaiB also crystallized as thin plates from PEG 400 under conditions resembling those used for crystallization of Anabaena KaiB (12), but these crystals diffracted poorly. The KaiB crystals grown from betaine belong to space group P2₁2₁2₁ with unit cell dimensions a = 52.8 Å, b = 66.0 Å, c = 114.8 Å, and four 105-residue KaiB polypeptide chains per asymmetric unit, giving a 41% solvent content (Table II). The four subunits (two dimers) in each asymmetric unit of the crystal compose half of two tetramers. Dynamic light scattering experiments (Fig. 1 and Table I) also indicated that Synechocystis KaiB (11.9 kDa) in aqueous solution is predominantly (~90%) in a single assembly state (polydispersity ~10%), corresponding to a tetramer (hydrodynamic radius of 3.25, apparent molecular weight of 53.2). In the crystal, pairs of KaiB dimers related by non-crystallographic symmetry interact to form a flattened tetramer (dimensions of 48 × 67 × 28 Å) (Fig. 2, A and B). Pairs of anti-parallel α-helices form each of the four sides of a square box, which resembles the Japanese character “Kai,” meaning cycle. This box is filled with four β-sheets and the top and bottom are each half-covered by two pairs of α-helices.

The electrostatic potential surface of tetrameric KaiB reveals a pattern of positive charges with functional implications (Fig. 2C). Positively charged residues are located around the perimeter of the box: Lys⁶, Arg²³, Lys²⁹, Lys⁴⁹, Arg⁷⁴, Lys⁷⁵, Arg⁸³, and Lys⁸⁵. Eight glutamates, Glu⁵⁵, Glu⁵⁶, Asp⁹⁵, and Glu⁹⁶ of two facing molecules, make a negatively charged cluster at the center of the tetramer. Along the diagonal, four aromatic residues, Tyr⁸, Phe³⁶, Tyr⁴⁰, and Tyr⁹⁴, line the interface between dimers (Fig. 3, A and B). At the end of these aromatic zipper residues, Tyr⁹⁴ packs against a cluster of three consecutive prolines (Pro⁷⁰-Pro⁷¹-Pro⁷²), forming additional hydrophobic interactions that connect the tetramer (see Fig. 5B).
We also found five trimethylglycine (betaine) molecules bound to the KaiB tetramer, primarily through interactions of the trimethyl moiety with electronegative groups on the protein. Pairs of betaine molecules bind in hydrophilic environments at the interface between KaiB dimers near Gln34-Glu35- (Phe36)–Gln37 at diagonally opposed corners of the box-shaped KaiB tetramer (Fig. 3C). One trimethylglycine was uniquely bound at the end of the aromatic zipper in a negatively charged pocket between two KaiB dimers (Fig. 3, D and E). This negative pocket is surrounded with positive residues.

KaiB Subunit Fold in the Tetramer—Each Synechocystis sp. PCC6803 KaiB subunit in the tetramer consists of three α helices and four β strands in the shape of a wedge (Fig. 4). The three longest β strands form a mixed β-sheet with N-terminal β1 in the center. β2 is parallel to β1, and β4 is anti-parallel to β1 and β2 (Fig. 4A). The two longest α helices (α1 and α3), which precede and are anti-parallel to β2 and β4, respectively, shield one side of this β-sheet. The shortest β strand (β3) hydrogen bonds with its symmetry mate across the dimer interface to form a small anti-parallel β-sheet, roughly perpendicular to the larger three-stranded β-sheet (Fig. 5A). Except for the five N-terminal and 10 C-terminal amino acids, the Synechocystis KaiB subunit is structurally well conserved with Anabaena KaiB (root mean square deviation of 1.27 Å for Ca) (Fig. 4B). This suggests that the core domain of KaiB homologues is structurally conserved throughout cyanobacteria (7).

In the Synechocystis KaiB tetrameric structure, the N termini have predominantly well ordered electron density (Fig. 4C). Based upon their location within the box-like tetramer (Fig. 2, A and B), the N termini are classified into two groups: inner and outer (Fig. 4D). The inner N termini protrude from the center of the Kai box (Fig. 2A), whereas the outer N termini hold the adjacent dimer, strengthening the tetrameric structure (Fig. 2B).

KaiB Oligomeric Interfaces—The tetrameric KaiB structure (Fig. 2B) reveals two different interfaces for KaiB (Fig. 5). Interface I exhibits non-crystallographic 2-fold symmetry (Fig. 5A) and is closely related to the dimer interface in the Anabaena KaiB dimer structure (12). In the Synechocystis complex structure, this dimer interface is centered on the main chain, antiparallel, β-strand hydrogen bonds between Ile59 residues of β3 in each subunit, and is mediated by a loop from Leu48 to Ala61, encompassing β3. The Gln62 side chain makes a hydrogen bond with the backbone oxygen of Leu87. The backbone nitrogen of Leu53 makes a hydrogen bond with the Ile88 backbone oxygen. The carbonyl oxygen atoms of Glu55 and Asp57 are linked through a water molecule to the nitrogen of Ala61. In addition to these hydrophilic interactions, several hydrophobic interactions occur between Leu53 and Ile88, Leu53, and Ile59, and Ile59 and Ile59. Overall each subunit of the dimer buries almost 800 Å² of surface area in Interface I.

To assemble dimers into the KaiB tetramer, the helix-turn-helix motif between α2 and α3 (Thr62–Asp62) fits into a cleft between β3 and β4 (Interface II, Fig. 5B). Pro70 and Pro72 are in van der Waals contact with Phe36 and Tyr94, respectively. The Lys57 and Glu55 side chains form a salt bridge, and the Ile68 backbone carbon interacts with Nζ of Lys68. Adding to these interactions between dimers, the Tyr40 hydroxyl forms hydrogen bonds with the backbone carbonyl oxygen of Leu92 and Nζ of Lys56 through a water molecule. Residues Phe46, Tyr40, Tyr4, and Tyr94 from all four subunits form a zipper of aromatic residues across this tetramer interface (Fig. 3, A and B).

At the edges of Interface II, the “outer” N termini stabilize the assembly of two KaiB dimers into the tetramer. The N terminus extends toward the outside of the Kai box and hooks to the adjacent dimer (Fig. 2, A and B). The Tyr8 hydroxyl makes a hydrogen bond with Glu35, and in one tetramer the Ser10 backbone oxygen and nitrogen make hydrogen bonds with the side chain of Glu34 (Fig. 5B). Overall, each dimer buries more than 1900 Å² of surface area in Interface II to form the tetramer.

Mutation at the Tetrameric Interface Disrupts the Circadian Clock—We identified an interesting kaiB mutant, with a substitution in the tetramer interface. This Asp77 to Gly mutant (position 90 in Synechococcus equivalent to position 91 in Synechocystis) exhibits arrhythmicity. In the tetrameric Synechocystis structure, a salt bridge between Asp91 and Lys56 orients Lys to form hydrogen bonds across dimer-dimer Interface II to Tyr40 and the backbone oxygen of Ile68. By precluding this salt bridge and leaving an unsatisfied positive charge, the D90G substitution likely weakens or disrupts KaiB tetrameric assembly.

To test the resultant phenotypic effect on circadian rhythm, we used the bioluminescence rhythm assay to monitor the mutant *S. elongatus* cells (Fig. 6). The parental wild type strain, which carries a luciferase reporter gene under control of the kaiBC promoter, exhibits normal circadian rhythmicity (Fig. 6A), whereas the kaiABC deletion mutant of this strain was arrhythmic (Fig. 6B, Ref. 5). Reintroduction of the wild type kaiABC locus restored normal rhythmicity (Fig. 6C), whereas introduction of the kaiABC locus carrying the D90G mutation in kaiB showed a severely impaired rhythmicity (a short free-running period; 19 h), which damped to arrhythmicity.

[two-column figure: A ribbon diagram illustrates secondary structure of Synechocystis sp. PCC6803 KaiB: α-helices, green; β-strands, yellow; coil, pink. B, overlay of Synechocystis KaiB (red) and Anabaena KaiB (yellow) shows structural conservation, except for the observed Synechocystis KaiB termini. C, 2Fobs – Fcalc electron density map, contoured at a level of 1 σ, for the N terminus of Synechocystis KaiB (molecule 1) in the tetrameric structure. D, N termini extending from the core domain suggest functional differences between the inner and outer portions of the Kai box.]
DISCUSSION

Structural Analysis of the KaiB Monomer—The structures of KaiABC proteins provide the basis for mutational mapping to help decipher protein function and protein-protein interactions (9–13, 15). Although like the KaiA and KaiC proteins, KaiB is an essential clock component, little is definitively known yet about the relationship between KaiB structure and function. Inside the Synechocystis KaiB molecule, nine leucines, two isoleucines, and one valine form a hydrophobic core, as also seen in Anabaena KaiB (12). In the Synechocystis KaiB structure, the Leu11 to Phe mutation of Synechoccus KaiB, which causes a short period circadian oscillation (6), maps to Leu12 on H9252 and is buried within this hydrophobic core. In contrast, the Synechococcus KaiB Arg74 to Trp mutant, which exhibits a similar short period phenotype (6), maps to solvent-exposed Lys75 on H9251. This mutation would decrease the positive charge around the rim of the KaiB tetramer, thus potentially disrupting intermolecular electrostatic interactions.

The N-terminal sensory domain of the SasA histidine kinase, isolated as a KaiC-associating protein, resembles KaiB in sequence and structure (Fig. 7), but has some provocative differences. The KaiB-like sensory domain of SasA (97 amino acids: 26% identity, 60% similarity) activates KaiBC expression and plays an important role in maintaining robust rhythmicity in cyanobacteria (16, 26). NMR structure determination and analysis for this domain, as a monomer in solution, revealed a thioredoxin topology (27, 28), as originally also proposed for KaiB (27). In fact, most KaiB homologues are annotated in sequence databases as thioredoxins and thiol-disulfide isomerases, because of their sequence similarities (Supplemental Materials Fig. 1), yet the topology of the KaiB structure differs from that of the thioredoxin family (27). KaiB shares with SasA and the thioredoxin family an initial β-α-β topology, matching the secondary structure predictions for these sequences (28), but after this, both topologies and predictions diverge.

Differences between the C-terminal topologies of KaiB and the KaiB-like domain of SasA may be intrinsic to their sequences, but might also arise from the different oligomeric states (tetramer versus monomer) in which their structures were determined. In the KaiB-like domain of SasA, key residues of the KaiB dimer interface (Interface I, Figs. 2B and 5A) are sequence-conserved or conservatively substituted: Gln52, Leu53, and Glu55 are unchanged, and Lys58 and Ile59 are substituted with Arg and Leu, respectively (Fig. 7, A and B). However, SasA and KaiB are not observed to associate in vivo.
through yeast two-hybrid analysis, or in vitro (16). Other hydrophilic residues that form a protruding turn on the loop forming this KaiB dimer interface are replaced in SasA by bulkier, more hydrophobic residues: Ala$_{54}$, Glu$_{66}$, and Asp$_{57}$ of KaiB are substituted with Val, Tyr, and Tyr in SasA. In the KaiB complex, these residues are surface-exposed, so the SasA substitutions need not necessarily disturb the dimerization interface. However, at the site of these sequence differences, the tetrameric KaiB and monomeric SasA structures begin to differ in secondary and tertiary structure. In SasA, these residues fold into a small interface. However, at the site of these sequence differences, substitutions need not necessarily disturb the dimerization complex, these residues are surface-exposed, so the SasA substitutions need not necessarily disturb the dimerization interface. Interestingly, in our Synechocystis KaiB structure, Asp$_{57}$ is the first of five residues comprising β3, which pairs with its symmetry mate to form a two-stranded antiparallel β-sheet at the center of the dimer interface (Interface I). In the middle of β3, Ile$_{59}$ interacts with its symmetry mate across the dimer interface. Flanking residues Lys$_{58}$ and Leu$_{60}$ of β3 point outward from the dimer, and participate instead in the tetramer interface (Interface II) between dimers. Thus, the conformation of this sequence might well be influenced by the oligomerization state of KaiB. In any case, the changes in sequence and/or structure would likely give very different binding specificities for KaiB and the KaiB sensory domain of SasA (16).

Interestingly, although the N-terminal sensory domain of SasA has been suggested to function as KaiB does (16), the sequences of KaiB and SasA are very divergent at the interface between dimers in the KaiB tetramer (interface II, Figs. 2B and 5B), as well as in the charged residues producing the characteristic electrostatic features of the KaiB tetramer (Fig. 7, A and C). Of the three consecutive Pro$_{70}$-Pro$_{72}$ of the helix-turn-helix motif of KaiB, only one remains Pro and one is entirely deleted. KaiB residues Gln$_{34}$ and Glu$_{56}$ are truncated to Ala; Lys$_{58}$ maintains the positive charge as Arg in SasA, but loses its hydrogen-bonding partners Tyr$_{40}$ (Ile in SasA) and Asp$_{91}$ (deleted). The aromatic zipper residues are also not conserved: Tyr$_{8}$, Phe$_{36}$, Tyr$_{40}$, and Tyr$_{94}$ of KaiB (Fig. 3, A and B) are converted to Pro, Glu, Ile, and Pro, respectively, in SasA (Fig. 7A). Furthermore, of nine charged residues contributing to the positive periphery of the KaiB tetramer, only Lys$_{29}$ and Arg$_{74}$ are conserved, whereas the other seven remain polar (four Gln, plus His, Asp, and Thr). Of the four negative cluster residues, Glu$_{55}$ and Asp$_{95}$ are conserved, but Glu$_{66}$ and Glu$_{66}$ become aromatic. Thus, residues leading to tetramer assembly and the characteristic electrostatic surface features of the KaiB complex (Fig. 2) are missing from SasA.

**Tetrameric Assembly of KaiB and Its Relationship to Function**—Our crystal structure reveals a tetrameric assembly state for KaiB, consistent with the oligomeric state of KaiB in aqueous solution, as measured by dynamic light scattering (Fig. 1 and Table I). Furthermore, biorhythm assays for a single-site cyanobacterial mutant (Fig. 6) support a critical role for this KaiB tetramer in the maintenance of the circadian clock. The wedge-shaped KaiB subunits form dimers like those of Anabaena KaiB (12), which further assemble into 2-fold symmetric, box-shaped tetramers (Fig. 2). The tetrameric structure reveals a new KaiB interface (Interface II) for protein-protein interaction (Figs. 3A and 5B). This predominantly (~60%) hydrophobic, dimer-dimer interface buries more than 1900 Å$^2$ of exposed surface area for each dimer, including a zipper of aromatic residues composed of Tyr$_{8}$, Phe$_{36}$, Tyr$_{40}$, and Tyr$_{94}$ (Fig. 3, A and B), which contribute almost ¼ of this buried area. As described under "Results," tetrameric oligomerization also produces characteristic electrostatic features at the molecular surface (Fig. 2C), positively charged residues around the edges and negative clusters at the center of the top and bottom faces present a hydrophilic interface for intermolecular interactions.

KaiB proteins from three different species of cyanobacteria (Synechocystis sp. PCC6803, Anabaena sp. PCC7120, Thermosynechococcus elongatus BP-1) share high amino acid sequence similarity (83–85% amino acid sequence identity, 92–95% similarity; Supplemental Materials Fig. 1; Refs. 12 and 25), yet crystallize differently. As described above, we crystallized Synechocystis KaiB from betaine in space group P2$_1$2$_1$2$_1$ (unit cell dimensions $a = 52.8$ Å, $b = 66.0$ Å, $c = 114.8$ Å with two dimers per asymmetric unit), although Anabaena KaiB was crystallized (12) from PEG 400 in space group C2 ($a = 104.8$ Å, $b = 36.4$ Å, $c = 54.1$ Å, $β = 98.8$° with one dimer per asymmetric unit) and T. elongatus KaiB was crystallized (25) from PEG 3350 in space groups P2$_1$ (unit cell dimensions $a = 89.6–90.1$ Å, $b = 67.3–71.2$ Å, $c = 105.8–106.8$ Å, $β = 100.1–100.3$° with 6–13 subunits per asymmetric unit) and P2$_1$ (unit cell dimensions $a = 63.7$ Å, $b = 33.4$ Å, $c = 93.7$ Å, $β = 101.1°$ with likely 4 subunits per asymmetric unit). Despite these significant differences in crystallization conditions and crystal forms, all these KaiB crystals likely contain the same tetrameric KaiB architecture that we have identified for Synechocystis sp. PCC6803 KaiB. In Fig. 5, C and D, we show by superposition that pairs of Anabaena KaiB dimers assemble into the same tetramer within their C2 crystal packing. The 36.4-Å length of the b edge of this unit cell accommodates the flattened tetramer layer, whereas the approximately doubled length (66.0 Å) of b in Synechocystis KaiB crystals accommodates 2 stacked tetramers. Preliminary analysis of Thermosynechococcus KaiB in two different crystal forms is also consistent with flattened tetramer building blocks packed in single (P2$_1$ $b = 33.4$ Å) and double (P2$_1$, $b = 67.3–71.2$ Å) (25). The consistent pattern of tetramer assembly in all four KaiB crystals argues that the tetramer is biologically relevant.

Furthermore, KaiB tetrameric self-assemble likely contributes to circadian rhythmicity in the cyanobacterial cell. KaiB and KaiC exhibit robust circadian rhythms, whereas KaiA remains constantly at low abundance in the cytosol. KaiB and
KaiC are most abundant at the early circadian night phase (circadian time 15–16), reaching a KaiA:KaiB:KaiC ratio up to 1:40:20 (17). Kageyama et al. (17) also showed that the Kai proteins dynamically assemble into heteromultimeric complexes in a circadian fashion. KaiB, unlike KaiA, is membrane-associated as well as cytosolic, with 50–80% of KaiB localized at the membrane (17). Under continuous illumination, the amount of membrane-associated KaiB peaks in the evening (at circadian time 12–16). In contrast, cytosolic KaiB peaks later (at circadian time 20).}

The self-assembly of KaiB tetramers from KaiB dimers may enable its transfer from the membrane to the cytosol and vice versa, by changing its equilibrium and/or molecular features. For example, the exposed surface of the KaiB dimer is more hydrophobic than that of the KaiB tetramer. KaiB lacks the hydrophobic sequence motif characteristic of a transmembrane anchor, and has been proposed to be a peripheral membrane-associated protein by Kitayama et al. (17). The stabilization of KaiB crystals by betaine (trimethylglycine) incorporation suggests a mode for membrane association, as trimethylglycine resembles the polar head groups of membrane phospholipids. Like trimethylglycine, the polar head groups of phospholipids contain a terminal positively charged group (amino (\(\text{NH}_3\)) or trimethylamino moiety ((\(\text{CH}_3\))\text{N})), followed by a negatively charged moiety (the carboxylate in betaine versus phosphate in phospholipids). Moreover, in the Synechocystis KaiB tetramer, we found four of five bound betaine molecules to be localized in pockets made between two KaiB molecules (Fig. 3C). Trimethylglycine binding thus illustrates both potential binding modes and oligomeric stabilization of KaiB by phospholipid membranes (Fig. 3, C–E). KaiB function may regulate or be regulated by the various metabolic activities, including electron transport, which takes place at the thylakoid or cell membranes of cyanobacteria.

Conclusions—The molecular mechanisms that orchestrate changes in the composition and stoichiometry of clock protein complexes hold a key to understanding circadian rhythms in organisms from cyanobacteria to humans (19, 29). The discovery and analysis of the KaiB tetramer reported here, together with the other recently determined structures for the KaiA and KaiB dimers (11, 12), the KaiC hexamer (15), and the monomeric N-terminal domain of the SasA kinase (28) provide a foundation for solving the mysteries of the assembly and disassembly of clock protein complexes.

Acknowledgments—We thank S. Clancy for assistance with protein purification, the SSRL staff for assistance in diffraction data collection, R. G. Garcia for Anaebaena KaiB coordinates, Y. Nakahira and S. Takeuchi for technical assistance with mutation analyses, J. Huffman for assistance with DLS, J. Tubbs for critical suggestions, Professors S. S. Golden and T. Kondo for generous encouragement, and B. R. Chapados and Professor J. A. Tainer for aiding in the initial steps of structure determination.

REFERENCES

1. Golden, S. S., Ishiura, M., Johnson, C. H., and Kondo, T. (1997) Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 327–354
2. Golden, S. S., and Canales, S. R. (2003) Nat. Rev. Microbiol. 1, 191–199
3. Golden, S. S. (2003)Curr. Opin. Microbiol. 6, 535–540
4. Ditty, J. L., Williams, S. B., and Golden, S. S. (2003) Annu. Rev. Genet. 37, 513–543
5. Johnson, C. H. (2004) Nature 430, 23–24
6. Ishiura, M., Kutsuna, S., Adachi, Y., Iwasaki, H., Andersson, C. R., Tanabe, A., Golden, S. S., Johnson, C. H., and Kondo, T. (1998) Science 281, 1519–1523
7. Devrny, V., Vinogradova. O., and Nevo, E. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 2495–2500
8. Iwasaki, H., Taniguchi, Y., Ishiura, M., and Kondo, T. (1999) EMBO J. 18, 1137–1145
9. Williams, S. B., Vakonakis, I., Golden, S. S., and LiWang, A. C. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 15357–15362
10. Usami, T., Fujita, M., Nakatsu, T., Hayashi, F., Shihata, H., Itoh, N., Kato, H., and Ishiura, M. (2004) Nat. Struct. Mol. Biol. 11, 623–631
11. Y. S. Vakonakis, I., Leewer, T. R., LiWang, A. C., and Sacchettini, J. C. (2004) J. Biol. Chem. 279, 20511–20518
12. Garcia, R. G., Wu, N., and Pill, E. P. (2004) EMBO J. 23, 1098–11098
13. Vakonakis, I., Sun, J., Wu, T., Holzenburg, A., Golden, S. S., and LiWang, A. C. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 1479–1484
14. Hayashi, F., Suzuki, H., Iwase, R., Usami, T., Miyake, A., Shen, J. R., Imada, K., Furukawa, Y., Yonekura, K., Namba, K., and Ishiura, M. (2003) Genes Cells 8, 287–296
15. Pattanayek, R., Wang, J., Mori, T., Xu, Y., Johnson, C. H., and Egli, M. (2004) Mol. Cell 15, 375–388
16. Iwasaki, H., Williams, S. B., Kitayama, Y., Ishiura, M., Golden, S. S., and
Kondo, T. (2000) Cell 101, 223–233
17. Kitayama, Y., Iwasaki, H., Nishiwaki, T., and Kondo, T. (2003) EMBO J. 22, 2127–2134
18. Xu, Y., Morii, T., and Johnson, C. H. (2003) EMBO J. 22, 2117–2126
19. Kagayama, H., Kondo, T., and Iwasaki, H. (2003) J. Bio. Chem. 278, 2388–2395
20. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
21. Navaza, J. (1994) Acta Crystallogr. Sect. A 50, 157–163
22. Bruenger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 905–921
23. McRee, D. E. (1999) J. Struct. Biol. 125, 156–165
24. Nishimura, H., Nakahira, Y., Imai, K., Tsuruhara, A., Kondo, H., Hayashi, H., Hirai, M., Saito, H., and Kondo T. (2002) Microbiology 148, 2903–2909
25. Iwase, R., Imada, K., Hayashi, F., Uzumaki, T., Namba, K., and Ishiura, M. (2004) Acta Crystallogr. Sect. D Biol. Crystallogr. 60, 727–729
26. Dvornyk, V., Deng, H. W., and Nevo, E. (2004) Mol. Biol. Biol. 21, 1468–1476
27. Klewer, D. A., Williams, S. B., Golden, S. S., and LiWang, A. C. (2002) J. Biomol NMR. 24, 77–78
28. Vakonakis, I., Klewer, D. A., Williams, S. B., Golden, S. S., and LiWang, A. C. (2004) J. Mol. Biol. 342, 9–17
29. Lee, C. Etchegaray, J. P., Cagampang, F. R., Loudon, A. S., and Reppert, S. M. (2001) Cell 107, 855–867