KaiB is a component of the circadian clock molecular machinery in cyanobacteria, which are the simplest organisms that exhibit circadian rhythms. Here we report the x-ray crystal structure of KaiB from the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1. The KaiB crystal diffracts at a resolution of 2.6 Å and includes four subunits organized as a dimer of dimers, each composed of two non-equivalent subunits. The overall shape of the tetramer is an elongated hexagonal plate, with a single positively charged cleft flanked by two negatively charged ridges whose surfaces includes several terminal chains. Site-directed mutagenesis of *Synechococcus* KaiB confirmed that alanine substitution of residues Lys-11 or Lys-43 in the cleft, or deletion of C-terminal residues 95--108, which forms part of the ridges, strongly weakens in vivo circadian rhythms. Characteristics of KaiB deduced from the x-ray crystal structure were also confirmed by physicochemical measurements of KaiB in solution. These data suggest that the positively charged cleft and flanking negatively charged ridges in KaiB are essential for the biological function of KaiB in the circadian molecular machinery in cyanobacteria.

The circadian clock is an endogenous biological mechanism that generates autonomous daily cycles of physiological activity that reflect daily environmental cycles of light and temperature (1). Cyanobacteria are the simplest organisms that exhibit circadian rhythms, and the circadian clock gene cluster, *kaiABC*, is essential for circadian rhythms in cyanobacteria such as *Synechococcus* sp. strain PCC 7942 (hereafter called *Synechococcus*) (2). KaiB and KaiC constitute an operon, whereas kaiA is transcribed as a single gene. KaiC down-regulates and KaiA up-regulates the *kaiBC* operon (2). Interactions between all three Kai proteins have been demonstrated *in vivo* and in the yeast two-hybrid system (3). The KaiA-KaiC interaction was studied in detail *in vitro* using highly purified enzyme preparations (4, 5). These studies revealed that two KaiA dimers interact with one KaiC hexamer (4). Amino acid substitutions in KaiA, KaiB, or KaiC alter the period length, phase, wave amplitude, or wave form (2, 5--9) of the circadian clock, but the one or more molecular mechanism by which these mutations alter circadian rhythms are not known.

KaiA has three domains: the N-terminal amplitude-amplifier domain, the central period-adjuster domain, and the C-terminal clock-oscillator domain (5). The structure of the N-terminal amplitude-amplifier domain of the KaiA dimer has been determined by NMR (10), and the structure of the C-terminal clock-oscillator domain has been determined by NMR (11) and x-ray crystallography (5, 12). The structure of full-length KaiA (13) has been determined by x-ray crystallography. KaiC forms a pot-shaped hexamer upon binding ATP (14, 15), with each subunit consisting of two approximately equal-sized subdomains. The KaiC structure has been determined by electron cryomicroscopy and single particle image analysis (15). Recently, the x-ray crystal structures of the KaiB dimer (12) and KaiC hexamer (16) were reported.

To improve our understanding of the structure and mechanism of the circadian clock machinery, we determined the x-ray crystal structure of *Thermosynechococcus elongatus* KaiB. KaiB from *T. elongatus* was chosen for this study because proteins from this thermophilic cyanobacterium tend to be highly stable. Furthermore, efficient procedures for gene transfer and manipulation in *T. elongatus* (17) have recently been developed and circadian rhythms in *T. elongatus* have been characterized (18). We report here the 2.6 Å x-ray crystal structure of *T. elongatus* KaiB, which reveals that KaiB is an unusual tetramer, composed of two asymmetrical dimers, that assumes the shape of an elongated hexagonal plate. The tetramer has a positively charged cleft flanked by two negatively charged ridges, suggesting that the cleft may play a role in ligand binding. The solution structure of KaiB was analyzed by gel filtration and analytical ultracentrifugation, and the presence of functional substructures in KaiB was tested by site-directed mutagenesis.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification—** *T. elongatus* KaiB and KaiB T64C were expressed and purified as described previously (19). Tag-free KaiB was cloned into pET-3a vector (Novagen) and expressed in *Escherichia coli* strain BL21(DE3) (Novagen). The protein was purified by ion-exchange chromatography on a DEAE-cellulose column (Wako), a Q-Sepharose High Performance column (Amersham Biosciences), and a Mono Q HR 5/5 column (Amersham Biosciences).
Structure Determination—The details of crystallization and data collection have been previously described (19). The KaiB crystals belonged to the monoclinic space group \( P2_1 \), with unit-cell dimensions \( a = 90.0, b = 67.0, c = 105.8 \, \text{Å}, \) and \( \beta = 101.3^\circ \). The \( T64C \) crystal belonged to the monoclinic space group \( P2_1 \) with unit-cell dimensions \( a = 63.7, b = 33.4, c = 93.7 \, \text{Å}, \) and \( \beta = 100.1^\circ \). The diffraction data were collected at SPring-8 beamlines, BL41XU and BL38B1, and the statistics of the diffraction data were reported previously (19). Initial phase was calculated from multiwavelength anomalous dispersion data of the osmium-derivative crystals of KaiB using the SOLVE program (20). After density modification, the protein backbone was traced using the graphics program O (21), but only 50% of the molecule could be constructed due to the low quality of the obtained map. To improve the map, experimental substructure and functional site of KaiB

**TABLE ONE**

| Summary of refinement statistics |
|----------------------------------|
| Resolution range (Å)            | 20.00–2.60 |
| Number of reflection            |            |
| Working set                     | 11736      |
| Test set                        | 622        |
| \( R \)-factor\(^a\)            | 22.7       |
| \( R_{free}\)^\(^b\)           | 28.9       |
| Root mean square deviation      |            |
| Bond length (Å)                 | 0.013      |
| Bond angle (°)                  | 1.659      |
| Ramachandran plot               |            |
| Most favored (%)                | 86.5       |
| Additionally allowed (%)        | 12.9       |
| Generously allowed (%)          | 0.6        |
| Disallowed (%)                  | 0.0        |
| Number of atoms                 |            |
| Protein                         | 3164       |
| Solvent                         | 93         |

\(^a\) \( R \)-factor = \( \sum (F_o - F_c) / \sum F_o \) where \( F_o \) and \( F_c \) are the observed and calculated structure factors, respectively.

\(^b\) \( R_{free} \) is the \( R \)-factor calculated using 5% of the reflections data chosen randomly and omitted from the start of refinement.

and introduced them into TS2 in the genomes of a kaiB-null strain of Synechococcus carrying a \( p_{kaiABC}::luxAB \) reporter gene (2). We measured the circadian bioluminescence rhythms of the transformed cells as previously described (5, 18) at 30 °C by using a bioluminescence monitoring apparatus (18, 25) and program RAP (26).

For \( in vivo \) rhythm assay in \( T. elongatus \), we replaced the kaiABC loci and its downstream \( tlr0484 \) locus (17) in the genome of a \( T. elongatus \) carrying a \( p_{kaiB}::Xi \ luxAB \) reporter gene (18) with a DNA segment containing both the \( T. elongatus \) kaiABC loci whose kaiB gene was mutated and a Cm-selective marker gene (17) inserted into the \( tlr0484 \) gene by homologous recombination (17). Therefore, the \( tlr0484 \) gene was disrupted in the transformed cells carrying the mutated kaiB gene. We measured the bioluminescence rhythms of the transformed cells at 41 °C as described previously (18).

**RESULTS**

X-ray Crystal Structure of the KaiB Tetramer: A Dimer of Dimers—KaiB is highly conserved among twelve cyanobacterial species, with the exception of a short C-terminal region (Fig. 1). KaiB from \( T. elongatus \) consists of 108 amino acid residues and has a deduced molecular mass of 12,025 Da. \( T. elongatus \) KaiB was crystallized (see “Experimental Procedures”), and the protein structure was determined at 2.6 Å by x-ray crystallography. The asymmetric unit of the KaiB crystal contains four KaiB monomers (labeled A–D in Fig. 2A). The final model includes Lys-6 to Ala-101 for subunit A, Leu-4 to Glu-108 for subunit B, Ala-2 to Ala-101 for subunit C, and Arg-5 to Leu-107 for subunit D. Subunits A and B and subunits C and D form two independent dimers (AB and CD), respectively, in each of which subunits are related by local 2-fold symmetry. Each of these two dimers, AB and CD, has a partner dimer related by a crystallographic

\(^4\) The abbreviation used is: CD, circular dichroism.
2-fold axis forming tetramers T1 (ABA’B’) and T2 (CDC’D’), respectively (Fig. 2A). These two tetramers are essentially identical, except for the N- and C-terminal regions of the A (A’) and C (C’) subunits (Fig. 2, A and C). Subunits A and B in tetramer T1 correspond, respectively, to subunits C and D in tetramer T2. The tetramer forms a slightly deformed, elongated hexagonal plate with a size of 85 mm 30 Å. The local 2-fold symmetry axes, which run approximately perpendicular to the crystallographic 2-fold axis, do not intersect the crystallographic 2-fold axis; thereby the four subunits are not equivalent in contrast to usual tetrameric structures. But each tetramer has two pairs of equivalent subunits: A-A’ and B-B’ (D-D’) (Fig. 2A). The environments of subunits A and B are completely different. Subunit A interacts directly with B and B’ but not with A’, whereas subunit B is in close contact with all the other subunits in the tetramer (Fig. 2A).

The globular core of the KaiB subunit (Tyr-8 to Tyr-94) has a two-layer a/β sandwich structure consisting of three β strands (β1, β2, and β4) and three α helices (α1, α2, and α3) (Fig. 2B). This region adopts a novel fold that has not been reported previously. A β-sheet comprising parallel strands β1 and β2 and anti-parallel strand β4 forms a wall on one side, while anti-parallel helices α2, α3, and α4 form a wall on the other side (Fig. 2B). The chain connecting β2 and α2 forms an L-shaped loop (Val-47 to Ala-61) that includes β3, which extends away from the core structure (Fig. 2B). β3 extends almost perpendicular to the core β-sheet and contributes to dimer formation by forming an intersubunit anti-parallel β-strand. The core structures of the four independent subunits in an asymmetric unit can be superimposed with root mean square displacements of 0.52–0.65 Å for corresponding Ca atoms (Fig. 2C).

In contrast to the core structure, both N- and C-terminal regions show large conformational variation. Subunits B and D, which are located in an equivalent position in each tetramer, exhibit similar N- and C-terminal structures (Fig. 2C). Their C-terminal regions from Tyr 94 fold back onto the surface of the core structure, making a loop and forming a short β-turn-β structure (β5 and β6) in the last stretch from Ala-101, while their N-terminal regions run along these C-terminal chains, although the first three or four residues are missing (Fig. 2, B and C). However, subunits A and C, which are also located in an equivalent position in each tetramer, have distinctly different terminal chain conformations from subunits B and D. Although the C-terminal chain of subunit A folds back in a conformation similar to subunits B and D, the C-terminal seven residues, which would form the short β-turn-β motif, are not visible and the N-terminal chain appears to extend away from the C-terminal chain, although the first five residues are not visible. The terminal chains of subunit C extend away from the core (Fig. 2, A and C), where the N-terminal chain is visible from Ala-2, whereas the C-terminal seven residues are not visible. The conformational variability of the terminal chains is displayed graphically in Fig. 2C. These data suggest that the N- and C-terminal regions of KaiB have significant flexibility and adaptability, and these regions may play important roles in the physiological function of KaiB.

**Intra- and Interdimer Interfaces**—The KaiB tetramer is a dimer of the dimeric unit AB or CD for tetramers T1 and T2, respectively (Fig. 2A). The dimer-dimer subunit interface is shown graphically in Fig. 3, which extends away from the surface of the core structure, making a loop and forming a short β-turn-β structure (β5 and β6) in the last stretch from Ala-101, while their N-terminal regions run along these C-terminal chains, although the first three or four residues are missing (Fig. 2, B and C). However, subunits A and C, which are also located in an equivalent position in each tetramer, have distinctly different terminal chain conformations from subunits B and D. Although the C-terminal chain of subunit A folds back in a conformation similar to subunits B and D, the C-terminal seven residues, which would form the short β-turn-β motif, are not visible and the N-terminal chain appears to extend away from the C-terminal chain, although the first five residues are not visible. The terminal chains of subunit C extend away from the core (Fig. 2, A and C), where the N-terminal chain is visible from Ala-2, whereas the C-terminal seven residues are not visible. The conformational variability of the terminal chains is displayed graphically in Fig. 2C. These data suggest that the N- and C-terminal regions of KaiB have significant flexibility and adaptability, and these regions may play important roles in the physiological function of KaiB.

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Important Substructure and Functional Site of KaiB

FIGURE 2. X-ray crystal structure of KaiB tetramer. A, crystal packing of KaiB represented as a Cα backbone diagram. Each asymmetric unit of the crystal of the mutant KaiB T64C contained two independent dimers (AB and CD). Each dimer formed a tetramer (T1 and T2) with a crystallographic 2-fold axis (black oval). Molecules related by crystallographic 2-fold axes are indicated by the same color. Other tetramers (pale blue) were also generated by the crystallographic symmetry. The black frame indicates a unit cell. B, structure of KaiB monomer represented as a ribbon diagram. Helices and strands are shown in orange and green, respectively. C, comparison of the structures of four independent KaiB subunits in the asymmetric unit. Co traces of all four subunits are superimposed in stereo diagram. Subunits A, B, C, and D are shown in green, orange, cyan, and blue, respectively. The figures were generated with MOLSCRIPT (28) and Raster3D (29).

The stability of the KaiB tetramer was examined during gel filtration or sedimentation. The tetramer was stable in solution from pH 4 to 10, from 4 to 40 °C and at very low ionic strength (data not shown). This is consistent with the observation that T. elongatus exhibits circadian rhythms in the temperature range 30 °C to 60 °C (18).

Surface Potential and Charge Distribution for the KaiB Tetramer—The following detailed description of the structure of the KaiB tetramer...
FIGURE 3. Molecular interfaces in KaiB tetramer. A, inter-dimer interface. B, intra-dimer interface. Interfaces are displayed by dividing a tetramer into two dimers in A, and each dimer into two monomers (B) by translation and rotation. Molecules are displayed in Corey-Pauling-Koltun representation with atoms involved in the intermolecular interactions (within an intermolecular atomic distance of 5 Å) color-coded as follows: red, charged oxygen; pink, polar oxygen; blue, charged nitrogen; light blue, polar nitrogen; yellow, carbon; and green, sulfur. The figures were generated with MOLSCRIPT (28) and Raster3D (29).

FIGURE 4. Measurements of the oligomeric state of KaiB. A, gel filtration chromatography of KaiB (red), T64C (blue), and tag-free KaiB (green). The positions of the molecular mass standards are indicated above the profiles. B, SDS-PAGE of chemically cross-linked KaiB. Cross-linking reaction was carried out for different time periods as indicated. C, sedimentation equilibrium by analytical ultracentrifugation. The upper panel shows the equilibrium profile (C) displayed as A280 versus radial distance and a theoretical concentration profile (red solid line) for a single molecular species with a molecular mass of 48.6 kDa (KaiB tetramer). The lower panel shows the residual plot of the curve fitting.

FIGURE 5. Measurements of the oligomeric state of Anabaena KaiB. A, gel filtration chromatography of Anabaena KaiB. The positions of the molecular mass standards are indicated above the profiles. B, SDS-PAGE of chemically cross-linked KaiB. Cross-linking reaction was carried out for different time periods as indicated. C, sedimentation equilibrium by analytical ultracentrifugation. The upper panel shows the equilibrium profile (C) displayed as A280 versus radial distance and a theoretical concentration profile (red solid line) for a single molecular species with a molecular mass of 47.4 kDa (KaiB tetramer). The lower panel shows the residual plot of the curve fitting.
The surface potential of the KaiB tetramer includes regions of non-random charge distribution. The most remarkable feature of the surface potential is two parallel ridges of highly concentrated negative charge surrounding a positively charged cleft. The ridges are related by a 2-fold axis running along the short diagonal of the elongated hexagonal plate (Fig. 6). However, this structure occurs only on one surface of the tetramer plate, SBB’T, but it does not form on the related surface, SAA’T, on which the terminal chains of the subunits are exposed.

The negatively charged ridges consist of the N- and C-terminal regions and the loop connecting β2 to β3 of subunits B and B’. Ten acidic residues of subunits B and B’ (Glu-55, Glu-56, Asp-57, Glu-95, Glu-96, Asp-99, Glu-102, Asp-103, Asp-104, and Glu-108) are clustered on each ridge (Fig. 6B). Three basic residues (Lys-11, Lys-43, and Lys-58) are on the bottom of the cleft, giving it a highly positively charged character (Fig. 6B). The major portion of each ridge is made up of the C-terminal region from Glu-95 to Glu-108. The segment from Glu-95 to Asp-99 extends away from the core and forms an anti-parallel β strand with the N-terminal chain of the same subunit. Asp-99 is at the peak of the ridge, moving downward through Glu-102, Asp-103, and Asp-104, which lies at the base of the ridge. The most C-terminal segment, Ala-101 to Glu-108, forms a β-turn-β structure, which is anchored onto the β sheet of the subunit core (Fig. 2B) by a main chain hydrogen bond between Leu-42 and Leu-107.

Another positive surface is observed in and around a hollow formed at the boundary between subunits A and B’ (or A’ and B) on the longest edge of the tetramer plate (Fig. 6B). Lys-34 and Lys-37 of subunit B (B’) and Lys-6, Lys-67, and Arg-74 of subunit A’ (A) contribute to this positive surface.

Interestingly, no significant charge distribution was observed on surface SAA’T (Fig. 6C). The N- and C-terminal chains of subunit A (A’) extend straight away from the edge of the tetramer plate on SAA’T, and

**FIGURE 6.** Electrostatic surface potential of the KaiB tetramer. A, stereo view of Cα backbone ribbon diagram, color-coded according to the amino acid sequence in rainbow color from the N terminus in blue to the C terminus in red. Side chains of residues for which substitution mutations were examined (Fig. 7) are displayed in ball and stick representation. The figure was generated with MOLESCRIPT (28) and Raster3D (29). B, electrostatic potential of surface SBB’T. The saturation threshold for the Grasp image is 10 and 10. C, electrostatic potential of surface SAA’T. D, electrostatic potential of surface SBB’T after C-terminal 14 residues are truncated (KaiBi-14). Electrostatic surface potential is color-coded: blue, positive; red, negative. The figures are made by using GRASP (30) and Raster3D (29). The negative ridges are outlined with dotted lines. The positive cleft and positive hollows are indicated by yellow and black arrowheads, respectively. The acidic and basic residues in the positive cleft, negative ridges, and positive hollow are labeled.
the C-terminal chain was not visible from Ala-101 onward. Lys-11 and Lys-43, which are in a pocket of positive charge at the bottom of the cleft on the SBB/H11032T surface, were fully exposed on surface SAA/H11032T. Structure-Function Analysis of Ridge and Cleft Regions of KaiB Tetramer—The overall structure of KaiB determined here shows little similarity to other known proteins, but the electrostatic surface potential of the tetramer (Fig. 6 B) provides clues as to possible structure-function relationships in KaiB. In particular, it seems likely that the positively charged cleft surrounded by negatively charged ridges plays a role in ligand binding and the biological function of KaiB. This idea is supported by the fact that Lys-11, Lys-43, and Lys-58 and a C-terminal cluster of negatively charged residues are highly conserved among twelve strains of cyanobacteria (Fig. 1).

The functional importance of KaiB residues in the ridges and cleft region was tested directly by site-directed mutagenesis of Synechococcus KaiB followed by in vivo analysis of circadian cycling. Alanine substitution mutants of Synechococcus KaiB were constructed and introduced into a targeting site (TS2) in the genome of a kaiB-null strain carrying a P_kaiBC::luxAB reporter gene (2). Using this system, the integrity of the circadian rhythms was assessed by conducting in vivo bioluminescence assays as described previously (2, 5). For convenience, in this discussion we refer to the amino acid coordinates of T. elongatus KaiB; when appropriate, the corresponding residues in Synechococcus KaiB are indicated.

Alanine substitution mutations of Lys-11 (K11A) or Lys-43 (K43A) disrupted circadian rhythms and mutation of Lys-58 (K58A) resulted in an unclear rhythm (Fig. 7 A), as deduced from in vivo bioluminescence assays in Synechococcus. Similar results were obtained with bioluminescence assays using mutants of T. elongatus KaiB (Fig. 8). In Synechococcus, Western blots were used to confirm that the Synechococcus KaiB mutants were expressed at a similar level as wild-type KaiB (data not shown). Furthermore, the CD profiles of the Synechococcus KaiB mutants were similar to the CD profile of wild-type KaiB (Fig. 9). These data demonstrate that the KaiB alanine substitution mutants studied here are stable and expressed efficiently in vivo in Synechococcus and support the hypothesis that mutations in the positively charged cleft residues in KaiB disrupt circadian rhythms. Therefore, we propose that the positively charged cleft and flanking negatively charged ridges in KaiB may be a functional site associated with a biologically important role in vivo.

The positively charged side hollow (black arrow in Fig. 6B) is another candidate functional site of KaiB. Although Lys-6, Lys-67, and Arg-74 of
subunit A (A') are conserved residues, Lys-34 and Lys-37 are not (Fig. 1). Alanine substitution mutations at these residues showed that K67A mutants have low amplitude and low bioluminescence, whereas K6A mutants have normal patterns of bioluminescence (Fig. 7A). However, Lys-67 of subunit B (B') contributes to the dimer-dimer interaction on surface SAA'T by forming an intermolecular salt bridge with Glu-55 of subunit A' (A), suggesting that Lys-67 may play a role in tetramer stability. This possibility was tested by analyzing the gel filtration properties of Synechococcus KaiB K66A (corresponding to T. elongatus K67A mutant). (Note that gel filtration and bioluminescence studies in Synechococcus were performed at 30 °C). The mutant KaiB eluted at almost the same position as wild-type KaiB at 4 °C or 30 °C (data not shown). Thus, it remains possible that Lys-67 plays a role on KaiB clock function. Additional experiments are needed to confirm this possibility.

Functional Analysis of the C-terminal Region of KaiB — The KaiB protein shows extensive sequence homology in twelve cyanobacterial strains (Fig. 1); however, the C-terminal region of KaiB is divergent, and it is possible that this variability confers species-specificity in KaiB function. This idea is supported by the fact that KaiB from Synechocystis sp. strain PCC 6803 (hereafter called Synechocystis) fully complements the null KaiB mutant of Synechococcus, whereas KaiB from T. elongatus and Anabaena partially complemented the mutant, generating a faint rhythm (Fig. 7B). To test this idea, T. elongatus-Synechococcus chimeric KaiB molecules were expressed in Synechococcus kaiB-null host cells, and the ability of the chimeras to complement the rhythm defect of the host was tested. Chimeric KaiB was generated with Synechococcus KaiB residues 1–93 and T. elongatus KaiB residues 95–108; alternatively, a second chimera contained residues 1–94 from T. elongatus KaiB and residues 94–102 from Synechococcus KaiB. The results show that the C-terminal region of Synechococcus KaiB is required for complementation of the rhythm defect in Synechococcus kaiB-null host cells (Fig. 7B). These data support the idea that the C-terminal region of KaiB plays a functionally important role and that variation in this region may confer species specificity to the circadian clock molecular machinery in vivo.

To further elucidate the role of the C-terminal region, we expressed a series of Synechococcus KaiB deletion mutants in kaiB-null Synechococcus cells and examined the circadian rhythm. Starting from KaiB 1–100, C-terminal residues were deleted sequentially one at a time. In rhythm assays, the results showed that the rhythm amplitude was disturbed and the rhythm period was lengthened significantly at every other residue (Fig. 7C). When C terminus was deleted up to residue 94, and the negative ridges of wild-type Synechococcus KaiB were completely removed (Fig. 6D, KaiB 1–94), the rhythm was strongly weakened and destabilized (Fig. 7C, KaiB 1–94). These results suggest that the conformation of the ridges in the Synechococcus KaiB tetramer may influence the ability of KaiB to interact with KaiA, KaiC, or some other ligands. Furthermore, the conformation of the ridges may also play a role in the species specificity of KaiB.

Whereas the C-terminal region of KaiB is poorly conserved among cyanobacterial species, the negatively charged character of this region is generally conserved (Fig. 1). To clarify whether the acidic property of the C-terminal region is essential for KaiB clock function, glutamine was substituted for Glu-95, and asparagine was substituted for Asp-98, Asp-100, and Asp-101 in Synechococcus KaiB (Fig. 7D). Each of these mutations alone had minor effects on circadian rhythm in vivo; however, when all four residues were mutated, the circadian rhythm was seriously disturbed (Fig. 7D). The electrostatic surface potential of the tetramer is altered completely in mutants that lack the C-terminal region (Fig. 6D), and a similar effect is likely for the quadruple mutant lacking negative charge in this region. These results suggest that the negative surface potential in the C-terminal region is necessary for KaiB clock function, and the disruption of circadian rhythm caused by truncation or mutation of the C-terminal region is likely to be due to the loss of this negative charge potential.

DISCUSSION

This study reports the 2.6 Å x-ray crystal structure of KaiB, which is an unusual tetramer composed of two asymmetric dimers. To our knowledge, the C-terminal domain of HrcQ\(_{ab}\) is the only other “dimer of dimers” structure that has been reported (31). In contrast, most tetrameric proteins are composed of four symmetrically related and equivalent subunits. For inherently symmetrical tetramers, non-equivalence among the subunits can be induced by substrate or ligand binding. In the KaiB tetramer, non-equivalence is intrinsic because the dimers bind in an asymmetric manner. This type of intrinsic asymmetry was observed in KaiB crystals in space group P2\(_1\) (with three independent tetramers) and in KaiB crystals in space group P2\(_2\) (with two tetramers) (19), which argues against the possibility that it is an artifact of crystal packing. In addition, Anabaena KaiB forms a similar dimer of dimers, although a recently reported study that it forms a dimer (12). Subunits A and B in the KaiB tetramer are tightly bound via an extensive and predominantly hydrophobic interface enclosed by an intermo-
lucular β strand and other hydrogen bonds (Fig. 3). In contrast, the inter-dimer interface between AB and A′B′ appears to be less stable and composed of more polar or charge-charge interactions; thus, the inter-dimer interface could potentially be destabilized by changes in pH or ionic strength (Fig. 3). This suggests that the KaiB tetramer might dissociate into two dimers, and such dissociation could play a role in regulating KaiB during cycles of circadian rhythms.

Previous studies identified two mutations in Synechococcus KaiB, L11F and R74W (corresponding to L12F and R75W, respectively, in T. elongatus KaiB), which alter circadian rhythms in Synechococcus (2). Leu-12 is in the KaiB core, and the adjacent residue, Lys-11, was identified here as essential for KaiB clock function (Fig. 6A). The impact of the L11F mutation may reflect altered side-chain packing induced by the bulky phenylalanine residue, resulting in structural change or instability. The role of Arg-75 is less clear; Arg-75 in subunits A, B, and C was exposed, but Arg-75 in subunit D formed an intermolecular hydrogen bond with Glu-96 in subunit C (Fig. 6A). Because this hydrogen bond is at the dimer-dimer interface, it may make a significant contribution to tetramer stability.

This study presents the results of in vitro mutagenesis and in vivo rhythm assays, which demonstrate that residues in the positive cleft and flanking negative ridges of KaiB are required for circadian rhythms in Synechococcus. Thus, we propose that this region is a functionally active site in KaiB. Amino acid substitutions are tolerated in the C-terminal region that form the negative ridges in KaiB, and the amino acid sequence of this region varies from species to species; however, the highly conserved property of this region is conserved and is likely to be functionally important in KaiB. It is possible that the negative ridges define the specificity of KaiB binding to target proteins, which may vary subtly from one species to another. Because the basic residues are located deep inside the cleft, ligand binding may be induced by structural changes in the more flexible ridges. This is consistent with the fact that the C-terminal region displays significant conformational flexibility and adaptability in the KaiB crystal structure. Therefore, we propose that this region may play an important role in defining and regulating KaiB binding specificity. This may be important to allow circadian rhythms to be maintained as the environment of cyanobacteria change. Thus, species-specific variation in the C-terminal region of KaiB may reflect adaptation of the organism to its changing environment.

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Note Added in Proof—The tetrameric structure of Synechoystis KaiB was reported (Hitomi et al. (2005) J. Biol. Chem. 280, 19127–19135).

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