Structural and Biochemical Characterization of a Cyanobacterium Circadian Clock-modifier Protein

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Circadian clocks are self-sustained biochemical oscillators. The oscillator of cyanobacteria comprises the products of three kai genes (kaiA, kaiB, and kaiC). The autophosphorylation cycle of KaiC oscillates robustly in the cell with a 24-h period and is essential for the basic timing of the cyanobacterial circadian clock. Recently, period extender (pex) mutants, of which show a short period phenotype, were classified as a resetting-related gene. In fact, pex mRNA and the pex protein (Pex) increase during the dark period, and a pex mutant subjected to diurnal light-dark cycles shows a 3-h advance in rhythm phase. Here, we report the x-ray crystallographic analysis and biochemical characterization of Pex from cyanobacterium Synechococcus elongatus PCC 7942. The molecule has an ($\alpha + \beta$) structure with a winged-helix motif and is indicated to function as a dimer. The subunit arrangement in the dimer is unique and has not been seen in other winged-helix proteins. Electrophoresis mobility shift assay using a 25-base pair complementary oligonucleotide incorporating the kaiA upstream sequence demonstrates that Pex has an affinity for the double-stranded DNA. Furthermore, mutation analysis shows that Pex uses the wing region to recognize the DNA. The in vivo rhythm assay of Pex shows that the constitutive expression of the pex gene harboring the mutation that fails to bind to DNA lacks the period-prolongation activity in the pex-deficient Synechococcus, suggesting that Pex is a DNA-binding transcription factor.

Organisms from cyanobacteria to mammals have a circadian rhythm, an adaptation to diurnal environmental changes such as light and temperature. The timing machinery of the rhythm is known as the “circadian clock” (1, 2). The clock has three representative properties (free running, resetting, and temperature compensation), which are essential to a system of time measurement. In cyanobacteria, circadian rhythms have been reported in amino acid uptake (3), cell division (4, 5), and various gene expressions (6–8) at each particular time.

After a photosynthesis gene (psbAI) in a unicellular strain, Synechococcus elongatus PCC 7942, was shown to be transcribed rhythmically with a circadian period under constant conditions (9), more than 100 mutants with defects, including arrhythmia and rhythms with a long or short period, were isolated (10). The mutations were mapped to a gene cluster named kaiABC (11), which is transcribed as kaiA and kaiBC mRNAs by the respective kaiA and kaiBC promoters. Transcription of either the kaiA or kaiBC operon is under circadian feedback regulation, whereby the kaiA and kaiC proteins (KaiA and KaiC) activate and repress the promoter of kaiBC (11), and this feedback functions to maintain normal circadian sustainability, but not the period (12). KaiC has autokinase activity essential to the rhythm, where the kinase reaction is accelerated and decelerated by KaiA and KaiB protein (KaiB), respectively (13–15). The phosphorylation of KaiC recurs with a circadian period in vivo (16, 17), which can be reconstituted in vitro (18). This observation indicates that various heteromultimeric complexes of KaiA, KaiB, and KaiC are formed in the cell (15, 19).

In cyanobacteria, the clock is reset by a light or dark pulse (20, 21). Resetting-related mutants have been isolated and found to lack normal circadian resetting. Period extender (pex), mutants of which show a short-period phenotype (22), was recently classified as a resetting-related gene (23). Insertion of pex into the genome of the clock mutant C22a (which has a kaiA1 mutation) causes extension of the 22-h period phenotype of C22a to 24 h, similar to that of the wild type (10, 11). Levels of pex mRNA and pex protein (Pex) increase in the dark period, and a pex mutant subjected to diurnal light-dark cycles shows an advance in the phase of the rhythm by 3 h, suggesting that Pex has a resetting function (23). Sequence analysis demonstrates that Pex has a PadR domain, which is conserved among PadR proteins in lactobacilli Pediococcus pentosaceus and Pediococcus plantarum (24). PadR is a transcriptional regulator related to multiple antibiotic resistance repressor (MarR) family proteins with DNA-

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The atomic coordinates and structure factors (code 2E1N) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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3 The abbreviations used are: MarR, multiple antibiotic resistance repressor; EMSA, electrophoretic mobility shift assay; dsDNA, double-stranded DNA; SeMet, selenomethionine; GST, glutathione S-transferase; PBS, phosphate-buffered saline; RTP, replication terminator protein.
binding activity (25), and binds to the promoter of the padA gene, which is essential for metabolizing environmental toxins such as p-coumaric acid.

Despite these structural indications, however, no experimental evidence has been obtained regarding the function of Pex. To understand the molecular mechanism involved in regulating the circadian clock oscillation in cyanobacteria, we have determined the crystal structure of a N-terminal deletion mutant of Pex, Pex-(15–148), from S. elongatus PCC 7942 at 1.8-Å resolution by x-ray diffraction. To our knowledge, this is the first structure determination of a protein of the circadian input system in the cyanobacteria circadian clock. Pex-(15–148) has a winged-helix motif and is likely to function as a dimer with a unique subunit arrangement. Electrophoresis mobility shift assay (EMSA) and mutation analysis demonstrate that Pex has specific affinity for double-stranded DNA (dsDNA) containing the kaiA upstream site and that the wing region in the winged-helix motif has a crucial role in interaction with the DNA.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Genes encoding full-length Pex, Pex-(1–148), and its N-terminal deletion mutants (Pex-(15–148) and selenomethionine (SeMet)-labeled Pex-(15–148)) were subcloned into expression vector pGEX6P-1 (GE Healthcare) at the 5’ BamHI-XhoI site, and expressed as fusion proteins of glutathione S-transferase (GST) in E. coli strain BL21(DE3) for Pex-(1–148) and Pex-(15–148), and in E. coli strain B834(DE3) for SeMet-labeled Pex-(1–148) and Pex-(15–148). The cells were grown at 37 °C to a cell density of 0.4–0.6 mg/ml in a Centricon-10 concentrator (Amicon). The concentration of the purified protein was determined by UV absorption.

The cells were harvested, resuspended in lysis buffer (20 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 30 mM reduced glutathione). The fusion protein was cleaved by 100 units of thrombin (Amersham) equipped with an A’ KTA Prime system (GE Healthcare), according to the manufacturer’s recommendations, and confirmed by DNA sequencing. The mutants were expressed as fusion proteins of GST from a pGEX6P-1 vector in E. coli strain BL21(DE3), and purified by the same protocol used for wild-type Pex-(15–148), except that a HiTrap Q column (GE Healthcare) was used instead of the HiTrap heparin column.

**Crystallization and Data Collection**—Pex-(15–148) was successfully crystallized at 20 °C by the hanging drop vapor-diffusion method using 0.5–1.0 M Li2SO4 as a precipitant in 0.1 M sodium acetate buffer (pH 3.8 – 4.6) containing 50 mM magnesium acetate. Crystals of SeMet-labeled Pex-(15–148) were obtained under the same conditions. All x-ray diffraction data were collected at 100 K on beamline BL-5 at Photon Factory, Tsukuba, Japan, using an ADSC Quantum 315 CCD detector. Before the x-ray experiments, crystals of Pex-(15–148) and SeMet-labeled Pex-(15–148) were each soaked in the crystallization buffer containing 20% ethylene glycol as a cryoprotectant. Diffraction data were processed with HKL2000 (26). The crystallographic data and data collection statistics of Pex-(15–148) and SeMet-labeled Pex-(15–148) are given in Table 1.

**Structure Determination and Refinement**—No successful solutions were obtained by the molecular replacement method, in which the structures of MarR family proteins were used as search models by Molrep (27) and Phaser (28). This is attributable to significantly large differences in the ternary structures between Pex-(15–148) and the search models. The structure of Pex-(15–148) was therefore solved by multiwavelength anomalous diffraction using the SeMet-labeled Pex-(15–148) crystal.

Experimental phases were calculated up to 2.0-Å resolution with SOLVE (29) and improved by solvent-flattening with RESOLVE (29). An initial model was build by ARP/WARP (30), followed by O (31), and refined with CNS (32). After several cycles of rebuilding with program O and refinement with CNS and REFMAC5 (33), the model finally converged, resulting in a crystallographic R value of 19.6% and a free R value of 23.4% for all diffraction data up to 1.8-Å resolution. The Ramachandran plot of the final model, containing 222 amino acid residues, 144 water molecules, and 2 SO4$^{2-}$ ions, shows that all of the amino acid residues have good stereochemistry, with 95.0% of residues in the most favorable regions and 5.0% in additional allowed regions defined by the program PROCHECK (34). The final refinement statistics are summarized in Table 1.

The figures were generated by PyMol (35). Coordinates for Pex-(15–148) have been deposited in the Protein Data Bank of the Research Collaboratory for Structural Bioinformatics (Protein Data Bank code 2E1N).

**Site-directed Mutagenesis and EMSA**—Alanine mutants of Pex-(1–148), Y82A, T83A, K86A, D90A, R104A, R106A, and R108A, were prepared using pGEX6P-1 wild-type Pex-(1–148) as a template with a QuikChange site-directed mutagenesis kit (Stratagene), according to the manufacturer’s recommendations, and confirmed by DNA sequencing. The mutants were expressed as fusion proteins of GST from a pGEX6P-1 vector in E. coli strain BL21(DE3), and purified by the same protocol used for wild-type Pex-(15–148), except that a HiTrap Q column (GE Healthcare) was used instead of the HiTrap heparin column.

The DNA binding capacities of wild-type and mutant Pex were examined by EMSA using a 25-bp complementary oligonucleotide containing the kaiA upstream sequence including the −60 to −36 region (kaiA upstream site), 5′-ATTTTTTCCCCAGAGATTAAAT-3′ (as the 1st codon of kaiA is +1), which was purchased from Invitrogen. The dsDNA was prepared by annealing at a 1:1 molar ratio in 0.1 M KCl solution.
Each solution (5 μl) containing 5 pmol of DNA oligomer (1 μM final concentration) and a 1.5-fold excess of each of wild-type and mutant Pex was separated by electrophoresis at 4 °C on a native 6% polyacrylamide gel at 100 V for 90 min. The running solution was 40 mM Tris–HCl buffer (pH 7.0) containing 20 mM acetic acid and 1 mM EDTA. The shifted bands stained by ethidium bromide in the gel were detected by an Image Analyzer (TOYOBO FAS-III).

We also purchased the 25-bp DNAs of a 5-nucleotide downstream sequence (kaiA-1) and 21-nucleotide upstream sequence (kaiA-2) of the kaiA upstream site (5′-TCCTTTGT-CCAGAGATTAATCTGTC-3′; kaiA-1, 5′-TGCAGTGCTATGC-3′; kaiA-2). Then, bioluminescence assays were performed.

In Vivo Rhythm Assay—The bioluminescence reporter S. elongatus PCC 7942 cell NUC42 was used as wild-type, in which a gene fusion of a kaiBC promoter and a gene set of bacterial luciferase, luxAB, was recombined into a genomic region, neutral site I (12). The pex-deficient mutant cell, YCC19, derived from the kaiA upstream site (kaiA-1) was used (23). The pex-deficient mutant harboring the pex with an E. coli inducible promoter, Ptrc, in the genomic region neutral site II was used as the constitutive induction cell of pex. Then, we constructed the plasmid by which Pex(R106A) protein was expressed with Ptrc inducible promoter. At first we amplified the DNA fragment of the pex gene with base pair substitutions at the 106th codon for Arg to Ala by PCR with KOD DNA polymerase (TOYOBO). The amplified pex DNA was digested with the BamHI restriction enzyme and inserted into unique BamHI site downstream of the Pptrc promoter in the pTS2KPptrc plasmid. The obtained plasmid was checked by sequencing and then introduced into YCC19. These cyanobacterial cells were cultivated on BG-11 agar medium containing 0.1 mM isopropyl β-D-thiogalactopyranoside under continuous light until the colonies with 0.2 mm in diameter appeared. After resetting of the clock by treatment of 12-h darkness, a lid of a microcentrifuge tube containing 30 μl of vacuum pump oil with 3% bioluminescence substrate (n-decanal; Wako) was placed on the agar medium with the colonies. Then, bioluminescence were monitored under continuous light at 30 °C using an automated system equipped with photon-multiplier tubes, as previously described (11).

Western Blotting Analysis—About 5000 of the Synechococcus colonies of each cell were formed on BG-11 agar medium containing 0.1 mM isopropyl β-D-thiogalactopyranoside for 4 days in a continuous light condition. Then those colonies were subjected to 12 h dark. The wild-type and pex-deficient mutant colonies subjected to 6 h dark were collected with a spreader. After the 12-h dark treatment, colonies were illuminated for 6 h, then, collected. The cell pellet was suspended in 200 μl of Tris-buffered saline and sonicated to extract soluble protein as described previously (23). The cell extract containing 18 μg of total protein was electrophoresed through a 20% SDS-acrylamide gel and blotted onto a polyvinylidene difluoride membrane. The membrane was blocked in phosphate-buffered saline-Tween (0.3%; PBS-T) for 1 h. The anti-GST-Pex antiserum was

### TABLE 1
Crystallographic data, data collection, and refinement statistics

| Crystallographic data and data collection statistics | Edge | Peak | High remote | Low remote |
|-----------------------------------------------------|------|------|-------------|------------|
| Wavelength (Å) | 0.97931 | 0.96408 | 0.98319 | 1.03973 |
| Space group | \(P_{21}2_{1}2_{1}\) | \(P_{21}2_{1}2_{1}\) | \(P_{21}2_{1}2_{1}\) | \(P_{21}2_{1}2_{1}\) |
| \(a\) (Å) | 56.7 | 61.5 | 57.2 | 61.6 |
| \(b\) (Å) | 75.1 | 75.1 | 75.1 | 75.1 |
| \(c\) (Å) | 50.0-1.8 | 50.0-1.8 | 50.0-1.8 | 50.0-1.8 |
| Resolution (Å) | 152,594 | 153,133 | 153,211 | 153,165 |
| Total observations | 24,165 | 24,193 | 24,237 | 24,212 |
| Unique reflections | 5.9 (50.4) | 6.7 (50.9) | 5.2 (50.8) | 4.3 (50.1) |
| Completeness (%) | 97.2 (75.8) | 97.3 (77.8) | 97.1 (75.1) | 98.2 (85.3) |
| Redundancy (%) | 6.3 (3.1) | 6.4 (3.2) | 6.4 (3.2) | 7.2 (4.1) |
| \(I/\sigma(I)\) | 12.4 | 12.5 | 12.4 | 12.5 |
| Mean figure of merit | 0.69 | | | |

#### Refinement statistics

| Resolution range (Å) | 32.2-1.80 |
| \(R\) (%) | 19.6 |
| \(R_{free}\) (%) | 23.4 |
| Mean B factor (Å²) | 33.3 |
| Root mean square deviations | |
| Bond length (Å) | 0.015 |
| Bond angle (°) | 1.461 |

#### No. of atoms

| Protein | 1900 |
| Water molecules | 144 |
| SO₄²⁻ ions | 10 (2 SO₄⁻) |

\(a\) Values in parentheses are for highest resolution shell. The resolution ranges of their outer shells are 1.86-1.80 Å for native data and 1.86-1.80 Å for Se-derivative data.

\(b\) Phasing power = root mean square heavy atom structure factor/residual lack of closure.

\(c\) Phasing power = root mean square heavy atom structure factor/residual lack of closure.

\(d\) \(R_{free}\) = \(\Sigma I(F_{o}) - \Sigma I(F_{c})/\Sigma I(F_{o})\), where the free reflections (5% of the total used) were held aside for \(R_{free}\) throughout refinement.
diluted 100-fold in PBS-T and used as primary antibody for 1 h. After washing the membrane in PBS-T, horseradish peroxidase-linked anti-rabbit Ig was diluted in PBS-T (1/1000) and used as the secondary antibody (GE Healthcare). These procedures were performed at room temperature except the blotting at 5 °C. The chemiluminescence substrate was Immun-Star HRP Luminol/Enhancer, used with the Fluor-S Multilimager chemiluminescence imaging system (Bio-Rad).

RESULTS

Crystal Structure—The crystal structure of Pex-(15–148) from cyanobacterium S. elongatus PCC 7942 was successfully solved by the multiwavelength anomalous diffraction method and refined at 1.8-Å resolution. The molecule has an (α + β) structure, which forms a winged-helix motif together with two additional α helices in the N-terminal (α1 helix, residues 25–33) and C-terminal (α4 helix, residues 120–131) regions (Fig. 1A). The winged-helix motif consists of helices α1 (residues 42–53), α2 (residues 60–70), and α3 (residues 78–90), strands β1 (residues 94–99) and β2 (residues 108–113), and the wing region that connects strands β1 and β2. The amino acid sequence of the molecule, together with assignments of the secondary structure elements, is shown in Fig. 2.

Two molecules (molecules A and B), which are related by a non-crystallographic 2-fold axis, are present in the crystal asymmetric unit (Fig. 3A). They have essentially the same structure with root mean square deviations of 1.1 Å for Cα atoms from residues 23 to 132. Residues 15–22 and 133–148 in molecule A and residues 15–22 and 135–148 in molecule B were disordered. Molecule A is in close contact with molecule B, forming a dimer in the crystal. The contact is hydrophobic in nature, with an interface of 1,150 Å² between helix α4 (Leu124, Leu127, and Tyr131) in molecule A and helix α0 (Met23, Phe25, Ile28, Tyr29, Phe31, and Phe32) in molecule B (Fig. 3B).

In addition to these structural features, the Fo − Fc map showed a higher peak with a contour level of more than 13 σ near the wing region (Fig. 1, A and B). On the basis of the chemical components of the crystallization solution and the fact that the peak was near basic residues such as Arg104, Arg106, and Arg108 in the wing region (Fig. 1A), this peak was assigned to SO₄²⁻. Interestingly, these three arginine residues are completely conserved among all cyanobacterial Pex proteins (Fig. 2).
Comparison of Pex with Other Winged-helix Proteins—Pfam (36) predicts that Pex belongs to the PadR protein family, which is related to the MarR family of proteins. In fact, a search of the Protein Data Bank using the Dali server (37) showed that Pex is similar to replication terminator protein (RTP) of Bacillus subtilis (PDB code 1BM9, Z-score 10.0) (38), metallothionine repressor (SmtB) of Synechococcus PCC 7942 (PDB code 1SMT, Z-score 9.0) (39), methicillin repressor (MecI) of Staphylococcus aureus (PDB code 1OKR, Z-score 8.8) (40), and MarR of E. coli (PDB code 1JGS, Z-score 8.3) (41). Although their amino acid sequences are not homologous to that of Pex (sequence identity 10–19%), these proteins all have winged-helix motifs for DNA binding.

The winged-helix motifs of Pex, RTP, SmtB, MecI, and MarR could be superimposed with root mean square deviations ranging from 2.4 to 4.0 Å. There is a large difference in a linker region between the α2 and α3 helices. The linker of Pex is significantly longer than those of the other four proteins and thus Asn73–Arg75 in this long linker of Pex can interact with His38–Leu40 in the N-terminal portion that precedes the winged-helix motif (Fig. 1, A and C). This interaction is not observed in the other four proteins due to conformational differences in the N-terminal region of the winged-helix motif. The arrangement of helix α4 in relation to the winged-helix motif also differs between Pex and the other four proteins (Fig. 4). In Pex, the α4 helix leans on the winged-helix motif, and Trp128, Tyr131, and Leu132 in the α4 helix form a hydrophobic core with Leu40, Leu44, Tyr48, Leu67, Trp71, and Tyr74 in the winged-helix motif (Fig. 4, A and D).

The four proteins that are similar to Pex (RTP, SmtB, Mecl, and MarR) are known to function as a dimer (38–41). The dimers of these proteins, however, form via different arrangements of the subunits. In RTP, a coiled-coil interaction between long C-terminal helices participates in dimerization (Fig. 4B) (38). In MarR and SmtB, interactions between long N- and C-terminal helices contribute to dimer formation (Fig. 4, C and D) (39, 41). In MecI, a dimerization domain consisting of a large N-terminal helix is involved in dimer formation (Fig. 4E) (40). In Pex, the N- and C-terminal helices (α0 and α4) of molecules A and B interact to form a dimer in the crystal (Fig. 4A). Therefore, the subunit arrangement in the Pex dimer is unique and has not been observed in the other winged-helix proteins. These differences in the subunit arrangements might correspond to variations in the manner of DNA recognition by these proteins.

DNA Binding—Pex has a winged-helix motif, which is a well known DNA-binding motif (Fig. 1A). The electrostatic surface potential of Pex, however, is not highly positively charged (Fig. 5). This feature contrasts with other DNA-binding proteins, in which positively charged areas are distributed over the molecular surfaces that interact with DNA. To establish whether or not Pex has affinity for DNA, EMSA was performed using dsDNA containing the kaiA upstream site. Fig. 6A clearly shows that both Pex-(1–148) and Pex-(15–148) have DNA binding activity (Fig. 6A).

Moreover, we also performed the EMSA experiment to examine whether or not Pex bind to the kaiA upstream site in a specific manner (Fig. 6C). The two 25-bp kaiA upstream DNAs, kaiA-1 and kaiA-2, and psbAII were tested. Pex completely abolished the binding affinity against kaiA-1, kaiA-2, and psbAII DNAs (Fig. 6B).

DNA Recognition—DNA-binding proteins with winged-helix motifs form complexes with dsDNA in different protein-DNA arrangements. In most cases, for example, in hepatocyte nuclear factor-3γ (43) and RTP (38), a recognition helix (α3 in Pex) mainly interacts with the major groove of the dsDNA, and the wing region interacts with the minor groove or the phosphate backbone. On the other hand, in a few cases, for example, in human regulatory factor X (44), the wing region interacts with the major groove, whereas the recognition helix forms a weak contact with the minor groove. The fact that the SO₃⁻ ion, which is known to mimic the phosphate backbone of DNA, is bound to the wing region in Pex (Fig. 1, A and B) supports the idea that Pex interacts with DNA via its wing region.

To determine experimentally whether the wing region or the recognition helix (α3) of Pex has a crucial role in DNA binding, alanine mutants of Tyr82, Thr83, Lys86, and Asp90 in the recognition helix (α3) and Arg104, Arg106, and Arg108 in the wing region were prepared and subjected to EMSA (Fig. 6B). On the one hand, the DNA-binding activities of the alanine mutants of Thr83 and Asp90 were not reduced as compared with wild-type Pex, but those of the mutants of Tyr82 and Lys86 were consid-
erably impaired. On the other hand, the DNA-binding activities of the alanine mutants of Arg\textsuperscript{104}, Arg\textsuperscript{106}, and Arg\textsuperscript{108}, which are located in the wing region and coordinated with SO\textsubscript{4}\textsuperscript{2−}, were completely abolished. These results demonstrate that Pex interacts with dsDNA mainly via its wing region.

**In Vivo Rhythm Assay**—To examine physiological significance of the DNA-binding activity of the Pex in vivo, the pex allele that corresponds to Pex(R106A) were introduced into the pex-deficient mutant cell, which showed an abnormal bioluminescence rhythm with a period 1-h shorter than that of the wild type (Fig. 7A). The constitutive induction of the wild-type pex gene in the deficient mutant resulted in rhythm with longer periods of about 3 h than that of the parental mutant (Fig. 7A), whereas the induction of the mutant gene that corresponds to
We have determined the crystal structure of Pex from cyanobacterium *S. elongatus* PCC 7942 using an N-terminal deletion mutant, Pex-(15–148), at 1.8-Å resolution. At first, we succeeded in crystallizing wild-type Pex, Pex-(1–148), but the crystals proved unsuitable for high-resolution x-ray crystallographic analysis. Several new constructs were therefore prepared on the basis of secondary structure prediction, and were subjected to expression, purification, crystallization, and preliminary x-ray diffraction experiments. As a result, crystals of the N-terminal deletion mutant Pex-(15–148) were obtained and found to diffract x-rays beyond 2-Å resolution. This finding indicates that the N-terminal portion of Pex-(1–148) affects crystallization for high-resolution x-ray analysis. In fact, the sequence alignment of the Pex protein among cyanobacteria (Fig. 2) shows that two other cyanobacterial Pex proteins lack N-terminal residues corresponding to residues 1–22 of Pex from *S. elongatus*. These residues precede the α0 helix and are not involved in the close dimerization contacts between molecules A and B (Fig. 3). Electron density corresponding to the eight N-terminal residues (residues 15–22) of Pex-(15–148) was not observed. These results strongly suggest that the N-terminal region that precedes the α0 helix has no physiological function in the cell and may be labile.

The two Pex molecules in the crystal asymmetric unit are in close hydrophobic contact with each other, and the residues that participate in this close contact (Fig. 3) are conserved among cyanobacterial Pex proteins (Fig. 2). This observation indicates that Pex functions as a dimer, as formed in the crystal. In fact, on size-exclusion chromatography, Pex-(1–148) eluted at a position corresponding to a globular protein with a molecular mass of 47,000, which is consistent with the mass of a dimeric or trimeric form of Pex-(1–148). Because Pex forms an elongated shape that flows fast in the gel, the molecular weight would be estimated higher. Furthermore, Pex-(39–139), in which helix α0 that contributes to dimer formation is deleted, did not adsorb to the HiTrap heparin column, which is known to have affinity for DNA-binding proteins (data not shown). These results demonstrate that the dimer formed in the crystal is the functional unit of Pex and plays a crucial role in DNA binding.

To examine the DNA-binding activity of Pex, at first we performed EMSA using 61-bp dsDNA containing the *kaiA* upstream sequence including the −81 to −21 region, the resulting Pex interacted with the DNA. Subsequently, we refined the sequence by deleting 5′- and 3′-terminal nucleotides, resulting in a 5′-ATTTTTCCTTTGTCCAGAGATT-AAT-3′ sequence requirement for Pex binding.

EMSA using a 25-bp dsDNA of the *kaiA* upstream site verified that Pex binds directly to dsDNA (Fig. 6A). Moreover, Pex completely abolished binding affinity against *kaiA*-1, *kaiA*-2, and *psbAII* DNAs (Fig. 6C), indicating that Pex specifically recognizes the *kaiA* DNA in our experimental condition, whereas the physiological function of Pex is unknown. Mutation analysis demonstrated that the wing region, which has a partial positive charge (Fig. 5), mainly interacts with the dsDNA, whereas the recognition helix (α3) forms weak contacts with the dsDNA. The mutated Pex (Y82A,K86A) in α3 helix shows a weak interaction with dsDNA, suggesting that these residues of α3 helix would be involved in DNA recognition. However, the binding affinity for DNA in the mutation of the wing region is completely abolished. This result suggests that Pex interacts with dsDNA.
with dsDNA mainly via its wing region, but Lys\textsuperscript{86} and Tyr\textsuperscript{82} in α3 helix contributes the DNA recognition. In fact, in the structure of the hRFX1-DNA complex, Lys in the α3 helix recognizes DNA, whereas the wing region makes most of the contacts with DNA via its major groove (44).

We performed the in vivo rhythm assay, resulting that the induction of the mutant pex (Pex(R106A)) in the Synechococcus cell had no physiological activity in the rhythm. This result strongly suggests the role of DNA binding in Pex function.

The distance between the two wing regions in the Pex dimer is ~70 Å, which is equal to twice the distance between adjacent major (or minor) grooves and corresponds to a distance of two turns of a double-helical DNA corresponding to 20-bp. This organization is favorable for the Pex dimer to interact at its two wing regions with the 25-bp dsDNA containing the kaiA upstream sequence. Fig. 5 shows a potential model of the Pex-DNA complex, in which each of the wing regions interact with a major groove of the dsDNA. It remains unknown, however, whether the two wing regions interact with the major grooves or the minor grooves of the dsDNA. A clear answer on the interaction between Pex and dsDNA will be obtained from x-ray crystallographic analysis of a Pex-DNA complex, which is in progress. In addition, further structural analysis, together with biochemical and cellular studies, will extend our understanding of the cellular functions of Pex.

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