ldpA Encodes an Iron-Sulfur Protein Involved in Light-Dependent Modulation of the Circadian Period in the Cyanobacterium *Synechococcus elongatus* PCC 7942

Mitsunori Katayama,[1,2]† Takao Kondo,[2] Jin Xiong,[1] and Susan S. Golden[1]*

Department of Biology, Texas A&M University, College Station, Texas 77843-3258,[1] and Division of Biological Science, Graduate School of Science, Nagoya University, and CREST, Japan Science and Technology Corporation, Chikusa-ku, Nagoya 464-8602, Japan[2]

We generated random transposon insertion mutants to identify genes involved in light input pathways to the circadian clock of the cyanobacterium *Synechococcus elongatus* PCC 7942. Two mutants, AMC408-M1 and AMC408-M2, were isolated that responded to a 5-h dark pulse differently from the wild-type strain. The two mutants carried independent transposon insertions in an open reading frame here named *ldpA* (for light-dependent period). Although the mutants were isolated by a phase shift screening protocol, the actual defect is a conditional alteration in the circadian period. The mutants retain the wild-type ability to phase shift the circadian gene expression (bioluminescent reporter) rhythm if the timing of administration of the dark pulse is corrected for a 1-h shortening of the circadian period in the mutant. Further analysis indicated that the conditional short-period mutant phenotype results from insensitivity to light gradients that normally modulate the circadian period in *S. elongatus*, lengthening the period at low light intensities. The *ldpA* gene encodes a polypeptide that predicts a 7Fe-8S cluster-binding motif expected to be involved in redox reactions. We suggest that the LdpA protein modulates the circadian clock as an indirect function of light intensity by sensing changes in cellular physiology.

Light is a major environmental signal that synchronizes circadian rhythms in various organisms to coordinate the daily oscillations of biological phenomena with the sidereal day. The input pathways that receive and transmit environmental signals to the central circadian oscillator of circadian systems vary among organisms, particularly in the mechanisms of perception of light signals. In mammals, light that sets the circadian clock is received by membrane-associated opsin proteins in the eyes that carry retinal chromophores (15, 39). In *Drosophila*, both opsin-based photopigments and the blue light photoreceptor cryptochrome function as photoreceptors for input pathways (39). In *Neurospora*, the putative transcription factors WC-1 and WC-2, which interact with FRQ, a critical element of the circadian system, are necessary for light perception (10, 25). In higher plants, phytochromes and cryptochrome transduce light signals to the clock (38).

Cyanobacteria are the only prokaryotes demonstrated thus far to have bona fide circadian systems that have the properties of those in eukaryotic organisms (13). In various cyanobacterial genera, the circadian clock has been shown to regulate such diverse biological activities as nitrogen fixation (14), amino acid uptake (8), and gene expression (22, 24). Several genes related to circadian clock function have been identified in the unicellular cyanobacterium *Synechococcus elongatus* PCC 7942, a strain for which many genetic tools are available.

These include the *kaiA*, *kaiB*, and *kaiC* genes, essential components for circadian rhythms (16).

One basic aspect of a circadian rhythm is the free-running period (FRP), which is the duration of the oscillation, peak to peak or trough to trough, under constant environmental conditions (4). Several loci in *S. elongatus* are known to affect the FRP under conditions of constant light and temperature. Many alleles of the *kaiA*, *kaiB*, and *kaiC* genes affect the FRP (16). An extra copy of the *pex* gene, which encodes a protein of unknown function, extends the circadian period by about 2 h (23). The *sasA* gene, which encodes a histidine protein kinase, amplifies circadian oscillation and may be a major conduit for temporal information out of the clock to the genes it controls (17). The residual rhythmicity in an *sasA* mutant has a short FRP. The only component known to be involved in an input pathway for resetting the clock is the histidine protein kinase CikA, which belongs to the bacteriophytochrome family of proteins (32). Mutants defective in *cikA* have a short FRP.

In this study, we identified the *ldpA* (light-dependent period) gene of *S. elongatus* PCC 7942 as encoding a new component of an input pathway to the cyanobacterial clock. Its predicted product is a previously undescribed protein that carries iron-sulfur cluster-binding motifs. Disruption of *ldpA* significantly attenuated the ability of *S. elongatus* to modulate the FRP under different light intensities. Our results suggest that the circadian clock is modulated by the redox state in cyanobacteria, acting at least in part through the iron-sulfur protein LdpA.

* Corresponding author. Mailing address: Department of Biology, Texas A&M University, 3258 TAMU, College Station, TX 77843-3258. Phone: (979) 845-9824. Fax: (979) 862-7659. E-mail: sgolden@tamu.edu.

† Present address: University of Tokyo, Meguro, Tokyo 153-8902, Japan.

MATERIALS AND METHODS

Strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All *Synechococcus* strains were grown in modified

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristic(s) or genotype
E. coli strains	
AM1452[a]	HB101 with helper plasmid pRL528 and transposon plasmid pAM1037
AM1460	HB101 with plasmid pRK2013[b] to provide conjugal transfer functions
DH10B	Host for plasmids
S. elongatus strains	
PCC 7942	Wild type
C22a[b]	Mutant of AMC149; point mutation in *kaiC* gene (C3535T) causes short circadian period (formerly called SP22)
AMC149	P*psbAI*::*luxAB* reporter inserted into NSI (Sp^r)
AMC403	P*psbAI*::*luxAB* reporter inserted into NSII (Km^r)
AMC408	P*purF*::*luxAB* inserted into NSII (Cm^r), P*psbAI*::*luxCDE* inserted into NSI (Sp^r)
AMC408-M1	Mutant of AMC408 with transposon insertion at nucleotide 729 of *ldpA* ORF
AMC408-M2	Mutant of AMC408 with transposon insertion at nucleotide 303 of *ldpA* ORF
AMC548	Derivative of C22a with P*psbAI*::*luxCDE* in NSII (Cm^r)
AMC664	Derivative of AMC408 with *ldpA* inactivated by recombination with pAM2188
AMC665	Derivative of AMC548 with *ldpA* inactivated by recombination with pAM2188
AMC693	P*purF*::*luxAB* reporter gene fusion inserted into NSI (Sm^r)
AMC694	Derivative of AMC693 with *ldpA* inactivated by recombination with pAM2180 and *psbAI*::*luxCDE* gene fusion introduced into NSII (Cm^r) by recombination with pAM1706
AMC695	Derivative of AMC693 with *ldpA* inactivated by recombination with pAM2181 and *psbAI*::*luxCDE* gene fusion introduced into NSII (Cm^r) by recombination with pAM1706
AMC696	Derivative of AMC693 with *ldpA* inactivated by recombination with pAM2180 and ectopic copy of *ldpA* and P*psbAI*::*luxCDE* gene fusion introduced into NSII (Cm^r) by recombination with pAM2186
AMC697	Derivative of AMC693 with *ldpA* inactivated by recombination with pAM2181 and ectopic copy of *ldpA* and P*psbAI*::*luxCDE* gene fusion introduced into NSII (Cm^r) by recombination with pAM2186
Plasmids	
pAM1037[c]	Tn5 plasmid derivative pRL1058 further modified by insertion of 0.7-kb fragment into *Xba*I site to add outward-reading promoters from *Anabaena* sp. strain PCC 7120 *glnA* and *rbcL* genes
pAM1706	Vector that transfers P*psbAI*::*luxCDE* fusion to NSII (subsite NS2.2, *Bgl*II) of *S. elongatus* chromosome
pAM2180	Tn5-containing plasmid derivative recovered from mutant AMC408-M1 by *Kpn*I digestion and circularization
pAM2181	Tn5-containing plasmid derivative recovered from mutant AMC408-M2 by *Kpn*I digestion and circularization
pAM2182	pBluescript II SK+ containing 3.7-kb *Eco*RI-*Hpa*I fragment of pAM2180 at *Eco*RI-*Sma*I sites
pAM2183	PBluescript II SK+ containing 2.3-kb *Hpa*I-*Bgl*II fragment of pAM2181 at *Sma*I-*Bam*HI sites
pAM2184	Entire ORF and 5'- and 3'-flanking regions of *ldpA* inserted into pBluescript II SK+ at *Eco*RI-*Bam*HI sites
pAM2185	pBluescript II KS+ containing 1.3-kb *Pvu*II fragment including *ldpA* ORF at *Eco*RV site in same orientation as *lacZ* promoter
pAM2186	Derivative of pAM1706 with P*lacZ*::*ldpA* fusion upstream of *psbAI*::*luxCDE*
pAM2188	Gentamicin resistance cassette inserted into *Alw*NI site of pAM2188

[a] Reference 20.
[b] Reference 16.
[c] Reference 2.

BG-11 medium (BG-11 M) (6) or on BG-11 M agar plates under continuous light (100 microeinsteins m^{-2} s^{-1}) at 30°C. Spectinomycin (5 μg ml^{-1}), chloramphenicol (7.5 μg ml^{-1}), kanamycin (5 μg ml^{-1}), or gentamicin (1 μg ml^{-1}) was used as needed for selection of *S. elongatus* transformants.

Transposon mutagenesis. A modified Tn5 transposon was introduced into wild-type AMC408 by conjugal transfer from *Escherichia coli* of pAM1037, a derivative of pRL1058, as described previously (20). AMC408 carries a P*purF*::*luxAB* reporter that exhibits class 2 circadian bioluminescence rhythms (24). Restriction enzymes and modifying enzymes were purchased from Promega and used as directed by the manufacturer. DNA flanking the transposon was recovered by extracting genomic DNAs from exconjugants, digesting them with *Kpn*I, circularizing them with T4 DNA ligase, and introducing them as plasmids into *E. coli* DH10B by electroporation (2, 20). Km^r plasmids were recovered, and nucleotide sequences of the genomic regions flanking the transposons were determined by cycle sequencing (dye terminator cycle sequencing ready reaction; ABI PRISM; PE Applied Biosystems, Foster City, Calif.) with primers AMO134 and AMO285, which are complementary to each end of the transposon.

Sequence analysis and Fe-S motif determination. Sequence motif determination was performed by using multiple approaches. The full-length LdpA sequence was first used to search against the nonredundant protein database and the protein structural database (PDB) at the National Center for Biotechnology Information by using BLAST (1) (http://www.ncbi.nlm.nih.gov/BLAST/). In this search, we used the BLOSUM45 amino acid substitution matrix, with a gap opening penalty of 11, an extension penalty of 1, an Expect value of 10, and a word size of 3. A more sensitive database search using a Bayesian algorithm (37)

was carried out with the BALSA program (http://bayesweb.wadsworth.org /balsa/balsa.html). Sequence motifs were further searched for the LdpA amino acid sequence by using the FingerPRINTScan (http://www.bioinf.man.ac .uk/fingerPRINTScan/), eMOTIF Search (http://motif.stanford.edu/emotif /emotif-search.html), Block (http://blocks.fhcrc.org/blocks/blocks_search.html), and InterPro (http://www.ebi.ac.uk/interpro/) servers. Structural fold prediction was done by using a threading server, BIOINBGU (http://www.cs.bgu.ac.il /~bioinbgu/form.html). Multiple-sequence alignment of LdpA with three ferredoxins with known 7Fe-8S structures was done by using the T-COFFEE server (http://www.ch.embnet.org/software/TCoffee.html), followed by manual refinement. To assess the statistical significance of sequence similarities between LdpA and 7Fe-8S ferredoxins, we used an alignment-independent program, PRSS, that calculates the probability of similarities of randomly shuffled and unshuffled sequences by using a distance matrix Monte Carlo procedure (29). The analysis was done in the Biology Workbench web server (http://workbench.sdsc.edu/). The gap-opening penalty was set at 12, and the gap-extending penalty was set at 2, and 1,000 global shuffles were performed by using the BLOSUM50 scoring matrix.

Construction of plasmids for complementation and disruption of *ldpA*. An intact copy of *ldpA* was reconstructed from the transposon insertion-containing alleles recovered from AMC408-M1 and AMC408-M2. We excised a 3.7-kb *Eco*RI-*Hpa*I fragment that includes the N terminus-encoding and upstream flanking regions of *ldpA* from pAM2180 (Km^r plasmid recovered from AMC408-M2; Fig. 2) and inserted it into *Eco*RI- and *Sma*I-digested pBluescriptII SK+ to create pAM2182. The 2.3-kb *Hpa*I-*Bgl*II fragment, which encodes the C-terminal

portion and downstream flanking region of *ldpA*, was excised from pAM2181 (Km^r plasmid recovered from AMC408-M1) and inserted into *Sma*I- and *Bam*HI-digested pBluescriptII SK+ to create pAM2183. After complete digestion of pAM2182 with *Eco*RI and its partial digestion with *Pst*I, a 2.9-kb *Eco*RI-*Pst*I fragment was cloned into *Eco*RI- and *Pst*I-digested pAM2183 to create pAM2184. pAM2184 contains the entire open reading frame (ORF) and the upstream and downstream flanking regions of *ldpA*. The 1.3-kb *Pvu*II fragment that contains *ldpA* was excised from pAM2184 and cloned into *Eco*RV-digested pBluescriptII SK+ to create pAM2185. A 1.7-kb *Pvu*II fragment in which *ldpA* is downstream of the *lacZ* promoter was excised from pAM2185 and cloned into *Sma*I-digested pAM1706 to create pAM2186, a vector that targets inserted genes to an *S. elongatus* locus called neutral site II (NSII; GenBank accession no. U44761). In this plasmid, *ldpA* is inserted upstream of the *luxCDE* genes (present to direct synthesis of the aldehyde substrate of the luciferase reporter) but transcribed divergently. The recreated transposon mutants AMC694 and AMC695 were transformed with pAM2186 to create complemented strains AMC696 and AMC697, respectively.

For directed disruption of *ldpA*, pAM2185 was partially digested with *Alw*NI and made blunt ended by using T4 DNA polymerase. Into the linearized plasmid, we inserted a 2.0-kb *Pvu*II-*Sma*I fragment from pAM2055 that includes a gentamicin resistance (Gm^r)-encoding gene to create pAM2188. We transformed wild-type P*purF*::*luxAB* reporter strain AMC408 and short-period (22 h) mutant AM548 with pAM2188 to create AMC664 and AMC665, respectively. Complete segregation of the mutant allele was confirmed by PCR with primers 5′-AGAA CTTCGGGATGGGC-3′ and 5′-GAACGTCTAACAGGACG-3′, which cross the insertion site of the gene encoding Gm^r.

Assay of bioluminescence rhythms. The original Tn*5* insertion mutants were identified by bioluminescence monitoring with a Packard TopCount luminometer (2). Briefly, mutant exconjugants grown on BG-11 M agar in continuous light (LL) were inoculated into BG-11 M and incubated on a rotary shaker for 2 weeks. Cyanobacterial suspensions (10 μl) at an optical density at 750 nm (OD$_{750}$) of 0.7 were used to inoculate BG-11 M agar pads in 96-well sample plates. These plates were incubated under standard LL conditions for 24 h and then subjected to a 12-h dark interval to synchronize the clocks of all of the cells in the population prior to bioluminescence monitoring on the TopCount luminometer. Illumination around the TopCount stackers results in a light gradient over the 96-well measuring plates, such that outer wells (columns 1 and 12) receive a fluence of approximately 225 microeinsteins m^{-2} s^{-1} and the inner wells (columns 6 and 7) receive approximately 50 microeinsteins m^{-2} s^{-1}.

For measurement of the bioluminescence rhythm under turbidostatic cultivation conditions, 150-ml cyanobacterial suspensions with an OD$_{730}$ of 0.7 to 1.0 were used to inoculate 1.4 liters of BG-11 M in flat bottles. The cultures were bubbled with air under standard LL conditions and incubated until the OD$_{730}$ reached 0.25. The cultures were then subjected to a 12-h dark interval to synchronize their circadian rhythms. Cell density was monitored by measurement of infrared light penetration into the cell suspension. An increase in OD$_{730}$ triggered a switch that activated a pump to add fresh medium and keep an OD$_{730}$ of 0.25. An aliquot (24 ml) was withdrawn every hour, and bioluminescence was measured by a photomultiplier tube apparatus. The output of the photomultiplier tube was amplified electronically and read by a computer analog-to-digital converter. The reading was averaged over the measurement period and plotted on the ordinate in millivolts as bioluminescence. Phase shifts and FRPs were calculated by regression procedures as described previously (32).

RESULTS

Screening for light input mutants. We generated random transposon mutations in a P*purF*::*luxAB* bioluminescent reporter strain of *S. elongatus* PCC 7942 (AMC408) and screened for mutants affected in light input pathways to the circadian system. In all screenings, a 12-h dark interval or a temperature cycle was used for synchronization of the circadian rhythm first, and then photic signals (5- or 12-h dark interval) were applied at specific times of the day that induce a circadian phase shift in the wild type. We chose exconjugants that showed phases different from that of the wild-type strain after the second dark pulse as candidate input mutants.

We used three screening protocols to accommodate mutants that might or might not be synchronized initially by photic
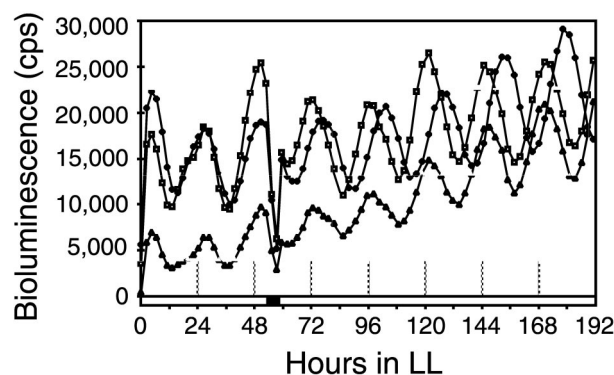

FIG. 1. Traces of circadian rhythms of bioluminescence from P*purF*::*luxAB* reporter strains. Symbols: diamonds, wild type; triangles, mutant AMC408-M1; squares, mutant AMC408-M2. Hour zero in LL indicates the time point of the end of the 12-h dark interval used for initial synchronization. The black bar indicates the time of application of a 5-h dark pulse for phase resetting.

signals. All of the protocols used a 5-h dark pulse that, for wild-type *S. elongatus*, would reset the relative phasing of circadian rhythms by as much at 10 h, depending on the stage in the circadian cycle at which it was administered (32). The protocols were as follows: (i) a 12-h dark interval to synchronize rhythms, followed by a transfer to LL and a 5-h dark pulse that started 53 h after the transfer to LL (56 mutant candidates among 700 exconjugants); (ii) a temperature cycle of 12 h at 40°C, 12 h at 30°C, and 12 h at 40°C in LL, followed by a 12-h dark interval that began 36 h after the end of the last 40°C pulse (234 mutant candidates among 2,600 exconjugants); and (iii) a temperature cycle of 12 h at 40°C, 12 h at 30°C, and 12 h at 40°C in LL, followed by a 5-h dark pulse that began 53 h after the end of the last 40°C pulse (93 mutant candidates among 700 exconjugants). From these candidate input mutants, we chose five exconjugants that reproducibly showed phases different from that of the wild-type strain after the phase-resetting dark pulse and recovered the transposons and flanking DNA as Km^r plasmids. The mutants were recreated by transforming AMC408 with the plasmids that had been linearized by cleavage with the restriction enzyme used for their recovery. Two mutants, AMC408-M1, which was originally isolated by protocol 3, and AMC408-M2, isolated by protocol 1, retained the phenotypes expected for input mutants (Fig. 1). These mutants also had a slightly shorter free-running circadian period than the wild-type strain under these screening conditions (Fig. 1).

Identification of transposon insertion sites. To identify the sites of mutations in AMC408-M1 and AMC408-M2, we determined the nucleotide sequences of the genomic regions flanking the transposons. Nucleotide sequencing revealed that the two strains carry independent insertions in the same ORF, as shown in Fig. 2. We designated this ORF the *ldpA* gene on the basis of phenotypic characteristics described below.

Translation of the *ldpA* ORF and comparison to similar sequences in public databases suggested that the start codon is a GTG and that LdpA is a soluble protein of 352 amino acid residues. Putative *ldpA* homologs (percent amino acid identity) are encoded in the genomes of *Synechocystis* sp. strain PCC
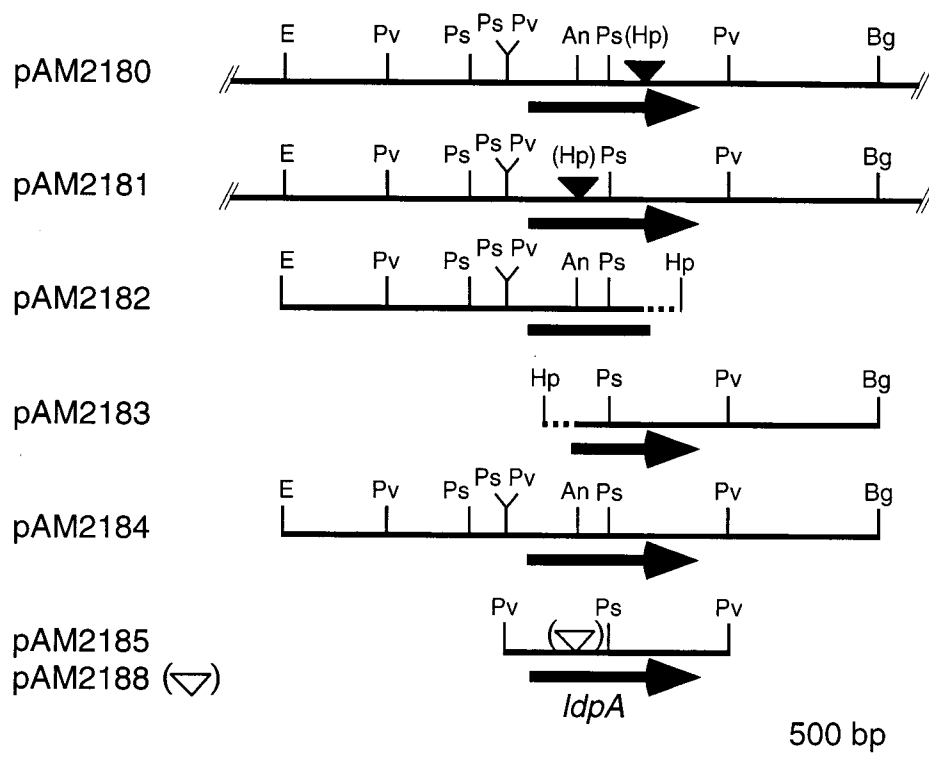
FIG. 2. Physical map of the *ldpA* region of the *S. elongatus* chromosome and plasmids used to reconstruct intact and disrupted *ldpA* alleles. Restriction sites are indicated by the following abbreviations: E, *Eco*RI; Pv, *Pvu*II; Ps, *Pst*I; An, *Alw*NI; Bg, *Bgl*II; Hp, *Hpa*I. Insertion sites of the transposon and Gm^r cassette are indicated by filled and open triangles, respectively. Broken lines indicate regions derived from the transposon. Plasmids pAM2180 and pAM2181 were recovered by restriction digestion and circularization from genomic DNA of mutants AMC408-M1 and AMC408-M2, respectively.

6803 (*sll0031*, 49%), *Anabaena* sp. strain PCC 7120 (*alr2308*, 44%), *Nostoc punctiforme* ATCC 29133 (44.0%), marine *Synechococcus* sp. strain WH8102 (43%), and *Prochlorococcus marinus* strain MED4 (35%), indicating conservation among diverse cyanobacteria.

Determination of an LdpA Fe-S motif. The deduced amino acid sequence of LdpA was first searched against the National Center for Biotechnology Information nonredundant database by using a gapped BLAST (1). Seven significant database hits ($E < 10^{-6}$) were recovered that were all annotated as hypothetical proteins from recently sequenced cyanobacterial genomes. A similar BLAST search against a more restricted database for protein structures (PDB) was done that generated

no significant hits. However, the best hit (PDB code 1H98) was a ferredoxin with 3Fe-4S and 4Fe-4S centers from *Thermus thermophilus* (Table 2). A more sensitive database search was conducted with a Bayesian algorithm (37) that identified significant hits as a ferredoxin from *Clostridium acidurici* (PDB code 1FCA) with two 4Fe-4S centers and another from *Azotobacter vinelandii* with 7Fe-8S centers (Table 2).

To determine more accurately the type of Fe-S centers likely to be present in LdpA, we analyzed the sequence by using multiple motif prediction programs that have different sensitivities for certain domains because of intrinsic differences in algorithms and database content (Table 2). Indeed, our searches indicated the presence of 4Fe-4S, 3Fe-4S, and 7Fe-8S

TABLE 2. Determination of Fe-S center types in LdpA by using multiple sequence motif search methods

Center type	Statistical support[a]						
	BLAST against PDB	BALSA	FingerPRINTScan	eMOTIF	Block	InterPro[b]	BIOINGBU[c]
4Fe-4S			5.10×10^{-6}		9.90×10^{-6}	+	
3Fe-4S				1.34×10^{-3}		+	
7Fe-8S	0.40	3.68×10^{-4}	4.40×10^{-8}		1.20×10^{-5}	+	14.8
8Fe-8S		1.70×10^{-6}					12.7

[a] Only the most significant (or best) hits are shown. Statistical support (*P* or *E* value) is indicated whenever available.
[b] For the InterPro search, + means a positive search result with no quantitative value.
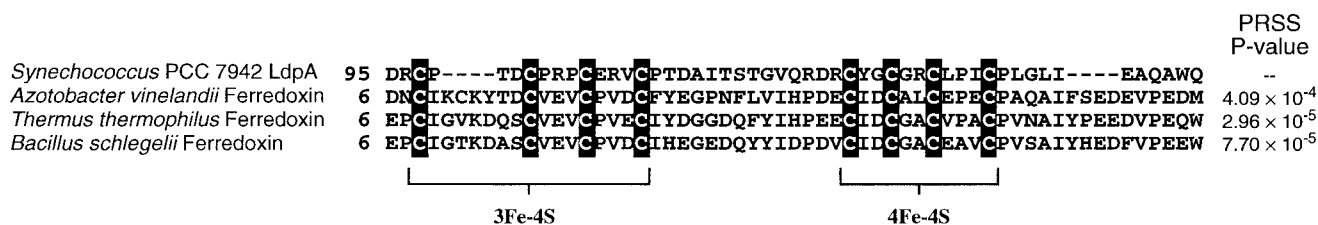[c] In the BIOINBGU threading search, the statistical confidence level is 12.

FIG. 3. Comparison of the deduced amino acid sequence encoded by the *ldpA* gene of *S. elongatus* PCC 7942 and ferredoxins with known 7Fe-8S centers. LdpA, GenBank accession no. AY136759; *T. thermophilus* ferredoxin, SwissProt accession no. P03942; *Bacillus schlegelii* ferredoxin, SwissProt accession no. Q45560; *A. vinelandii* FdI, SwissProt accession no. P00214. Only the Fe-S center domain is shown. Conserved Cys residues are highlighted, and corresponding Fe-S centers are indicated by brackets. The PDB codes for the ferredoxins from *A. vinelandii*, *T. thermophilus*, and *B. schlegelii* are 1F5B, 1H98, and 1BC6, respectively. Pairwise sequence similarities between LdpA and other ferredoxins were assessed by an alignment-independent PRSS test. Statistical significance (*P* values) for the pairwise sequence comparison is indicated on the right of the sequence alignment.

types of clusters with significant statistical support. Furthermore, a structural fold recognition analysis carried out for LdpA generated a most significant hit for a 7Fe-8S ferredoxin (PDB code 5FD1 from *A. vinelandii*) and second most significant hit for an 8Fe-8S ferredoxin (PDB code 1CLF from *C. pasteurianum*). The result demonstrates a high probability that the LdpA protein adopts a tertiary structure similar to that of the 7Fe-8S ferredoxin in *A. vinelandii*. In reviewing all of the available evidence, the consensus appears to be that LdpA has two Fe-S centers, one for 3Fe-4S and another for 4Fe-4S. A more refined sequence alignment between LdpA and proteins with known 7Fe-8S centers (3, 7, 26) showed full conservation of all Cys residues at the putative Fe-S center domain (Fig. 3). Statistical support (*P* values) for each pairwise comparison (29) between LdpA and known 7Fe-8S proteins is shown in Table 2. The entropy-based analysis without influence of the alignment shows that the sequences are indeed significantly similar, with 100% of the comparisons having *P* values smaller than 0.001, which is significant enough to establish a direct homologous relationship.

Insertional inactivation of *ldpA* and complementation of the mutant phenotype by a wild-type allele. We created a Gm^r interruption allele of *ldpA* by introducing the drug resistance cartridge into an *Alw*NI site that is near the insertion site of the transposon in mutant AMC408-M2. This reconstructed mutant had the short-period phenotype observed in the original transposon mutants (Table 3). To confirm that *ldpA* alone is responsible for the phenotype, we introduced a wild-type *ldpA* gene into *ldpA* Tn5 mutants. For this experiment, we used strains AMC694 and AMC695, which carry the M1 and M2 Tn5 mutations in a background with a reporter configuration different from that of AMC408. A heterologous promoter,

P*lacZ*, was provided in the vector because sequence data suggested that *ldpA* is downstream of a thioredoxin reductase gene with which it might be cotranscribed; thus, the *ldpA* fragment might not have its own promoter (data not shown). As shown in Table 3, the FRPs of both AMC694 and AMC695 were restored to the wild-type FRP by the introduction of *ldpA* (AMC696 and AMC697, respectively). We concluded that loss of function of *ldpA* is responsible for the short-period phenotypes in the collection of *ldpA* mutants.

Characterization of the light input phenotype of *ldpA* mutants. To elucidate the presumed input pathway defect of the mutants, we performed a series of phase-resetting experiments. A dark pulse administered at different times in the circadian cycle reproducibly causes phase shifts that differ with respect to magnitude and direction in *S. elongatus* (32). We compared the relationships between the timing of the 5-h dark pulse relative to the circadian cycle and the resultant phase shift in the wild type and in *ldpA* mutants. The results are plotted as phase response curves (PRCs) in Fig. 4. The shapes of the PRCs of AMC694 and AMC695 are almost identical to that of the PRC of the wild-type strain. This indicates that both mutants retain the wild-type ability to shift the phase of the circadian rhythm.

The FRPs of *ldpA* mutants were shorter than that of the wild-type strain by approximately 1 h under light conditions of 25 microeinsteins m^{-2} s^{-1} (Table 3). Light is known to affect circadian systems not only with respect to phase resetting but also by influencing the FRP (4). Generally, increasing light intensity shortens the FRP in diurnal organisms and phototrophic organisms, including unicellular algae (9) and higher plants (34). As shown in Fig. 5A, the FRP of wild-type *S. elongatus* decreases with increasing light intensity by about 1 h. However, *ldpA* mutants show no substantive change in period over this range of light intensities (Fig. 5A).

To examine this phenotype in more detail, we inactivated *ldpA* in the short-period mutant C22a (22 h), which has a point mutation in the *kaiC* gene (16). The FRP of C22a is more sensitive to the change in light intensity than is the wild-type strain, varying by about 3 h over the light gradient tested (Fig. 5B). Inactivation of *ldpA* decreased the sensitivity of the period to light intensity in C22a as well (Fig. 5A). These results suggest that the primary effect of inactivation of *ldpA* is attenuation of the ability to increase the period length with decreasing light intensity, not a defect in the ability to phase shift in

TABLE 3. FRPs of *ldpA* mutants and complemented derivatives

Strain	Avg FRP ^{a} (h) ± SD ($n = 6$)
Wild type	25.6 ± 0.06
AMC694 (*ldpA*)	24.6 ± 0.18
AMC665 (*ldpA*)	24.5 ± 0.31
AMC695 (*ldpA*)	24.7 ± 0.18
AMC696 (*ldpA$^+$*)	25.8 ± 0.28
AMC697 (*ldpA$^+$*)	25.5 ± 0.49

^{a} FRPs were measured under white light (25 microeinsteins m^{-2} s^{-1}).
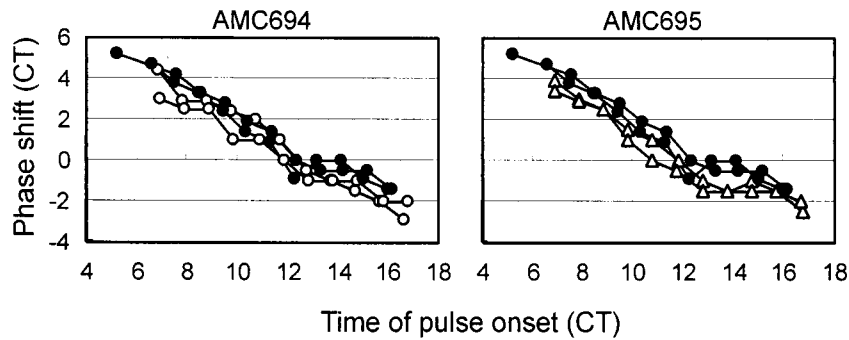
FIG. 4. PRCs for 5-h dark pulses in the wild-type strain (solid circles) and *ldpA* mutants AMC694 (○) and AMC695 (△). PRCs for duplicate samples of each strain are shown. Circadian time (CT) is a normalization for the difference between the circadian periods of the strains; 1 circadian h = 1/24 of a full circadian cycle. Positive values represent phase advances, and negative values represent phase delays.

response to a dark pulse. This specific phenotype led us to name the locus light-dependent period gene A.

Effect of a photosynthesis inhibitor on the circadian period. The expectation that LdpA carries Fe-S clusters suggests involvement in electron transfer and redox reactions (5). One possibility is that LdpA mediates a redox signal from the photosynthetic apparatus to the circadian oscillator. To test this, we examined the effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of the photosystem II reaction center, on the free-running circadian period. If the putative

LdpA protein transmits photosynthetic information, addition of DCMU would be expected to have a smaller effect on the FRP of an *ldpA* mutant than on that of a wild-type strain. As shown in Fig. 5C, addition of a sublethal concentration of DCMU increased the FRP in C22a but did not block sensitivity to different light intensities. DCMU also increased the FRP of the C22a-*ldpA* mutant. The period of the C22a-*ldpA* mutant was still shorter than that of C22a in the presence of DCMU (Fig. 5D). These results indicate that photosynthetic activity contributes to the light input pathways that modulate FRP, but
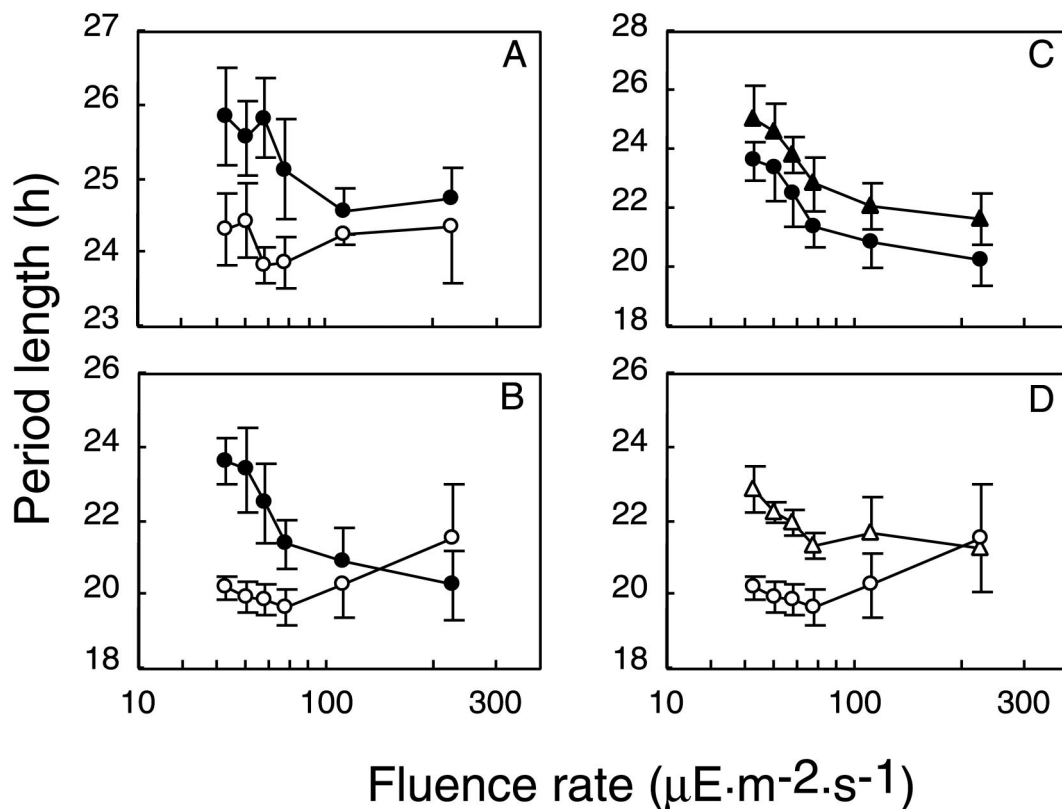

FIG. 5. Effect of white light fluence on the FRP. Panels A and B show the FRPs of the wild type (A) and C22a (B) (●) and those genetic backgrounds with *ldpA* disrupted (○). Panels C and D show the FRPs of C22a (C) and C22a with *ldpA* disrupted (D) with (△, ▲) and without DCMU addition (○, ●). DCMU was added to media at a final concentration of 0.2 μM at the beginning of a 12-h dark interval. Error bars indicate standard deviations (n = 8).

the putative LdpA protein is not the sole component that transmits light intensity signals to the circadian clock.

Disruption of *ldpA* had pleiotropic effects. The *ldpA* disruptants grew slightly more slowly than the wild type, with a doubling time of 7.3 h instead of the 6.1 h of the wild-type strain under a light fluence of 34 microeinsteins $m^{-2} s^{-1}$. *ldpA* mutants were also yellowish green in color because of a phycocyanin content that was only 50 to 70% of that of the wild-type strain. Because a slower growth rate would lead to a lower cell density and greater light penetration into the culture, we wanted to exclude the possibility that the short-period phenotype of *ldpA* mutants is an indirect effect of the slow growth rate. We compared the FRPs of an *ldpA* mutant and the wild-type strain under turbidostatic culture conditions in which the OD_{730} was maintained at 0.25. The mutant showed a consistently shorter period (24.2 h) than the wild-type strain (25.2 h) under 16 microeinsteins of white light $m^{-2} s^{-1}$.

DISCUSSION

Mutants identified by a screen for circadian-resetting defects revealed the involvement of a previously undescribed Fe-S protein in the modulation of the circadian period of *S. elongatus*. The primary defect in the mutants is not an inability to reset per se, as is seen in mutants that lack the CikA histidine protein kinase (32). Rather, *ldpA* mutants are insensitive to the period lengthening under low light fluence that occurs in the wild-type strain. As a result, even under a gradient of light intensities, *ldpA* mutants always exhibit an FRP that is at the short end of the spectrum observed for the wild type. Thus, at the highest light intensities we use routinely in the laboratory, the mutant and wild-type strains have equivalent FRPs (Fig. 5). The screen for phase-resetting mutants used a monitoring setup in which a light gradient was present across the 96-well plates; we do not know the fluence received by the controls with which these mutants were compared when they were originally identified. Our current understanding of the phenotype suggests that they were running with a 1-h shorter FRP than the controls, such that the timing of the resetting dark pulse fell at a slightly different point during the circadian cycle for the wild type and the *ldpA* mutants. With these data in mind, we now pair mutants and controls in the same columns of sample plates for screening (27). Despite the serendipitous identification of these mutants in the resetting screen, *ldpA* does seem to be involved in an input pathway to the clock but affecting a parametric (recurrent period-modulating) rather than nonparametric (single phase-resetting) aspect. Identification of LdpA provides a means by which to gain insight into the biological mechanisms behind Aschoff's rule, the observation that circadian periods tend to decrease with an increase in light intensity (4).

Several *Arabidopsis thaliana* mutants have conditional FRP changes depending on the light intensity. Deficiency in *phyA* (defect in one of the phytochrome photoreceptors) causes an exaggerated increase in FRP relative to that of the wild type in response to decreased red light fluence (34). A *cry1* (cryptochrome blue light receptor) mutation similarly affects the response to decreasing blue light (34). The *gi-1* weak allele of *GI*, which encodes a membrane-spanning protein, shortens the FRP and reduces the rate of period lengthening with decreas-
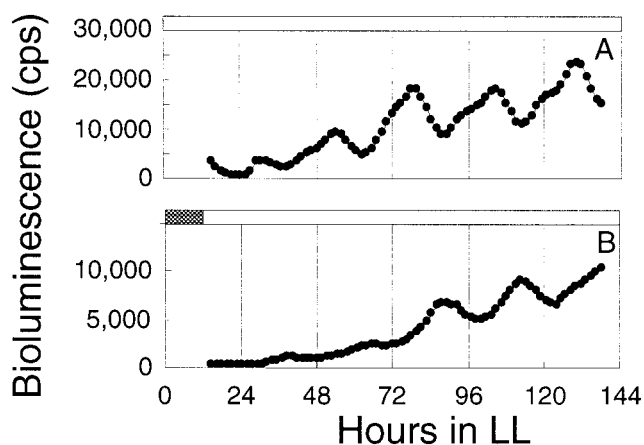

FIG. 6. Resetting of circadian rhythms by DCMU. Cyanobacterial suspensions (3 ml) with an OD_{730} of 0.3 were exposed a synchronizing 12-h dark interval, and then 1 μM DCMU (A) or 0.05% ethanol (solvent used for DCMU; B) was added. After incubation for 12 h under standard light conditions, cells were collected by centrifugation, washed twice with fresh BG-11 M, and suspended in 3 ml of BG-11 M. Samples (10 μl) were inoculated onto sample plates, and circadian rhythms were measured. The interval of DCMU treatment is indicated by the gray bar in panel B.

ing fluence (28). Conversely, mutation of *ZTL* lengthens the FRP and increases the rate of period lengthening with decreasing light intensity (35). Among these, the *phyA* mutant also has a resetting defect, taking a longer time that the wild type for re-entrainment under 10-h light–10-h dark cycles (34).

In contrast to *phyA* or *cry1 Arabidopsis* mutants, disruption of *ldpA* decreased the rate of change in FRP over a range of light intensities. This is most similar to *Arabidopsis GI* mutants (28). It is likely that the putative LdpA protein has a function that represses the light sensitivity of the circadian clock such that it tends to run slower at decreased light intensity. Another mutation that causes a short-period phenotype in *S. elongatus* PCC 7942 is in *pex*, a gene that encodes a protein of unknown function (23). We expect that cyanobacteria have several input pathways that modulate the FRP, as do plants.

Addition of a sublethal concentration of the photosystem II inhibitor DCMU also increased the FRP. This suggests that photosynthesis can be a part of the light input pathway of the circadian system in cyanobacteria. This is consistent with the finding that pulsed addition of a lethal concentration of DCMU at various times during the circadian cycle resets the rhythm similarly to a dark pulse (K. Okamoto and T. Kondo, unpublished results), as shown here for one time point (Fig. 6). In the eukaryotic alga *Lingulodinium polyedrum* (formerly *Gonyaulax polyedra*), a sublethal concentration of DCMU shortens the FRP (30). DCMU also inhibits phase shifting in response to a light pulse in *L. polyedrum* (19) and in *Chlamydomonas reinhardtii* (18). Thus, a connection between photosynthetic electron transport and light input pathways of the circadian system is a common characteristic among some phototropic organisms.

The deduced sequence of LdpA predicts that it is a 7Fe-8S protein similar to some ferredoxins (3, 7, 26). This suggests that LdpA is involved in reduction, oxidation, or electron transfer (5). This biochemical function is consistent with a role

that represses the sensitivity of the circadian clock to light. We propose that photosynthetic activity (favors reduction) negatively regulates the activity of LdpA, repressing the sensitivity of the circadian clock to light. This model can explain the observation that the short-period phenotype of *ldpA* mutants is prominent only at low light intensity (Fig. 5).

Alternatively, the putative LdpA protein may function as a redox sensor unrelated to light per se (5). In the SoxR protein of *E. coli*, oxidative stress changes the redox state in the [2Fe-2S] cluster and leads to a change in its activity as a transcription factor (11, 12). In the FNR protein, exposure to oxygen causes the conversion of [4Fe-4S] to [2Fe-2S] and this conversion causes a change in protein conformation that represses its transcription factor activity (21). The cellular redox state was recently suggested to modulate the mammalian circadian system (31). The reduced phycocyanin content in an *ldpA* mutant, like the short period, is typical of the phenotype of wild-type *S. elongatus* cells incubated at a high light intensity. However, in the mutant, the antenna pigment does not increase when cells are incubated under low-light conditions (data not shown). Recent reports indicate that responses to a high light intensity and nutrient limitation in this organism share pathway components, indicating an integration of photoreception and metabolic signals (33, 36). LdpA may lie within such a pathway. Whether the connection is direct or indirect, LdpA seems to adjust the cyanobacterial clock to changes in the light environment.

ACKNOWLEDGMENTS

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