Quinone sensing by the circadian input kinase of the cyanobacterial circadian clock

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Edited by J. Woodland Hastings, Harvard University, Cambridge, MA, and approved September 26, 2006 (received for review August 2, 2006)

Circadian rhythms are endogenous cellular programs that time metabolic and behavioral events to occur at optimal times in the daily cycle. Light and dark cycles synchronize the endogenous clock with the external environment through a process called entrainment. Previously, we identified the bacteriophytochrome-like circadian input kinase CikA as a key factor for entraining the clock and the redox state of the plastoquinone pool, which, in photosynthetic organisms, varies as a function of the light environment. Furthermore, CikA associates with the Kai proteins of the circadian oscillator, and it influences the phosphorylation state of KaiC during resetting of circadian phase by a dark pulse. The abundance of CikA varies inversely with light intensity, and its stability decreases in the presence of the quinone analog 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB). The pseudo-receiver domain of CikA is crucial for sensitivity to DBMIB, and it binds the quinone directly, a demonstration of a previously unrecognized ligand-binding role for the receiver fold. Our results suggest that resetting the clock in Synechococcus elongatus is metabolism-dependent and that it is accomplished through the interaction of the circadian oscillator with CikA.

Results

CikA Is Present in a High-Molecular-Mass Complex and Copurifies with Oscillator Proteins. The three Kai proteins and SasA have been shown to form a periodosome, a high-molecular-mass protein complex essential for sustaining circadian rhythmicity in Synechococcus elongatus (19, 20). Previously, we demonstrated that LdpA and CikA might also be a part of the periodosome (18). To test directly for evidence that CikA forms a complex with components of the circadian oscillator, we used both copurification and gel-filtration methods.

Either CikA or KaiC was affinity-tagged with 6 His residues, expressed in cyanobacterial cells at wild-type (WT) levels, and recovered under mild conditions that allow copurification of the three Kai proteins and SasA. Previous work showed that CikA associates with KaiC in vitro, similar to that in vivo, that is sustained for at least 72 h (7).

In contrast to the depth of understanding of oscillator performance (7), the pathway of signal transduction from the environment to the Kai proteins has not been clearly established. An environmental signal that universally affects circadian clocks is light. Usually, light is sensed by a photoreceptor, which affects the cellular level of a clock component (15, 16). In cyanobacteria, the bacteriophytochrome-like protein CikA was identified as a key constituent of the input pathway because cikA mutants lack the ability to reset the phase of the rhythm after a dark pulse (8).

Our results suggest that resetting the clock in Synechococcus elongatus is metabolism-dependent and that it is accomplished through the interaction of the circadian oscillator with CikA.
proteins with which they interact. The fraction that coeluted with His-tagged KaiC contained KaiA (whose interaction with KaiC is known) and CikA (Fig. 1A, lane 2). None of these proteins was present in elute from extracts from the WT cells, which do not express a His-tagged protein (Fig. 1A and B, lanes 1). The sigma factor RpoD1, which was not expected to interact with Kai proteins, did not copurify with any of these samples either when the tagged proteins were expressed at native levels or when overexpressed (see Fig. 6, which is published as supporting information on the PNAS web site). KaiA coeluted with tagged CikA (Fig. 1A, lane 2). We did not detect KaiC in the fraction that copurifies with CikA-His expressed at the WT level (21), probably because the yield of eluted CikA-His is an order of magnitude lower than that of KaiC-His expressed at its WT level based on silver-stained SDS/PAGE (data not shown). However, KaiC was found in the eluted fraction when CikA-His was overexpressed (Fig. 6, lane 3). Overexpression of His-tagged KaiC to a similar extent reproduced the associations that were detected when it was expressed at WT levels (Fig. 1A, lane 2, and Fig. 6, lane 1), suggesting that the data obtained with overexpressed CikA are reliable. KaiB and SasA, known to interact with KaiC, were not tested.

To test whether the composition of native complexes in vivo is consistent with the physical interaction of Kai proteins and CikA detected in the copurification assay, cyanobacterial proteins and protein complexes were separated by gel filtration. Two samples were analyzed from cells grown in a 12-h light/12-h dark cycle (LD), where time points are designated by zeitgeber time (ZT5 and ZT17), indicating 5 or 17 h after light onset. In both samples, CikA is present in fractions that correspond to a molecular mass of just under 440 kDa (Fig. 1C); this value is larger than the active CikA dimer of 166 kDa (21), which indicates that CikA participates in a protein complex.

KaiC fractionated in two populations as reported previously (19). The first is that in which CikA was found, ~440 kDa; KaiC forms a hexamer (~360 kDa) as a part of the periodosome (19). At ZT17, this population of KaiC molecules shifted to fractions that correspond to just under 669 kDa. The second population corresponds to an estimated molecular mass ~<100 kDa, which likely includes monomeric KaiC (~61 kDa).

At ZT5, KaiA, whose dimer is 65 kDa, mostly eluted in the fractions that include monomeric KaiC; however, a small amount of KaiA was present across most fractions analyzed. At ZT17, most KaiA eluted in high-molecular-mass fractions that also contain CikA and some KaiC. Taken as a whole, the data from gel-filtration analysis and copurification experiments allow us to suggest that CikA forms a high-molecular-mass complex and, along with Kai proteins, is a part of the periodosome.

**CikA Affects the Phosphorylation State of KaiC.** A striking phenotypic feature of the cikA mutant is its inability to reset the phase of the circadian rhythm after a 5-h dark pulse, whereas WT cells respond in the next cycle with a peak that is offset by up to 12 h relative to the initial daily peak time (8). The greatest difference in circadian resetting between the WT and cikA strains is detected when the dark pulse is given at ZT8 (21). To establish the role CikA plays during the dark pulse, cikA-null and WT cells were collected before, during, and after a pulse administered at ZT8, and protein extracts were subjected to immunoblot analysis. The amount of CikA increased in the dark, and it decreased once cells were returned to light (Fig. 2A), but it did not change in cells not subjected to the dark pulse (ZT8–ZT14; data not shown). KaiA was constant both during the dark pulse and in constant light conditions (LL, data not shown). KaiC is known to autophosphorylate, and the phosphorylation pattern varies during the circadian cycle (22). This pattern differed strikingly between WT and cikA strains. In WT, the KaiC pool was mostly phosphorylated at ZT8, and it became progressively dephosphorylated during the dark pulse and after the return to light (Fig. 2B). When WT cells were kept in LL, the proportion of protein bands corresponding to phosphorylated and unphosphorylated forms of KaiC, respectively (47), and they were verified by lambda phosphatase treatment (data not shown). \( \triangledown \text{cikA} \) is a mutant defective for CikA.
unphosphorylated KaiC increased more slowly. In the cikA strain at ZT8, KaiC was equally divided between phosphorylated and unphosphorylated forms. The proportion of phosphorylated KaiC slowly increased whether or not cells were subjected to a dark pulse. These data indicate that CikA affects the phosphorylation state of KaiC and its dynamics in response to an environmental stimulus.

CikA Is Sensitive to Light and an Inhibitor That Affects Redox State. Immunoblot analysis was performed to see whether the abundance of CikA is regulated during the LD cycle. In WT cells, the CikA level decreased in the light and increased in the dark, and in LL it decreased in the subjective day and increased in subjective night, indicating that abundance of CikA is regulated by the circadian clock (Fig. 3A). To separate circadian from environmental control, CikA abundance was examined in a kaiC mutant that lacks a functional clock. In the absence of light, CikA levels were still regulated in an LD cycle but not in LL. Thus, the abundance of CikA is modulated by light and by the circadian clock, decreasing in light (day) and increasing in the dark (night).

In photosynthetic organisms, some processes that appear to be regulated by light are actually regulated by the redox state of the cell, particularly by that of the PQ pool, which varies in relation to the intensity of light (23). To establish whether the amount of CikA is regulated by redox state, cyanobacterial cells were treated briefly with commonly used photosynthetic electron transport (PET) inhibitors and proton ionophores (24). Previously, we demonstrated that the quinone analog DBMIB affects the levels of CikA, LdpA and, to a lesser extent, KaiA, but it does not affect other proteins, such as PsdC (an iron–sulfur cluster protein involved in PET within photosystem I) and D1 (a key component of photosystem II) (18). Fig. 3B demonstrates that after 15 min of treatment with the DBMIB, CikA was undetectable, whereas treatment with other classes of inhibitors (DCMU, CCCP, methylamine, and hydroxylamine) had no effect. The level of RpoD1, which is not specifically related to the clock, was not affected by any of the treatments. Additional experiments showed that CikA disappears within the 3-min sample preparation time when cells are harvested immediately after 10 μM DBMIB treatment (Fig. 7, which is published as supporting information on the PNAS web site).

DBMIB and other PET inhibitors block translation in S. elongatus (25). To determine whether DBMIB reduces CikA levels by blocking synthesis or by accelerating its degradation, cells were treated with the translation inhibitor chloramphenicol, which did not cause a reduction in CikA levels or alter the effect of DBMIB on CikA (Fig. 3C). The data indicate that the effect of DBMIB is posttranslational, that DBMIB probably actively affects the stability of CikA, and that translation of new proteins is not required for CikA degradation in the presence of the quinone analog.

The PsR Domain of CikA Is Crucial for DBMIB Sensitivity of the Protein. To identify the domain of CikA responsible for CikA sensitivity to DBMIB, we examined a collection of strains that carry defective variants of CikA encoded by point or deletion mutations in cikA (21). When various concentrations of DBMIB were added to the panel of mutants, those CikA variants that lack the GAF domain (ΔGAF), a kinase-defective missense mutant (H393A), or a variant missing the PsR domain (ΔPsR), or the PsR domain alone (PsR), showed that CikA disappears within the 3-min sample preparation time when cells are harvested immediately after 10 μM DBMIB treatment (Fig. 7, which is published as supporting information on the PNAS web site).

Membrane-Associated Factors Are Not Necessary for CikA Sensitivity to DBMIB. Native quinones, including the PQ of cyanobacteria and other photosynthetic organisms, are lipid-soluble molecules, so all of their known targets are proteins associated with membranes (26). CikA is a soluble protein; however, in vivo CikA
is localized to the poles of the cell (21). To see whether specific membrane-associated or soluble factors are necessary for the effect of DBMIB on CikA, we separated cyanobacterial proteins into membrane and soluble fractions; CikA was present in both (Fig. 8B, which is published as supporting information on the PNAS web site), as might be expected if it is peripherally associated with the membrane at the cell pole and some CikA molecules detach during the fractionation. CikA in both fractions was still sensitive to DBMIB, indicating that specific soluble or membrane-associated factors either are not necessary for the DBMIB effect or are present in both fractions (Fig. 8B). Furthermore, when CikA-His was purified under mild conditions that allow copurification of interactants (Fig. 6), CikA in the eluate fraction was also sensitive to DBMIB (Fig. 8C), suggesting that the specific factor responsible for the DBMIB effect on CikA is closely associated with CikA or that DBMIB affects CikA directly.

The PsR Domain of CikA Binds Quinones Directly. The sensitivity to DBMIB of partially purified CikA (Fig. 8C) and the fast disappearance CikA in the presence of DBMIB (Fig. 7) suggested that the quinone might affect CikA directly rather than through an intermediate step. Analysis of mutants (Fig. 4) pointed to a special role for the PsR domain in this process. To see whether DBMIB directly binds the PsR domain, purified recombinant PsR was subjected to two-dimensional NMR analysis in the presence or absence of equal molar DBMIB (Fig. 5). In the presence of DBMIB, a subset of NMR peaks changed significantly, and other peaks changed little or not at all, which suggests that the interaction with the PsR domain is specific at this 1:1 ratio. Addition of the PET inhibitor DCMU, which had no effect on CikA stability in vivo, did not affect the NMR spectrum (Fig. 9B, which is published as supporting information on the PNAS web site), nor did ethanol, which was used as solvent for DBMIB and DCMU stocks, at the relevant concentrations (data not shown). The NMR spectrum of another protein involved in circadian regulation (SasA) did not change in the presence of DBMIB, supporting specificity in the DBMIB–PsR interaction (Fig. 9F).

Discussion

In the clocks of multicellular eukaryotes, the transcription–translation feedback loop requires cellular and sometimes tissue-level compartmentalization of input, oscillator, and output pathways. Here, we present additional evidence that, in contrast to eukaryotic systems, cyanobacteria colocalize most of the known clock components in a large protein complex, the periodosome. Previously, it was shown that the three Kai proteins of the oscillator, and SasA of the output pathway, are part of this complex (19). Recently, we demonstrated that Kai proteins also copurify with LdpA, a component of the input pathway responsible for adjusting circadian period length according to light intensity (18). Here, we provide evidence that CikA, a key element of the input pathway, is also a part of the periodosome.

We demonstrate that CikA affects the ratio of phosphorylated to unphosphorylated KaiC (Fig. 2). A kaiC mutant with altered phosphorylation rhythms has a resetting phenotype similar to that of the cikA mutant (28). It is still not clear how CikA affects the phosphorylation state of KaiC. Yeast two-hybrid assays do not detect direct interaction of CikA with any of the Kai proteins (S.R. Mackey and S.S.G., unpublished data). The addition of DBMIB to Kai proteins in vitro does not affect the phosphorylation rate of KaiC (S.B. Williams and S.S.G., unpublished data). Thus, the interaction of CikA and Kai proteins probably includes one or more yet-unknown proteins, which serves as a bridge between them and which is absent from both yeast and in vitro assays.

It was proposed initially that CikA acts similarly to plant phytochromes by sensing light with a chromophore bound in the GAF domain. The data shown here pose an alternative paradigm in which the cyanobacterial clock receives information about light and darkness not through a photoreceptor (as is done in most circadian systems) but by CikA detecting quinones, small molecules whose presence and redox state depend not only on light but also on the metabolic state of cyanobacterial cells. Others have shown that the metabolic state of the organism affects the circadian clock in mammals, in which the phasing of circadian rhythms in the liver is reset by feeding (29). A possible molecular mechanism of feeding entrainment is that the redox state of nicotinamide adenine dinucleotide cofactors affects the DNA-binding capacity of CLOCK/BMAL1 and NPAS2/ BMAL1 heterodimers, which are oscillator components in the mammalian clock (30). In plants, there is no evidence yet for metabolic control of the circadian clock; however, redox control of gene expression and some posttranscriptional processes is well established (31, 32).

Here, we report that quinones directly affect the structure of CikA (Fig. 5). It is known that ubiquinone Q0 affects kinase activities of the purified soluble histidine kinases BvgS (regulator of virulence in Bordetella pertussis) and EvgS (transcriptional regulator in E. coli) (27). Another kinase from E. coli, ArcB, which regulates gene expression during the switch between aerobic and anaerobic growth conditions, is sensitive to quinones such as ubiquinone (Q0) and menadione (MK-3) (33). These three kinases contain the PAS domain, a fold that is responsible...
for the quinone binding; in other proteins, PAS binds a variety of small ligands. However, the ArcB ortholog in *Haemophilus influenzae* lacks the PAS domain and nevertheless is able to mediate signal transduction in response to redox conditions, suggesting that domains other than PAS can be involved in quinone redox sensing (34).

In CikA, the PsR domain binds a PQ analog (Figs. 4 and 5). The founding members of the receiver family act as recipients of phosphoryl groups from histidine kinases by a reaction at a conserved active-site aspartyl residue (35). The PsRs of CikA and KaiA in the *S. elongatus* clock lack the aspartyl residue, and they are incapable of phosphoryl transfer based on structural analyses (8, 14). PsRs are present in other bacteria and in plants, and the biochemical functions of many are unknown. The current work suggests that diverse ligand-binding functions may be played by PsRs, as is true for PAS and GAF domains (36–38).

The PsR of CikA is responsible for localization of the protein at the cell pole (21). We show here that part of the CikA population copurifies with the membrane fraction, indicating that CikA is localized within close proximity of quinones, which are lipid-soluble molecules. Previously, we proposed that the redox status of the PQ pool affects the stability of CikA (18) because DBMIB causes reduction of the PQ pool, and it is widely used for altering the redox state of the cell (24). The current data show that DBMIB affects CikA directly, regardless of the effect that it has on the redox state of native PQ by blocking PET. Indeed, the quinone analog DCBQ, which accepts electrons directly from photosystem II and should compete with native PQ for reduction (such that the PQ pool is oxidized) (39), behaves in a manner similar to DBMIB, which binds to the cytochrome *b*/*f* complex and prevents PQH$_2$ reoxidation (such that the PQ pool is reduced). We conclude that the exogenously added quinone itself binds to the PsR domain of CikA; if native oxidized and reduced PQ have different binding affinities for PsR, this distinction is overcome at the concentrations of DBMIB and DCBQ that are commonly used. This technical difference could explain why there is such a dramatic effect on CikA stability in the presence of DBMIB, whereas during the dark/light transition in *vivo* the CikA depletion is more modest. After DBMIB treatment the CikA antiseraum sometimes detects a band of reduced mobility (Fig. 5C) that comigrates with a band detected by antiserum raised against ClpP (data not shown), a protease whose depletion affects circadian period (40); we propose that binding of DBMIB by CikA targets the protein for immediate degradation, accounting for its rapid disappearance.

TOC1, a light-sensitive component of the circadian oscillator in plants (41), also contains a PsR domain. It remains to be seen whether components of the clock in plants and other kingdoms are able to sense the light environment in a manner similar to that used by CikA.

**Materials and Methods**

**Bacterial Strains, Culture Conditions, and DNA Manipulations.** All *S. elongatus* PCC 7942 strains were grown as described previously (17). Cyanobacterial and *E. coli* strains used in this study are listed in Table 1, which is published as supporting information on the PNAS web site. Null strains for *cikA* (AMC1426) or *kaiC* (AMC1511) were constructed by insertion of an inactivation cassette (42) or a Mu transposon (40). Cyanobacterial transformations were performed as described earlier (42). *E. coli* strains DH10B and BL21DE3 hosted plasmids, and they were grown as described previously (38).

**Expression and Purification of Tagged Proteins.** Expression and purification of tagged proteins from *S. elongatus* under low-stringency conditions were performed as described previously (18) with minor modifications. KaiC-His (Fig. 1A) was induced with 7 µM isopropyl β-D-thiogalactoside (IPTG) for 24 h. This level of expression complemented a *kaiC* mutant, as judged by restoration of rhythmicity in a bioluminescent reporter gene assay (44). Expression of CikA-His at the WT level was induced with 10 µM IPTG for 24 h (Fig. 1B). For the experiment shown in Fig. 6, cultures were incubated for 24 h in the presence of 100 µM IPTG. Eluted fractions were analyzed directly by 12.5% SDS/PAGE (43), with the exception of the CikA-His fraction used to detect KaiA (Fig. 1B), which was concentrated 25-fold with trichloroacetic acid precipitation. Each experiment was repeated at least three times, yielding essentially the same results. The PsR domain was purified from *E. coli* AM3677, as described earlier (38).

**Gel-Filtration Chromatography.** Chromatographic analysis was performed as previously (19) with some modifications. WT *S. elongatus* (400 ml at OD$_{750}$ = 0.7) was collected at 1,500 × g for 10 min and washed once in 2 ml of BB5 buffer. The pellet was resuspended in 0.25 ml of BB5 buffer containing 1 mM ATP, 5 µg/ml DNase I, 10 µg/ml RNase, and a protease inhibitor mixture for use with bacterial cell extracts (Sigma, St. Louis, MO) according to the manufacturer’s recommendations. The soluble fraction was prepared as described (45), brought to a total volume of 3 ml, and loaded on a HiPrep 26/60 Sephacryl S-300 HR (GE Healthcare, Piscataway, NJ) equilibrated with BB5 buffer with 1 mM ATP by using an ÄKTA Explorer chromatography system (Amersham Biosciences, Piscataway, NJ). Protein fractions were eluted by using BB5 buffer with 1 mM ATP. To detect CikA and KaiA proteins, the fractionated protein samples were analyzed directly by SDS/PAGE (12.5% gel) (43). To detect KaiC, 1 ml of each fractionated protein sample was precipitated with trichloroacetic acid to concentrate the sample ~25-fold before SDS/PAGE separation. Protein standards for calibration of the column included thyroglobulin (669 kDa), ferritin (440 kDa), and lactose dehydrogenase (140 kDa) (GE Healthcare). At least three independent experiments were performed for each time point, yielding essentially identical results.

**Immunoblot Analysis.** Immunoblot analysis was performed as described earlier (18, 46). Proteins were transferred to nitrocellulose membranes by using either capillary (43) or electrophoretic transfer with a Transblot SD semidry transfer cell according to the manufacturer’s recommendations (Bio-Rad, Hercules, CA). Phosphorylated and unphosphorylated bands of KaiC (47) were confirmed by treatment of samples with lambda protein phosphatase (New England Biolabs, Beverly, MA).

**Light Treatment.** Cyanobacterial cultures (100 ml) were synchronized by two LD cycles. One set of cultures was kept in LL, and the second set was subjected either to dark-pulse treatment or to LD. For Fig. 2 samples (1 ml, OD$_{750}$ = 0.7) were collected at ZT8 (8 h after coming out of LD entrainment), at 1 h, 3 h, and 5 h from the start of the dark pulse, at 1 h after the dark pulse ended (+1), and at corresponding times of LL. For Fig. 3, samples (1 ml) were collected every 4 h from cells in LD or LL. Cells were collected for 2 min at 16,000 × g, and pellets were frozen at −80°C before processing. The total protein fraction was prepared as described (48) with minor modifications. Pellets were resuspended in 70 µl of ice-cold IA lysis buffer (18) and kept on ice for the remainder of the procedure. Cells were broken with glass beads (100 µg) for 1.5 min in a MiniBeadbeater-8 (BioSpec Products, Bartlesville, OK). Ice-cold IA lysis buffer was added (60 µl). Samples were mixed by vortexing and spun at 1,000 × g to remove beads, and the supernatant fractions were collected, yielding whole-cell extract. A sample (8 µg of protein) was loaded for each lane of SDS/PAGE.
Treatment with Inhibitors. For Fig. 3B, the following inhibitors were added to the cyanobacterial culture to a final concentration of 10 μM: DBMIB, DCMU, CCCP, methylamine hydrochloride, and hydroxyamine hydrochloride. All inhibitors were from Sigma except DCMU (Serva, Germany). Because inhibitors were dissolved in ethanol (except hydroxylamine, dissolved in water), an equal volume of ethanol was added to the control sample. Cells at OD750 = 0.7 (1 ml) were treated for 15 min and collected for 2 min at 16,000 × g. The total protein fraction was prepared as described above. For Fig. 3C, chloramphenicol was added to samples (250 μg/ml) 5 min before the addition of DBMIB. For Fig. 4, samples were treated as for Fig. 3B, except a gradient of DBMIB concentrations was used (3, 5, and 10 μM).

NMR Analysis. NMR samples of 15N-enriched PsR domain were prepared as described earlier (38). Final protein concentrations were 0.2 mM. NMR spectra of the PsR domain alone and in the presence of DBMIB (0.2 mM) were acquired on an Inova spectrometer (Varian, Palo Alto, CA) operating at 600-MHz proton frequency. An equal volume of ethanol was added to the control sample to a final concentration of 0.1%. The sample temperature was maintained at 25°C during data acquisition. Two-dimensional 1H-15N correlation spectra were collected as described previously (48). Acquisition times were 64 ms in both the t2 and t1 dimensions. All chemical shifts were referenced to internal DSS (49).

We thank Dr. T.C. Hall for use of the chromatography system, Dr. K. Tanaka for RpoD1 antisera, and members of the clocks journal club at Texas A&M for insightful suggestions. Our laboratory members, Y. Chen, G. Dong, Dr. K. Holtman, and Dr. S. Mackey, contributed strains and helpful advice. The Gene Technologies Laboratory (Texas A&M University) provided sequencing and oligonucleotide services. This work was supported in part by National Insts. of Health Grants GM62419 and NS39546 (to S.S.G.) and GM064576 (to A.C.L.). N.B.I. was supported by a National Science Foundation–North Atlantic Treaty Organization postdoctoral fellowship. The NMR instrumentation in the Biomolecular NMR Laboratory at Texas A&M University was supported by National Science Foundation Grant DBI-9970232.