Oxidized quinones signal onset of darkness directly to the cyanobacterial circadian oscillator

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Synchronization of the circadian clock in cyanobacteria with the day/night cycle proceeds without an obvious photoreceptor, leaving open the question of its specific mechanism. The circadian oscillator can be reconstituted in vitro, where the activities of two of its proteins, KaiA and KaiC, are affected by metabolites that reflect photosynthetic activity: KaiC phosphorylation is directly influenced by the ATP/ADP ratio, and KaiA stimulation of KaiC phosphorylation is blocked by oxidized, but not reduced, quinones. Manipulation of the ATP/ADP ratio can reset the timing of KaiC phosphorylation peaks in the reconstituted in vitro oscillator. Here, we show that pulses of oxidized quinones reset the cyanobacterial circadian clock both in vitro and in vivo. Onset of darkness causes an abrupt oxidation of the plastoquinone pool in vivo, which in contrast to a gradual decrease in the ATP/ADP ratio that falls over the course of hours until the onset of light. Thus, these two metabolic measures of photosynthetic activity act in concert to signal both the onset and duration of darkness to the cyanobacterial clock.

The cyanobacterial circadian oscillator is composed of three proteins: KaiA, KaiB, and KaiC (4). The phosphorylation state of KaiC oscillates with a 24-h rhythm both in vivo (8) and in a mixture with the other Kai proteins in vitro (hereafter, referred to as the in vitro oscillator mixture) (Fig. 1A). KaiA enhances autophosphorylation of KaiC, whereas KaiB activates KaiC autodephosphorylation by inhibiting KaiA function (9–11). Previous work showed that the ATP/ADP ratio affects KaiC phosphorylation directly and that manipulation of this ratio in the in vitro oscillator mixture can mimic resetting of the circadian phase of rhythms in vivo (12). The C-terminal domain of KaiA is known to enhance KaiC phosphorylation by binding to the A-loops of KaiC (11, 13); the N-terminal pseudoreceiver domain of KaiA is related to signal transduction receiver domains, suggesting a regulatory role (9). We previously showed that the pseudoreceiver domains present in both CikA and KaiA bind quinones (9, 14–16). Importantly, only oxidized quinones bind to the pseudoreceiver domains of KaiA, forming KaiA aggregates that cease to stimulate KaiC phosphorylation (15); this redox selectivity suggests that reversible binding and aggregation is a sensory mechanism for entraining the oscillator. Here, we show that oxidized quinones, markers of the onset of darkness in S. elongatus, reset the clock both in vivo and in vitro. We propose that this sensory mechanism, acting through KaiA, works in concert with [ATP]/[ADP] sensing by KaiC to signal both the onset and duration of darkness to the clock.

Results

Oxidized Quinones Applied in the Phosphorylation Phase of Kai Induce a Phase Shift in the in Vitro Oscillator Mixture. A circadian rhythm is, by definition, sensitive to environmental cues (2), and treatments given at different times in the clock cycle generate different outcomes for the behavior under study [known as a phase response curve (PRC)] (17). In S. elongatus, a 4- to 8-h dark pulse applied during the phosphorylation phase of the KaiC cycle induces striking phase shifts in gene expression rhythms, but it has little effect if applied during the dephosphorylation phase (Fig. 2) (12). We used this property to test the phase-dependent effect of KaiA quinone sensing in the in vitro oscillation mixture by applying quinones (15). We found that 4.8 μM oxidized 2,3-dimethoxy-5-methyl-p-benzoquinone (Q0) was sufficient to change both complete dephosphorylation of KaiC, and the addition of the reducing agent dithionite recovered KaiC phosphorylation activity (Fig. 1 B and C). Application of oxidized Q0 for different durations during both phosphorylation and dephosphorylation phases, expected to mimic a dark pulse, had

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phase-dependent effects. The addition of oxidized Q₀ for 2–12 h, only during the phosphorylation phase of the KaiC cycle, induced a shift in the peak positions of a control in vitro oscillation mixture to which no Q₀ was added to aid comparisons of the timing of peaks in each reaction. Addition of oxidized Q₀ during the dephosphorylation phase of KaiC (Fig. 1C) or dithionite alone at any time during the cycle (Fig. S1) did not induce a phase shift. The decrease in peak phosphorylation is characteristic of Q₀ treatment and likely reflects the inability of dithionite to fully disaggregate KaiA (15).

**Quinone-Induced Phase Advances, but Not Delays, Were Observed in Vitro.** This phase-selective effect corresponds to the previously reported PRC for gene expression rhythms generated by dark-pulse treatment of cyanobacterial cultures (18) and predicted that a 4-h addition of oxidized Q₀ should be equivalent to a 4-h dark pulse. To test this hypothesis, 4-h pulses of oxidized Q₀ were administered at different points throughout the cycle to generate a PRC (Fig. S2). The data from Q₀ pulses in the oscillator mixture matched well with previously reported PRCs from bioluminescence rhythms of reporter genes for phase advances, but phase delays were not observed in vitro (Fig. 2). In contrast, the previously reported method of entrainment by modulating the ratio of ATP/ADP in the oscillator mixture (12), which acts through KaiC, induces both phase advances and delays.

**PRC Generated by Administration of Oxidized Quinones in Vivo Corresponds to the PRC Induced by 4-h Dark Pulses.** It is possible that the effect of quinone modulation is limited in vitro, because only KaiA, and not CikA, is affected in the in vitro oscillator mixture. Thus, Q₀ administration was used in vivo to determine the PRC of oxidized quinone in the intact system. Q₀ was added to cyanobacterial reporter strains at different circadian times, and the timing of subsequent bioluminescence peaks was determined. Phase shifts were observed on addition of a 20-μL drop of Q₀ solution at or above 3 mM (Fig. S3). At circadian times (CTs) 9 and 17, phase advances were observed, whereas administration at CT 24 resulted in a phase delay, which is in agreement with the previously reported PRC for 4-h dark pulses (Fig. 2) (18). Thus, oxidized quinones can reset the clock in both directions, but the isolated minimal oscillator is limited in its response to this signal.
In Vitro Oscillator Mixtures of Different Phases Can Be Synchronized by Applying Oxidized Quinones. Cyanobacterial cultures that have been entrained to different phases of peak gene expression by offset light–dark (LD) cycles can be synchronized by one 12-h L to 12-h D cycle (19). We tested the ability of oxidized Q0 to mimic the effect of dark synchronization in oscillator mixtures by administering it for 10 h, the duration for which the maximum phase shift was observed in Fig. 1B, to three oscillator mixtures with peaks that were offset by 8 h (Fig. 3A). After the treatment, two, but not all three, of the oscillating rhythms were set to the same phase (Fig. 3B). This result is consistent with the observation that repeated LD cycles are required to change the phase entrainment of a cikA null mutant (14), which is missing one quinone-sensing component.

PQ Pool in S. elongatus Is Oxidized on Onset of Darkness. As previously hypothesized (15), lipid-soluble quinones may interact with KaiA at the periphery of a cellular membrane (20). Thus, the redox state of the PQ pool, which is modulated by light-dependent photosynthetic electron transport, could signal an L/D transition. To test the premise that oxidized quinones signal the onset of darkness, we measured the status of the PQ pool continuously in S. elongatus during LD transitions that simulated diurnal cycling. Redox poise was measured indirectly by monitoring chlorophyll (Chl) fluorescence emission associated with PSII (Fv; variable yield) and total Chl emission intensity using fast repetition rate fluorometry (21) in accordance with previous studies in plants (22). Changes in Chl fluorescence quenching are attributed to the redox state of QA, which is in rapid equilibrium with the PQ pool (23) ([ST Text, Fig. S4–S8, Scheme S1–S2]). The PQ pool became substantially more oxidized immediately after the LD transition, changing 40.9 ± 3.6% (n = 3) of the total observed range in Fv/Fm (Fig. 4). The redox state of the PQ pool partially recovered quickly and then, slowly became more reduced throughout the 12-h dark period, consistent with the expected transition from photosynthetic to respiratory electron flow through the pool.

Discussion

Our data are consistent with a model in which the diurnal L/D solar cycle is sensed by cyanobacteria through the change in electron flux through shared electron carriers of photosynthesis and respiration, which affect both the ATP/ADP ratio and the oxidation state of the PQ pool. The former affects the status of KaiC directly, and the latter affects the ability of KaiA to stimulate KaiC autophosphorylation, providing two sources of metabolic input to the circadian oscillator (Fig. 5).

As previously reported, manipulation of the ATP/ADP ratio can also reset the phase of KaiC phosphorylation in the in vitro oscillator mixture (12). When cells enter darkness, the ATP/ADP ratio drops gradually rather than acutely and reaches a threshold...
ratio, which induces a phase shift, after ~2 h of darkness (12). In contrast, the PQ pool becomes oxidized abruptly on onset of darkness (Fig. 4), and a 2-h administration of oxidized quinones was sufficient to induce a phase shift (Fig. 1B). Together, the quinone oxidation state could provide an acute stimulus marking an L/D transition, whereas the ATP/ADP ratio, changing slowly and steadily, would measure the duration of darkness.

The in vitro oscillator mixture has only the three Kai proteins, and it is not connected to additional components with which the oscillator is likely to interact in vivo, such as CikA, which also is quinone- and L/D-sensitive (14, 24) and necessary for normal resetting of the clock by dark pulses (5). The quinone-induced PRC for the in vitro oscillator mixture is limited relative to the PRC in vivo that is generated either by pulses of darkness or oxidized quinones (Fig. 2). These data may reflect the role of CikA in vivo, which is absent from the isolated oscillator. Consistent with this idea, a cikA mutant requires several L/D cycles to reset and synchronize circadian phase in a cell population compared with a single cycle to reset the WT. Similarly, synchronization of three out-of-phase in vitro oscillator mixtures would require multiple cycles of quinone oxidation reduction (Fig. 3). Overall, the data are consistent with environmental sensing and jetlag in the cikA mutant (5), which occurs at the single-cell level. KaiA and CikA share structural similarity in the pseudoreceiver domain (Fig. 6) (9, 16), and abundance of the CikA protein is affected by the oxidation state of the PQ pool (14). Thus, a possible function of CikA in phase resetting is to work cooperatively with KaiA to generate phase delays in the PRC, which are quinone-induced in vivo but not in the in vitro oscillator mixture (Fig. 2).

The onset of darkness initiates both acute and gradual responses in various metabolites of photosynthesis, and components of the cyanobacterial oscillator have evolved to sense an L/D transition using at least two, the ATP/ADP ratio and the redox state of the quinone pool, through KaiC and KaiA, respectively. Therefore, we propose that the photoreceptive signal transduction input pathway of the cyanobacterial circadian clock is in the well-characterized photosynthesis machinery and the metabolic consequences of its activity.

Materials and Methods

Purification of KaiA and KaiB. The genes encoding KaiA and KaiB from S. elongatus were amplified using PCR and cloned in-frame with Small ubiquitin-related modifier (SUMO) into the pET-28b expression vector using Ndel and HindIII cloning sites. The resulting plasmids were used to transform Escherichia coli BL21(DE3). Transformed E. coli cultures in log phase in LB at 37 °C were induced to overexpress recombinant KaiA or KaiB with 1 mM isopropyl-β-D-thiogalactopyranoside (Calbiochem). Cells were harvested after 6 h, and pellets were resuspended in 50 mM NaCl and 20 mM Tris HCl (pH 7.5). Cells suspensions were passed two times through a chilled French pressure cell, and lysates were clarified by centrifugation at 20,000 × g for 60 min at 4 °C. Tagged proteins were isolated on an Ni-charged chelating column. Proteases and ATPases were removed by anion-exchange chromatography (buffer A: 20 mM NaCl, 20 mM Tris HCl, pH 7; buffer B: 1 M NaCl, 20 mM Tris HCl, pH 7; gradient: 0–80% buffer B over 80 mL). The SUMO fusion KaiA and KaiB was cleaved after incubation at 4 °C overnight with the Ulp1 protease to generate a single cycle to reset the WT. Similarly, synchronization of three cycles of quinone oxidation reduction (Fig. 3). Overall, the data are consistent with environmental sensing and jetlag in the cikA mutant (5), which occurs at the single-cell level. KaiA and CikA share structural similarity in the pseudoreceiver domain (Fig. 6) (9, 16), and abundance of the CikA protein is affected by the oxidation state of the PQ pool (14). Thus, a possible function of CikA in phase resetting is to work cooperatively with KaiA to generate phase delays in the PRC, which are quinone-induced in vivo but not in the in vitro oscillator mixture (Fig. 2).

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Purification of KaiC. The gene encoding KaiC from S. elongatus was amplified using PCR and cloned in-frame with the PreScission Protease (GE Healthcare) cutting site into the pET41a(+) vector (Novagen) between Ncol and Xhol sites; the resulting plasmid was used to transform E. coli BL21(DE3). Transformed E. coli cultures in log phase in LB at 37 °C were cooled to room temperature for 1 h and induced to overexpress recombinant KaiC with 0.1 mM isopropyl-β-D-thiogalactopyranoside (Calbiochem). Cells were harvested after 16 h, and pellets were resuspended in 50 mM Tris HCl (pH 7.3) with 150 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, and 5 mM ATP. Tagged KaiC proteins were separated from the supernatant fraction on a GSTrap HP column (GE Healthcare) by washing the column with 90 mL of buffer. PreScission Protease (GE Healthcare) was used (1 unit/mL in 12 mL to cut the GST tag. KaiC was separated from the cleaved GST tag, tagged KaiC, and protease by passage a second time through a GSTrap HP column. Purity was assessed using SDS/PAGE and diazoylated against phosphorylation assay buffer (20 mM Tris HCl, 150 mM NaCl, 0.5 mM EDTA, 5 mM MgCl₂, 1 mM ATP, pH 8.0). Protein solutions were concentrated, passed through a sterile 0.2-μm filter, and stored at −80 °C. Protein concentrations were determined by using Coomassie Plus-The Better Method Assay Reagent (Pierce).

KaiC Phosphorylation Assay. The in vitro KaiC phosphorylation assays were performed in sterile 2-mL glass vials in a 30 °C water bath and included KaiA and KaiB at 1.2 μM and 3.5 μM final concentrations in the phosphorylation assay buffer. The in vitro oscillator mixture was divided from the same stock mixture immediately after KaiC (3.5 μM) was added and frozen at −80 °C. Here, 10 mM ATP was used to remove previously reported [ADP]/[ATP] effect on the phase (12). To generate different phases, each reaction mixture was melted at 30 °C at the desired time points. Q₀ solution (4.8 mM) was prepared by dissolving Q₀ into 200-proof ethanol. Dithionite was dissolved into water immediately before use. All of the in vitro reactions were performed under the nitrogen environment to avoid oxygen. Periodically, 20-μL aliquots were removed and denatured at 65 °C for 10 min with 2 μL SDS/PAGE gel loading dye (100 mM Tris HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 400 mM β-mercaptoethanol). A sample (5 μL) of each was loaded onto SDS polyacrylamide gels (4% stacking, 6.3% running) with 15 wells. Densitometry was performed to record KaiC phosphorylation using ImageJ (National Institutes of Health).

Bioluminescence Assay. S. elongatus AMC 462 is a derivative of a WT isolated PCC 7942 carrying a bacterial luciferase reporter that consists of two neutral Bioluminescence Assay. Materials and Methods

Fig. 6. The superimposed structures of the pseudoreceiver domains of CikA and KaiA. The pseudoreceiver domains of CikA (magenta; Protein Data Bank ID code 2J48) and KaiA (blue; Protein Data Bank ID code 1M2E) were superimposed using the Matchmaker function of UCSF Chimerera (27).
from the PQ pool (26). Bioluminescence assays were performed on a Packard TopCount scintillation counter (PerkinElmer Life Sciences) according to a previous protocol (25).

Fast Repetition Rate Fluorometry. S. elongatus FCC 7942 was grown in BG-11 medium on a 12-h/12-h L/D cycle (100 μL samples taken from the turbidostat 4 h before the L to D transition and concentrated previously described (21) with the following specifications: the cells were continuously illuminated at 60 to 20 μL samples were monitored within the turbidostat every 2 min after a 627-nm measuring pulse (Δt = 1 με m−2 s−1). Chl fluorescence was detected by a built-in positive-intrinsic-negative (PIN) photodiode equipped with 665- to 750-nm bandpass filters.

Fast repetition rate (FRR) fluorometry studies were performed as previously described (21) with the following specific alterations. Aliquots were taken from the turbidostat 4 h before the L to D transition and concentrated to 20 μg Chl ml−1; 50-μL samples (1 μg Chl total) were loaded into the humidified FRR fluorometer sample chamber. During the first 4 h of the experiment, the sample was continuously illuminated at 60 με m−2 s−1 by a blue light emitting diode (LED). This actinic light source was switched off at t = 4 h and turned on again for 4 h at t = 16 h. This lighting schedule was coordinated to conditions under which the cells were acclimated in the turbidostat. During the entire 20-h experiment, variable Chl fluorescence was monitored by subjecting the sample to five 60-μs single turnover flashes (515 nm) at 10 Hz every 2 min. The average light intensity of five SFFs over the 2-min period is 0.08 με m−2 s−1. These conditions are illustrated in Scheme S1. During the periods when the actinic light source was on, fluorescence parameters are defined as light-adapted origin or instantaneous fluorescence (Fp), light-adapted maximum fluorescence (Fm′), and light-adapted variable fluorescence yield (Fv/Fm′, Fm′ − Fp/Fm′). For the dark period, fluorescence parameters are defined as origin fluorescence (Fo), maximum fluorescence (Fm), and maximal variable fluorescence yield (Fv/Fm′ = (Fm′ − Fo)/Fm′).

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**Supporting Information**

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**SI Text**

**Cells Maintained in the Turbidostat Exhibit Similar F<sub>i</sub> Behavior Compared with Cells Transferred to the Fast Repetition Rate Fluorometer.** The PSI FMT 150 photobioreactor allows for the measurement of F<sub>i</sub> within the growing chamber. However, these data have poor signal to noise because of convection from bubbling. Nonetheless, the general trends observed in Fig. S5 are consistent with the fast repetition rate (FRR) fluorometry results in Fig. 4 and Fig. S4. Fluorescence is significantly more quenched after the light–dark (LD) transition and less quenched after the DL transition.

The origin and maximum fluorescence traces in Fig. 4 exhibit an underlying baseline with a positive slope. This finding is mirrored in the variable fluorescence yield measurements. Given that such a feature is not present in F<sub>i</sub> measurements in the turbidostat (Fig. S5), we attribute the sloping baselines of Fig. 4 to the physiological adaptation of the cells during the 20-h measurement period. Note that the baseline is present in both L and D periods of the experiment. This finding suggests that this behavior is not the result of photoinhibition, which contributes to the photohinating proton fluorescence quenching (qI) component of nonphotochemical quenching (qN) (1). qI contributions can be excluded given that the actinic light source in FRR fluorometry experiments (60 μE·m<sup>-2</sup>·s<sup>-1</sup>) is less intense than the light source in the turbidostat (100 μE·m<sup>-2</sup>·s<sup>-1</sup>).

**Changes in Fluorescence Quenching After the LD and DL Transitions Are the Result of Photosystem II Activity.** qE resulting from changes in the thylakoid membrane proton gradient is denoted qE (1). To study the effects of qE after the LD and DL transitions, the blue actinic source in the FRR fluorometer sample chamber was replaced with a 735-nm near-IR LED; 735 nm only excites photosystem I (PSI) and does not affect photosystem II (PSII). During 735-nm illumination, cyclic electron transfer around PSI is active, thus enhancing the thylakoid membrane proton gradient and enabling photophosphorylation (2). As shown in Fig. S6, no significant change in fluorescence quenching occurs after either LD or DL transition. This observation suggests that the dramatic changes in fluorescence quenching observed in Fig. 4 and Figs. S4 and S5 are photochemical in nature and not the result of qE.

**Increased Fluorescence Quenching During the Dark Period Is the Result of the Acceptor Side of PSII.** Data in Fig. 4 and Fig. S4 represent average values over five single turnover flashes (STFs). Full data for each STF number are shown in Fig. S7. During the periods when the actinic light is on, chlorophyll (Chl) fluorescence is not dependent on STF number. However, during the dark period, origin and maximum Chl fluorescence levels are more quenched during the first STF. We attribute this observation to donor- and acceptor-side contributions to photochemical quenching (qP). On the donor side of PSII, Chl fluorescence is most quenched by the dark-stable S<sub>1</sub> intermediate of the PSII water-oxidizing complex (3). On the acceptor side, after a 2-min dark adaptation, the primary electron acceptor, Q<sub>A</sub>, is more likely to be oxidized.

The STF number dependence of fluorescence quenching in Fig. S7 does not show indications of oscillations. Given the cyclic nature of the water-oxidizing complex turnover cycle, significant contributions of donor-side qP would be present as a damped period four oscillatory pattern in the variable yield of Chl fluorescence with minimum yield on the third STF (3). F<sub>v</sub>/F<sub>m</sub> values in Fig. S7 are lower on the fourth and fifth STFs than the third STF. We rationalize this behavior by suggesting that acceptor-side contributions of qP are more significant than S-state quenching. In other words, the relative population of Q<sub>A</sub> to Q<sub>A</sub> decreases during the five STFs in this FRR fluorometry experiment.

**Fluorescence Quenching Does Not Change After the LD Transition When the Plastoquinone Pool Is Fully Reduced.** Introducing the cyt b<sub>f</sub> inhibitor 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) to illuminated cells results in a fully reduced plastoquinone (PQ) pool (PSII reduces PQ, which cannot be reoxidized by cyt b<sub>f</sub>) (Scheme S2). The LD transition was specifically probed in the presence of DBMIB as shown in Fig. S8. No change in fluorescence quenching is observed when the PQ pool is fully reduced. This observation supports our prior assertion that the primary quenching mechanism involves acceptor-side contributions to qP (i.e., Q<sub>A</sub>).

**Fig. S1.** The effect of dithionite addition in the in vitro oscillator mixture. (A) Dithionite (300 μM final concentration) was added on the phosphorylation phase of KaiC in 2-h intervals (open triangle). The dotted lines were aligned on the peak positions of a control in vitro oscillation mixture to which no Q0 was added to aid comparisons of the timing of peaks in each reaction. (B) Same as A except that dithionite was added on the dephosphorylation phase.

**Fig. S2.** The effect of the 4-h addition of oxidized Q0. Phase shift was monitored by addition of 4-h oxidized Q0 with every 2-h interval. The oxidized Q0 was added at the solid triangle, and dithionite was added at the open triangle to reduce Q0. The observed phase shift was marked with an asterisk.

**Fig. S3.** The phase response with Q0 addition in vivo at constant light. The oxidized Q0 was added at circadian times (A) 9, (B) 17, and (C) 24. For the control experiment, ethanol was added at the open triangle. The oxidized Q0 concentrations applied to all experiments are shown to the right to C. The phase advance (red asterisk) and delay (blue asterisk) peaks used on Fig. 2 are marked on the graph.
Fig. S4. Full FRR fluorescence traces measured during the simulated diurnal cycle as described in Fig. 4.
Fig. S5. Instantaneous fluorescence during the diurnal cycle as measured within the turbidostat by pulse amplitude modulation (PAM) fluorometry.
**Fig. S6.** FRR fluorescence traces during a modified diurnal cycle in which only PSI is activated during the light periods; 735-nm light (90 μE m⁻² s⁻¹) illuminated the sample during hours 0–4 and 16–20.
Fig. S7. Changes in FRR fluorescence parameters based on STF number.

Fig. S8. Origin and maximum fluorescence before and after the LD transition in the presence of DBMII. The sample was illuminated with blue actinic light during the first 20 min.
Scheme S2. Key pathways involved in photosynthetic and respiratory reduction and oxidation of the PQ pool in *Synechococcus elongatus* PCC 7942. Green, photosynthesis only; red, respiration only; orange, both processes. Modified from Vermaas (1) and Skizim et al. (2).