Circadian Rhythm | Hot Paper

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Regulation of the Cyanobacterial Circadian Clock by Electrochemically Controlled Extracellular Electron Transfer**

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Abstract: *There is growing awareness that circadian clocks are closely related to the intracellular redox state across a range of species. As the redox state is determined by the exchange of the redox species, electrochemically controlled extracellular electron transfer (EC-EET), a process in which intracellular electrons are exchanged with extracellular electrodes, is a promising approach for the external regulation of circadian clocks. Herein, we discuss whether the circadian clock can be regulated by EC-EET using the cyanobacterium Synechococcus elongatus PCC7942 as a model system. In vivo monitoring of chlorophyll fluorescence revealed that the redox state of the plastoquionone pool could be controlled with EC-EET by simply changing the electrode potential. As a result, the endogenous circadian clock of S. elongatus cells was successfully entrained through periodically modulated EC-EET by emulating the natural light/dark cycle, even under constant illumination conditions. This is the first example of regulating the biological clock by electrochemistry.*

The circadian clock is a universally conserved endogenous biochemical pacemaker that controls biological processes with a 24 h rhythm, and is found in organisms as diverse as cyanobacteria, fungi, algae, plants, and mammals.[1] Entrainment of an endogeneous circadian clock to environmental fluctuations requires a complicated molecular timing system, which involves numerous cellular machinery components that vary by species. In particular, the intracellular redox state, which influences various biological processes, is also known to have an intimate relationship with circadian rhythms. For example, the redox state of suprachiasmatic nuclei (SCN), which is a master circadian pacemaker of the endogenous circadian rhythm of mammals,[2,3] exhibits day/night changes that directly influence SCN neuronal activity. Moreover, recent research has shown that peroxiredoxins, enzymes found in virtually all living organisms,[4] have daily rhythms of oxidation/reduction in a range of cell types, including those with no nucleus or DNA, such as human blood cells.[5] Emerging evidence in multiple systems suggest that the cellular redox state is essential for the function of biological clocks.

Extracellular electron transfer (EET) is a process in which the intracellular electrons of living cells are exchanged with an extracellular electron donor/acceptor across the cell membrane.[6–8] In the presence of electron mediators, an electrode can serve as the extracellular electron donor/acceptor for the EET process. The direction (electron injection or ejection) and rate of electron transfer can be controlled simply by changing the applied potential. This process, termed electrochemically controlled EET (EC-EET), could be a promising approach for external regulation of the redox state, and hence, circadian clocks as well, because the intracellular redox state is determined by the exchange of the redox species.

Cyanobacterium *Synechococcus elongatus* PCC7942 is one of the model species for investigation of the circadian clock.[9] The circadian rhythm of *S. elongatus* cells is due to the combined function of three circadian clock proteins, KaiA, KaiB, and KaiC.[10,11] KaiA and KaiB proteins regulate the circadian pacemaker by enhancing and attenuating the autokinase activity of KaiC.[12] It was recently reported that oxidized (not reduced) quinones reset the phase of the clock by suppressing KaiA function.[13] As the redox balance of intracellular quinones is affected by environmental light intensity, the redox-responsive phosphorylation function of KaiA bridges environmental light/dark cycles and endogenous molecular oscillation. Although it was shown that the addition of oxidized quinones could reset the clock in a similar manner to thermal perturbations, the effect is only transient, and continuous regulation of the circadian clock has not been demonstrated to date.

Herein, we discuss our attempts to regulate the circadian clock of *S. elongatus* by periodic tuning of the redox state of plastoquinones (PQs) by EC-EET under constant illumina-

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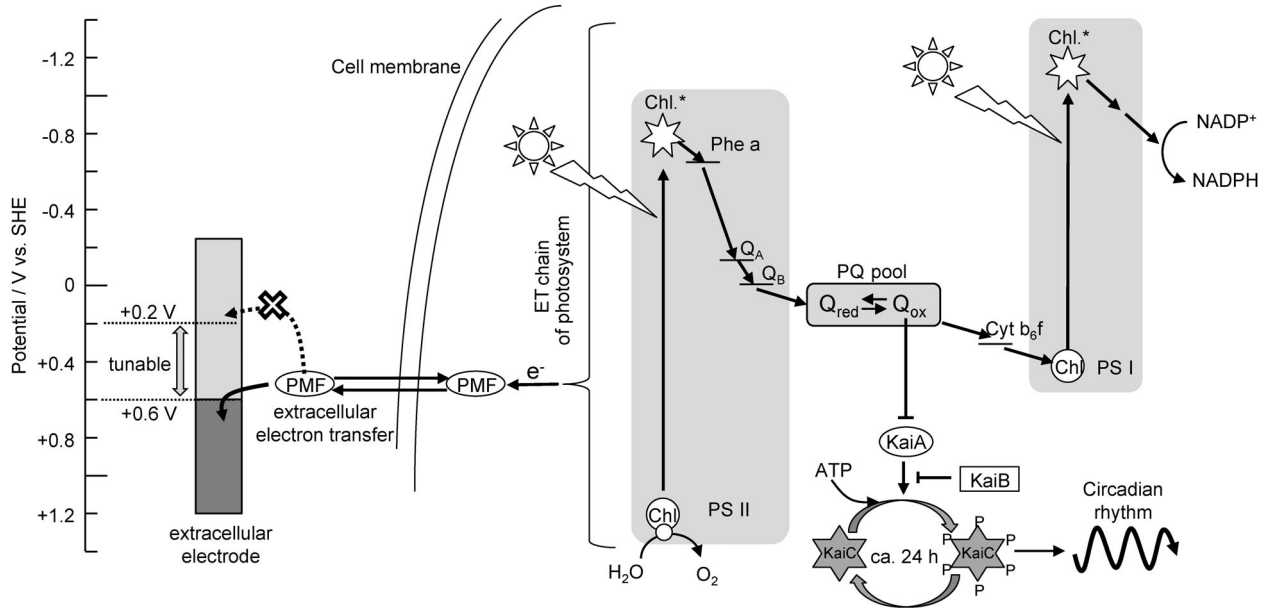

Figure 1. The relationship between electrochemically controlled extracellular electron transfer, the intracellular electron transfer chain, and the cyanobacterial circadian clock.

tion conditions (Figure 1). We first constructed an EC-EET pathway in *S. elongatus* cells using a transmembrane biocompatible electron mediator, poly(2-methacryloyloxyethyl phosphorylcholine-co-vinylferrocene) (Poly(MPC-co-VF); PMF), which is composed of biocompatible MPC[14] and redox-active VF (midpoint potential (E_M): +0.5 V vs. SHE; 2 mM VF is contained in 1 g L^{-1} PMF). We recently reported that PMF molecules mediate EET across cell membranes without apparent cytotoxicity, even for long-time electrochemical analysis.[15] In the present experiments, *S. elongatus* cells were introduced into an electrochemical chamber containing BG11 medium supplemented with 1 g L^{-1} PMF molecules. The redox potential of PMF allows it to receive electrons from intracellular redox species, such as nicotinamide adenine dinucleotide (E_0': −0.3 V vs. SHE) and plastoquinone (PQ; E_0': +0.1 V vs. SHE). Therefore, we can expect that intracellular electrons will be passed to the electrode through EC-EET by applying a potential more positive than the E_M at which PMF is electrochemically oxidized.

Figure 2 shows the anodic microbial photocurrents generated by *S. elongatus* cells plotted against light intensity with a wavelength of 680 nm, which excites the chlorophyll molecules of photosynthetic systems (see the Supporting Information for experimental details). The electrode potential was set at +0.6 V to promote the electrochemical oxidation of PMF. It can be seen that the photocurrent increased with the increasing intensity of the irradiated light. Because of the fact that the photosynthetic electron transfer chain is located in the thylakoid membrane, and that a microbial photocurrent was not observed in the absence of *S. elongatus* cells, or without PMF, even in the presence of cells (Figure S1), we concluded that PMF molecules mediated the transfer of intracellular electrons to the anode, and thus, an EET system was constructed. Importantly, even in the presence both of PMF and *S. elongatus* cells, a photocurrent

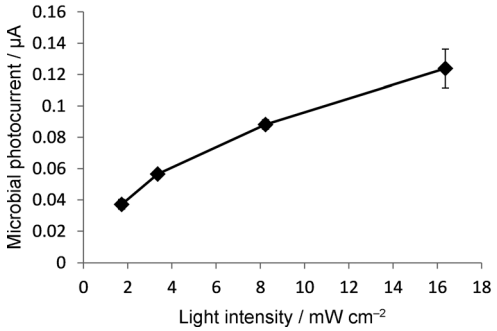

Figure 2. Microbial photocurrent from *S. elongatus* cells in the presence of PMF at +0.6 V. Error bars indicate the standard error ($n=2$).

was not observed at +0.2 V, a potential at which PMF is electrochemically reduced, indicating that through PMF EET can be regulated by the electrode potential.

We next investigated the potential dependency of the fluorescence intensity of chlorophyll in photosystem II (PS-II) to determine if the redox state of PQ can also be regulated by the EC-EET process. Photoexcited electrons in PS-II have four different pathways to relax, which quantitatively compete with each other: 1) heat dissipation, 2) energy transfer to adjacent pigments, 3) electron transfer to the downstream electron transfer chain, and 4) fluorescence emission. In contrast to pathways 1 and 2, only the activity of pathway 3 changes based on the redox state of PQ. Therefore, although the quantitative relationship between pathways 3 and 4 is not strictly valid, the redox state of PQ can be qualitatively estimated by observing fluorescence intensity. Following previously reported procedures,[16] a modulated fluorometer was used to monitor in vivo chlorophyll fluorescence of *S. elongatus* cells (see the Supporting Information for the experimental details). For this technique, living cells in the

electrochemical system are periodically irradiated with 620 nm light to excite the chlorophyll involved in PS-II, and the generated fluorescence is then measured. Notably, only fluorescence that follows periodic excitation was extracted using a lock-in-amplifier. The redox state of PQ can be estimated from the ratio of variable fluorescence (F_v') to maximum fluorescence (F_m').[16] As a reference, F_v'/F_m' was first measured under light/dark cycles with a periodicity 1 h using 635 nm actinic light in the absence of PMF molecules and without applied potential (Figure 3a). In accordance with

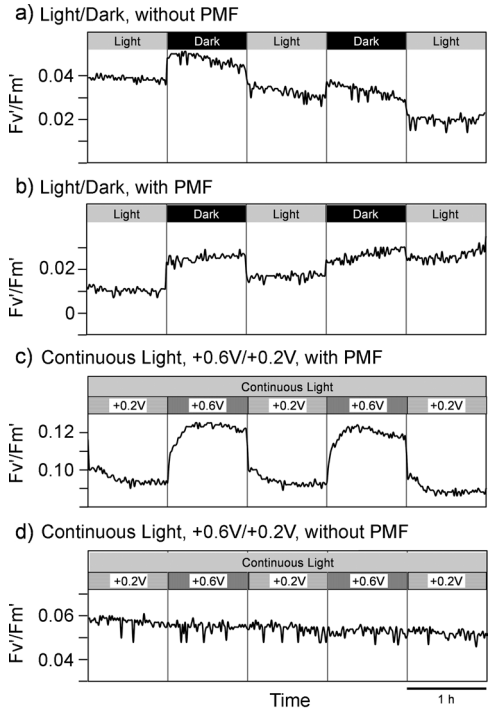

Figure 3. Time courses of F_v'/F_m' from PS-II under light/dark (L/D) cycles (a,b) or +0.2 V/+0.6 V (N/P) cycles (c,d). The samples were continuously illuminated under the N/P cycles.

the literature,[16] the F_v'/F_m' decreased (or increased) under light (or dark) conditions, where the PQ molecules are predominantly in the reduced (or oxidized) state. As the fluorescent intensity reversibly responded to the environmental light/dark conditions within 10 min, it is likely that the redox state of PQ is the dominant factor for the change in fluorescence intensity. Notably, addition of the reduced form of PMF had no effect on the chlorophyll fluorescence under the periodic light/dark stimulation (Figure 3b), indicating that the reduced form of PMF does not influence the redox state of PQ.

Figure 3c,d shows the time courses of F_v'/F_m' against periodic +0.6 V/+0.2 V shifts of the electrode potential under constant illumination conditions in the presence and absence of PMF molecules. In the presence of PMF, the F_v'/F_m' changed in response to the periodic electrochemical perturbation (Figure 3c). Specifically, the F_v'/F_m' increased at +0.6 V, the potential at which the PMF molecules were electrochemically oxidized, whereas the intensity correspondingly decreased when the potential was set at +0.2 V. Such potential dependency of the F_v'/F_m' was not observed in the absence of PMF (Figure 3d). In addition, the same tendency was observed even without actinic light irradiation (Figure S2). The slower response to F_v'/F_m' in Figure 3c compared to those in Figure 3a,b might be because of the diffusion process of the PMF mediator. These results provide strong evidence that PMF molecules transfer the intracellular electrons of the PQ to the anode in this electrochemical system (Figure 1). Together, these results clearly indicate that the redox state of PQ corresponds to that experienced under dark conditions (i.e., oxidative), even under illumination, when a positive electrode potential is applied in the presence of PMF.

Based on the results shown above, we anticipated that the PQ redox state, which is of essential importance for the cyanobacterial circadian clock, is regulated by EC-EET constructed with the PMF. To monitor the circadian rhythm of *S. elongatus* in vivo, we used an engineered strain of *S. elongatus* in which a bioluminescent reporter was introduced downstream of the promoter of the *kaiBC* gene, whose activity exhibits circadian rhythms with a large amplitude. Bioluminescence re-presenting clock-driven transcription of kaiBC was monitored as a measure of circadian clock output. To confirm whether the transcription of *kaiBC* responded to periodically modulated EC-EET, we monitored bioluminescence changes during alternate +0.6 V (12 h)/+0.2 V (12 h) perturbation with 24 h periodicity under constant illumination conditions (Figure 4a, phase I). The intensity of the bioluminescence decreased at +0.6 V and increased at +0.2 V (Figure 4a1,a2, respectively), indicating that the transcription of circadian-clock-driven genes can be regulated by adjusting the electrode potential.

After subjecting *S. elongatus* cells to three cycles of +0.6 V/+0.2 V with 24 h periodicity, the cells were then exposed to open circuit conditions without applied potential. Time courses of the bioluminescence produced under these constant environmental conditions are shown in Figure 4a1,a2 (phase II). Bioluminescence with circadian oscillation continued for more than three days without any periodic environmental input. Importantly, the oscillatory phase was determined by the phase of the potential cycles in phase I, which clearly indicates that the endogenous rhythm was entrained by the periodically modulated EC-EET. Note that clear 24 h oscillation did not occur when cells were not subjected to the phase I period (Figure S3). For an endogenous rhythm to qualify as a circadian rhythm, it is necessary that the oscillation exhibits temperature-compensation character, in which the period length is only slightly affected by temperature changes. We then further confirmed that the length of the rhythm cycles in phase II, which were entrained with the potential cycles in phase I, was not markedly different from those at 24 h at various temperatures. The Q_{10} value, which represents the factor by which the oscillation frequency increases for every ten degree rise in the temperature, and can be an index for the temperature insensitivity, was estimated to be 1.03 (Figure 4b).

Based on the present experimental findings, it can be concluded that we have successfully regulated the circadian

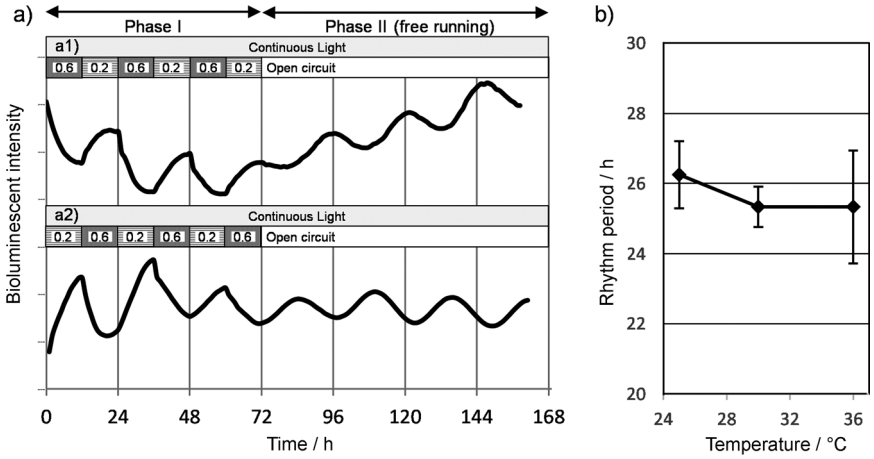

Figure 4. a) Time courses of bioluminescence from the engineered strain of *S. elongatus* expressing a bioluminescent reporter under electrochemical anaysis with constant light irradiation. Phase I is the period for electrochemical entrainment. The cells were then exposed to open-circuit conditions (phase II). b) Periods of the circadian rhythm at different temperatures. Error bars indicate the standard deviation ($n = 3$ or 4).

clock of the cyanobacterium *S. elongatus* by EC-EET. Importantly, the redox state of PQ can be periodically modulated by electrochemically alternating the redox state of PMF, which might be the entraining cue of cyanobacterial circadian rhythm. This speculation is evidenced by the fact that environmental light/dark conditions can be emulated by externally controlling the redox state of PQ by periodically modulated EC-EET. Although the machinery of biological clocks varies by species, the intracellular redox state is frequently involved in clock regulation. We propose that tuning the redox state of PQ through EC-EET is a potential method to directly or indirectly regulate the biological clock across a range of species.

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