Hourglass Model for a Protein-Based Circadian Oscillator

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(Received 15 September 2005; published 24 January 2006)

Many organisms possess internal biochemical clocks, known as circadian oscillators, which allow them to regulate their biological activity with a 24-hour period. It was recently discovered that the circadian oscillator of photosynthetic cyanobacteria is able to function in a test tube with only three proteins, KaiA, KaiB, and KaiC, and ATP. Biochemical events are intrinsically stochastic, and this tends to desynchronize oscillating protein populations. We propose that stability of the Kai-protein oscillator relies on active synchronization by (i) monomer exchange between KaiC hexamers during the day, and (ii) formation of clusters of KaiC hexamers at night. Our results highlight the importance of collective assembly or disassembly of proteins in biochemical networks, and may help guide design of novel protein-based oscillators.

DOI: 10.1103/PhysRevLett.96.038303 PACS numbers: 82.39.Rt, 87.15.Rn, 87.17.Aa

Circadian rhythms coordinate an organism's activity to the day-night cycle of its environment. In complex organisms such as humans, the circadian oscillator consists of a set of proteins that directly regulate their own production [1]. In these organisms, positive and negative regulation of gene expression is an essential part of the oscillatory mechanism [2,3]. Circadian oscillations also occur in some photosynthetic bacteria (cyanobacteria) [4]. Surprisingly, these oscillations do not require gene regulation, and can be reconstituted in the test tube with only three proteins, KaiA, KaiB, and KaiC, with ATP as an energy source [5]. Thus, the Kai system presents a tractable biological network to explore and define the physical mechanisms leading to oscillatory behavior.

Studies of the Kai proteins of the cyanobacterium *Synechococcus elongatus* have revealed the following: KaiC monomers bind ATP and form ringlike hexamers [6]. KaiC then autophosphorylates at a slow rate ($\sim 0.05\ h^{-1}$) at two of three possible sites per monomer [7]. Spontaneous dephosphorylation of KaiC is fast enough that at steady state only about 20% of KaiC monomers are singly or doubly phosphorylated [8]. Phosphorylation of KaiC is accelerated by KaiA, which forms dimers, and this acceleration is suppressed by KaiB [9]. *In vitro,* when all three proteins and ATP are present, the phosphorylated fraction of KaiC oscillates persistently with a period of approximately 24 hours [5]. *In vivo,* KaiC phosphorylation increases during the day and decreases during the night. The peak of KaiC phosphorylation at the beginning of the night coincides with the formation of bound complexes of KaiA and KaiB with hexamers of KaiC [10]. Complexes open early in the day. If each KaiC hexamer is following its own stochastic cycle of phosphorylation and dephosphorylation, how are these cycles synchronized to form a coherent clock?

This Letter provides a quantitative model for coherent 24 h Kai-protein oscillations (see Fig. 1). We call this the hourglass model: during the day KaiC hexamers become increasingly phosphorylated (sand fills the hourglass), then large clusters of KaiC hexamers form in complex with KaiA and KaiB (the hourglass is turned over), KaiC hexamers in clusters are unaffected by KaiA, so during the night the clusters dephosphorylate (sand empties from the hourglass), until the clusters break up, releasing KaiA, KaiB, and KaiC hexamers. The hourglass model incorporates the known biochemical interactions of the Kai proteins and two new features that seem to be vital for robust oscillations, namely, exchange of KaiC monomers during the day, to synchronize hexamer phosphorylation, and formation of clusters of KaiC hexamers at night, to synchronize release of dephosphorylated hexamers. There is some experimental evidence for monomer exchange *in vivo* [10], but, to our knowledge, there is no direct evidence regarding clustering of ternary complexes of KaiA, KaiB, and KaiC.

The Kai-protein oscillator is insensitive to fluctuations of protein concentrations and ratios, since these are observed to vary significantly *in vivo* [9]. We hypothesize that there is a saturating amount of KaiA *in vivo* to assist phosphorylation of KaiC, since even a $\sim$1:7 ratio of KaiA dimer:KaiC hexamer is sufficient to nearly fully phosphorylate all hexamers [9]. Similarly, we assume there is saturating KaiB *in vivo* to complex with phosphorylated KaiC hexamers and form clusters.

To quantitatively study the hourglass model, we numerically solve a set of time-dependent equations describing the kinetics of KaiC oligomers, each composed of exactly N_o KaiC monomers. The physiological case of hexamers corresponds to $N_o = 6$. KaiC oligomers are assumed to be either free or in clusters of fixed size N_c. Exchange of

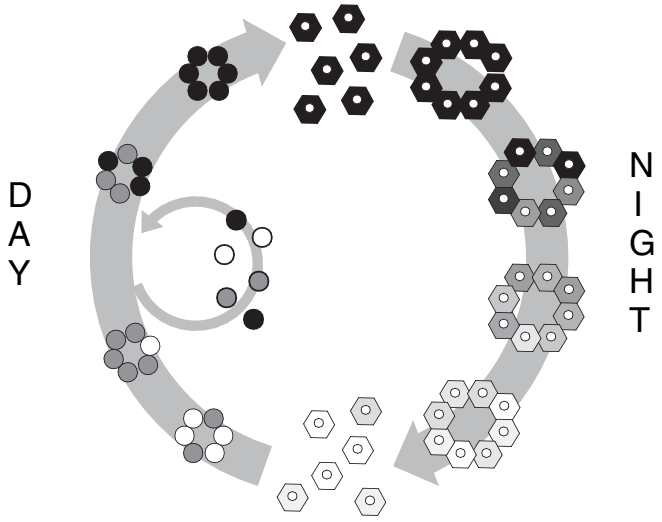

FIG. 1. Schematic of model circadian oscillator in cyanobacteria, including proposed KaiC-monomer exchange during the day and cluster formation at night. KaiC monomers (small circles) self-associate in the presence of ATP to form hexamers. During daylight hours, KaiA (not shown) accelerates phosphorylation of KaiC, which can have 0 (white), 1 (gray), or 2 (black) phosphoryl groups. During this period, KaiC-monomer exchange helps synchronize hexamer phosphorylation levels. Fully phosphorylated KaiC hexamers (hexagons) can complex with KaiA and KaiB (not shown), allowing KaiC to form clusters of N_c hexamers ($N_c = 8$ shown). During the night, KaiC in clusters dephosphorylates (lighter shaded hexagons). When a cluster's phosphorylation level reaches $P_{\min}$, the cluster breaks apart, freeing KaiC hexamers to begin the day cycle again.

monomers among free oligomers is assumed to be fast. Therefore, instead of following the phosphorylation states of free oligomers, we follow only their monomer constituents. Specifically, $M_0(t)$, $M_P(t)$, and $M_{2P}(t)$ are, respectively, the concentrations of unphosphorylated, singly phosphorylated, and doubly phosphorylated KaiC monomers in free oligomers. Each of two modification sites on a KaiC monomer is phosphorylated at a rate α and dephosphorylated at a rate γ. Since KaiC oligomers are assumed to be random mixtures of the three types of monomers, the concentration of free, fully phosphorylated oligomers is $(m_{2P})^{N_o} O_f$ where $m_{2P} = M_{2P}(t)/[M_0(t) + M_P(t) + M_{2P}(t)]$ is the fraction of doubly phosphorylated monomers and O_f is the concentration of free oligomers given by $O_f = [M_0(t) + M_P(t) + M_{2P}(t)]/N_o$. We assume that only fully phosphorylated oligomers are able to form clusters, at a rate β.

Upon formation, each cluster of N_c oligomers is fully phosphorylated; i.e., it has all $2N_oN_c$ possible sites phosphorylated. Since dephosphorylation of each site occurs independently, at a rate γ, the equation tracks only the concentration of clusters of each total phosphorylation level, C_i. We assume that when the phosphorylation level of a cluster reaches $P_{\min}$ the cluster breaks up. Thus there are $i = P_{\min} + 1, \ldots, 2N_oN_c$ possible phosphorylation states of a cluster. When a cluster breaks up, N_c free oligomers are released, which increases the levels of $M_0(t)$ and $M_P(t)$ accordingly.

These processes are described by the following equations:

$$\frac{dM_0(t)}{dt} = -2\alpha M_0 + \gamma M_P + (N_oN_c - P_{\min})(P_{\min} + 1)\gamma C_{P_{\min}+1}, \quad (1)$$

$$\frac{dM_P(t)}{dt} = 2\alpha M_0 + 2\gamma M_{2P} - \alpha M_P - \gamma M_P + P_{\min}(P_{\min} + 1)\gamma C_{P_{\min}+1}, \quad (2)$$

$$\frac{dM_{2P}(t)}{dt} = \alpha M_P - 2\gamma M_{2P} - N_oN_c\beta[(m_{2P})^{N_o} O_f]^{N_c}, \quad (3)$$

$$\frac{dC_{2N_oN_c}(t)}{dt} = \beta[(m_{2P})^{N_o} O_f]^{N_c} - 2N_oN_c\gamma C_{2N_oN_c}, \quad (4)$$

$$\frac{dC_i(t)}{dt} = -i\gamma C_i + (i + 1)\gamma C_{i+1}, \quad i = P_{\min} + 1, \ldots, 2N_oN_c - 1. \quad (5)$$

The solutions to these equations yield stable oscillations in the phosphorylation of KaiC over a fairly broad range of parameters, for sufficiently large oligomers and clusters. In general, the oscillations constitute a limit cycle; i.e., the oscillatory solution is the only solution, independent of initial conditions. In Fig. 2, we show the fraction of either single or doubly phosphorylated KaiC (solid lines) and the fraction in clusters (dashed lines) for parameters chosen to reflect wild-type behavior and possible period mutants. Figure 2(a) shows oscillations for hexamers ($N_o = 6$) with cluster size $N_c = 12$ for the parameters yielding the largest amplitude oscillations. The fraction of phosphorylated KaiC oscillates between 38% and 70%. To produce a period of 24 h, the dephosphorylation rate is set to $\gamma = 0.129\ \mathrm{h}^{-1}$, which is within the measured experimental range [5]. The formation of clusters lags the rise in phosphorylation by ~8 h. A similar lag is seen *in vivo,* where the maximum association of KaiA and KaiB with KaiC follows the peak in KaiC phosphorylation by around 6 h [9,10].

In Figs. 2(c), 2(c), and 2(d), we show oscillations for several theoretical "period mutants." Experimentally, mutations in KaiA or KaiC that lead to a slower rate of phosphorylation produce long-period oscillations both *in vivo* [11] and *in vitro* [5]. In Fig. 2(b), the rate of phosphorylation α is reduced by 25% compared to Fig. 2(a). This has the effect of lengthening the period by 5% as it takes longer for the free oligomers to become fully phosphorylated. In Figs. 2(c) and 2(d), we show oscillations for two theoretical short-period mutants. In Fig. 2(c),

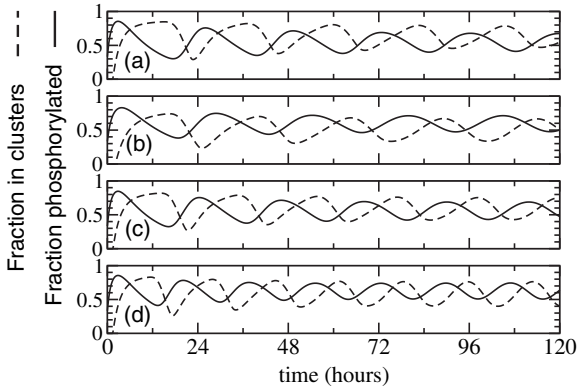

FIG. 2. Model circadian oscillations showing fraction of phosphorylated KaiC (solid curves) and fraction of KaiC in clusters (dashed curves) from Eqs. (1)–(5). In (a)–(d) $N_o = 6$, $N_c = 12$, $\gamma = 0.129\ \mathrm{h}^{-1}$, the total number of oligomers, $O_0 = 500$, and with initial conditions $M_0(t=0) = (0.7)2N_oO_0$, $M_{\mathrm{P}}(t=0) = (0.3)2N_oO_0$, and $M_{2\mathrm{P}}(t=0) = 0$. (a) Wild-type behavior, with parameters $\alpha/\gamma = 8.0$, $\beta O_0^{N_c-1}/\gamma = 4.88 \times 10^{14}$, $P_{\min} = 14$. (b) Long-period mutant with parameters as in (a) except for a lower phosphorylation rate $\alpha/\gamma = 6.0$. (c) Short-period mutant with parameters as in (a) except for a higher dephosphorylation rate $\gamma'/\gamma = 1.1$. (d) Short-period mutant with parameters as in (a) except for earlier cluster opening, $P_{\min} = 29$.

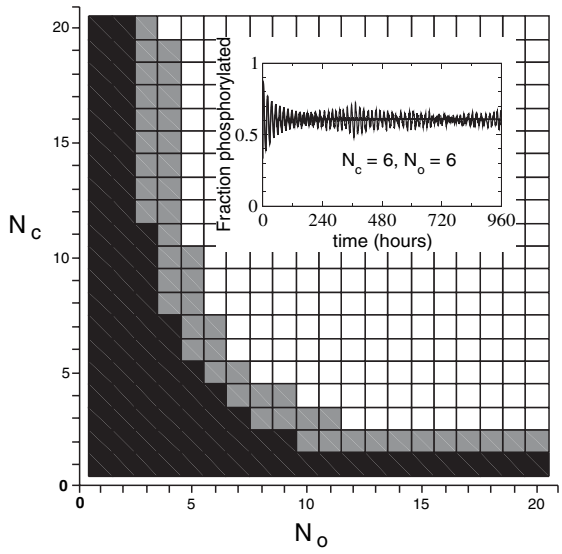

FIG. 3. Oscillation phase diagram for different sized oligomers N_o and different sized clusters N_c. White squares are values of N_o and N_c for which Eqs. (1)–(5) produced a limit-cycle oscillator for some choice of parameters. For other N_o and N_c values, gray squares indicate that oscillations occurred in a stochastic simulation with $O_0 = 500$ oligomers. Black squares indicate no oscillations. Inset: Oscillations of KaiC phosphorylated fraction for a stochastic simulation with 500 KaiC hexamers, $N_o = 6$, $N_c = 6$, $\alpha/\gamma = 9.5$, $\beta O_0^{N_c-1}/\gamma = 3.125 \times 10^7$, and $P_{\min} = 14$.

the dephosphorylation rate γ is raised by 10% compared to Fig. 2(a), which leads to an overall shorter lifetime of clusters and reduces the oscillation period by 9%. A similar shortening of the period is achieved in Fig. 2(d) by increasing the level of phosphorylation at which clusters break up, from $P_{\min} = 14$ to 29, which decreases the oscillation period by 26%. Importantly, we find that changing the concentration of KaiC by a factor of 2 affects the period by only $\sim \pm 5\%$.

Cluster formation in the model is necessary to synchronize the release of dephosphorylated oligomers. Without cluster formation, we find that the time it takes oligomers to dephosphorylate has too large a stochastic variation to support stable oscillations. For example, the standard deviation of the time it takes a single hexamer to fully dephosphorylate is 40% of the average time. In contrast, the standard deviation of the time it takes a cluster of $N_c = 12$ hexamers to dephosphorylate to $P_{\min} = 14$ is 10% of the average time [as in Figs. 2(a)–2(c)]. For a given cluster size there is a unique, nonzero $P_{\min}$ that minimizes the standard deviation over the mean of cluster opening times [12]. In the model, how big do clusters have to be to produce stable oscillations? We performed a numerical search over the space of oligomer sizes N_o and cluster sizes N_c to find the regions where stable oscillations exist for some choice of parameters (see Fig. 3). For the physiological case of hexamers, stable oscillations are possible down to a cluster size of $N_c = 8$. In general, larger oligomers allow stable oscillations with smaller clusters. Roughly speaking, oscillations are possible if the standard deviation of the cluster lifetime is $\simeq 15\%$ of the average, or less. The cluster size N_c also enters as an exponent in the rate of cluster formation. However, we find that this exponent, which represents the number of oligomers required to nucleate a cluster, can be reduced by at least a factor of 2 without strongly affecting the oscillations [13]. Similarly, oscillations are little affected by a moderate spread of cluster sizes (up to $\pm 30\%$ for $\langle N_c \rangle = 14$).

Besides cluster formation, we found that monomer exchange between free oligomers, which synchronizes their phosphorylation levels, is necessary for stable oscillations. To study the model without exchange, we modified Eqs. (1)–(3) to follow the concentrations of free oligomers of different total phosphorylation levels. As before, only fully phosphorylated oligomers were allowed to form clusters [14]. In this nonexchange version of the model, no stable oscillations were found over the full range of N_o and N_c values shown in Fig. 3.

None of the kinetic equations we studied includes noise. Neglect of noise is valid for a large, freely mixing volume of interacting proteins, such as in the *in vitro* experiments [5]. However, within the cytoplasm of a single cell the number of Kai proteins is limited to $\sim$500 KaiA, $\sim$20 000 KaiB, and $\sim$10 000 KaiC [9]. We have found that stochastic effects due to a finite number of KaiC monomers can increase the region of oligomer and cluster space where oscillations are found. Stochastic simulations of Eqs. (1)–(5) were performed using the Gillespie algorithm [15].

With 5000 KaiC oligomers, the results of the stochastic simulations simply reproduced the results of the kinetic equations. However, with only 500 KaiC oligomers, the stochastic simulations produced oscillations for some N_o and N_c values where the kinetic equations did not (gray squares in Fig. 3). For example, the inset of Fig. 3 shows persistent oscillations for a stochastic simulation with $N_o = 6$ and $N_c = 6$. The oscillations are not merely noise, as a Fourier transform of the data for $t > 240$ h shows a narrow peak at a fixed nonzero frequency (data not shown). Such behavior has been observed in simulations of other circadian oscillators, where biochemical noise perturbs the system away from the stable fixed point [3].

For stable oscillations, Kai hexamers must remain synchronized as they periodically phosphorylate and dephosphorylate. Our model relies on monomer exchange and cluster formation to achieve synchrony. This need not be the only way to generate oscillations in the Kai system, and we briefly consider two alternative mechanisms: (1) an autocatalytic process of assembly and disassembly of ternary complexes of KaiA, KaiB, and KaiC could synchronize the population of hexamers, or (2) monomer exchange could synchronize phosphorylation, and the time it takes each hexamer to dephosphorylate could be very sharply peaked. Alternative (1) is similar to our model of cluster formation, but requires stronger assumptions, namely, that individual hexamers be kinetically trapped in long-lived metastable states and then rapidly escape from these states due to transient interactions only when nearly phosphorylated or dephosphorylated. In contrast, in our model, dephosphorylating hexamers are stabilized by direct contact with other hexamers in clusters. A relevant analogy is the collective assembly or disassembly of microtubules, in which polymers of GDP-bound tubulin are stabilized by a GTP-bound cap [16]. In alternative (2), in addition to long-lived metastability, the dephosphorylation time of each hexamer must be accurate to better than $\simeq 15\%$. It is not clear how phosphorylation-state-dependent dephosphorylation rates could become so fine-tuned, since oscillations do not occur for lesser accuracy.

In summary, we have presented a working model for one of the simplest biochemical networks that displays oscillations, namely, the circadian oscillator of cyanobacteria. Our model has two significant experimentally testable predictions: monomer exchange during the day, and cluster formation at night. These predictions are in concord with the importance of collective protein assembly or disassembly in biochemical networks such as bacterial cell division, which relies on the assembly of FtsZ [17], a homolog of tubulin, cell polarization that is governed by the dynamics of MreB, a homolog of actin [18], procapsid formation by viral scaffold and coat proteins [19], and the collective assembly of RecA on DNA for damage repair [20]. Novel oscillators based on transcription have been demonstrated [21], and we hope that our analysis may help guide the design of novel protein-only oscillators.

We thank Morten Kloster and Nancy Forde for valuable suggestions. One of us (E. E.) acknowledges the support of NSERC and CIAR in carrying out this research.

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[12] The average dephosphorylation time of a cluster is $t_{\mathrm{avg}} = \sum_{i=P_{\mathrm{min}}+1}^{2N_oN_c} 1/(i\gamma)$ with a variance of $\sigma^2 = \sum_{i=P_{\mathrm{min}}+1}^{2N_oN_c} 1/(i\gamma)^2$. σ/t_{avg} has a minimum at nonzero P_{min}.
[13] The time interval during which clusters form is approximately independent of N_o and N_c: Significant cluster formation begins when the fraction of doubly phosphorylated monomers reaches a threshold value $m_{2\mathrm{P}} \simeq 0.5$. Then $m_{2\mathrm{P}}$ remains pinned at this threshold until the source of new doubly phosphorylated monomers is depleted. So the time interval for cluster formation is just the time it takes to phosphorylate the pool of M_{P}, which is $\simeq 1/\alpha$.
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