

Mixtures of opposing phosphorylations within hexamers precisely time feedback in the cyanobacterial circadian clock

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Short Abstract — Circadian oscillations are generated by the cyanobacterial clock proteins, KaiA, KaiB, and KaiC, through rhythmic interactions that depend on multisite phosphorylation of KaiC. However, the mechanisms that allow the oscillatory period to remain roughly 24h over a wide range of protein stoichiometries are not clear. We show using biochemical techniques and mathematical modeling that the hexameric organization of KaiC allows the two KaiC phosphosites to oppose each other within a hexamer in determining the hexameric affinity for both KaiA and KaiB. The opposition produces an ultrasensitive switch in the strength of negative feedback, which generates period robustness across component stoichiometries.

Keywords — allostery, circadian rhythms, modeling, robustness

I. INTRODUCTION

ALMOST all light-sensing organisms have circadian or near 24hr period rhythms in gene expression to anticipate diurnal environmental changes [1]. These rhythms are driven by cell-autonomous oscillators that maintain a circadian period in the absence of environmental cues [1]. In these clock systems, period robustness against intrinsic and extrinsic noise emerges as a general requirement given the fitness defects and health problems that result from mismatches between the environmental and internal clock period [2, 3]. The cyanobacterial core oscillator consists of just three proteins, KaiA, KaiB, and KaiC, but is capable of generating robust single cell rhythms that remain in phase for several generations [4, 5]. The KaiABC system is the only circadian clock that can be fully reconstituted *in vitro*, making it an ideal system to study the biochemical mechanisms of period robustness [6]. Previous studies show that phosphorylation oscillate with a near 24h period on two residues in KaiC, Ser431 and Thr432 [7]. These oscillations are generated by rhythmic interactions with KaiA, which stimulates KaiC autokinase activity, and KaiB, which sequesters KaiA in ternary complexes [8]. However, the biochemical mechanisms that allow KaiC phosphorylation to oscillate with a circadian period over a wide range of protein stoichiometries *in vitro* are not clear.

II. RESULTS

We show that KaiC hexamers consist of a mixture of differentially phosphorylated subunits, and that the two phosphorylation sites have opposing effects, but only when mixed together in a KaiC hexamer, on its affinity for KaiB. Phosphorylation on Thr432 opposes KaiB binding while phosphorylation on Ser431 enhances KaiB binding. We likewise show that the ability of KaiA to simulate KaiC

kinase activity negatively correlates with increasing levels of Ser431 phosphorylation within a KaiC hexamer and that KaiA directly opposes the binding of KaiB to KaiC in a fixed phosphorylation state. Given this experimental evidence, we propose a model where KaiA and KaiB recognize alternative allosteric states of the KaiC hexamer, and where the stability of either state is determined by the combined phosphorylation state of all six subunits. To investigate the role of subunit phosphostates on the allosteric transition, we built an ODE model describing the phosphorylation, dephosphorylation and complex formation reactions of KaiC hexamers constrained by experimental rate constants, and used a Monte Carlo search procedure of free energies assigned to each monomeric KaiC phosphostate the hexameric sum of which determine the free energy difference between the two allosteric states. We find that in order to generate stable oscillations over the observed component stoichiometries, phosphorylation on the two KaiC phosphosites must oppose each other in the context of sufficiently coupled KaiC molecules that undergo allosteric transitions concertedly. This opposition produces an ultrasensitive switch in the strength of negative feedback with respect to time, allowing the oscillator to maintain the correct period across a wide range of component concentrations. Similar strategies based on opposing modifications in oligomeric systems may be used to support robustness in other timing systems and in cellular signaling.

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