Circadian clocks must be entrainable to keep their oscillations in phase with the day-night rhythm. On the other hand, they must also exhibit input compensation: the period must remain about one day in different constant environments. The post-translational oscillator of the Kai system can be entrained by transient or oscillatory changes in the ATP fraction, yet is insensitive to constant changes in this fraction. We study in three different models of this system how these two seemingly conflicting criteria are met: the Van Zon model (Van Zon et al., PNAS, 2007), the Rust model (Phong et al., PNAS, 2013), and our new model presented in chapter 4. We find that the new model exhibits the best trade-off between input compensation and entrainability: on the footing of equal phase-response curves, it exhibits the strongest input compensation. Performing stochastic simulations at the level of individual hexamers allows us to identify a new mechanism, which is employed by the new model to achieve input compensation: At lower ATP fraction, the individual hexamers make a shorter cycle in the phosphorylation state space, which compensates for the lower pace at which they traverse the cycle.
5.1. Introduction

Circadian clocks help organisms to coordinate their metabolism and behavior with the daily changes in the environment [145]. These clocks are prevalent in a wide range of organisms from bacteria to humans, but all have three important features in common: First, a circadian clock is a self-sustained oscillator, meaning that oscillations persist even in the absence of any external cue, with a rhythm of about 24 hours. Second, the clock is entrainable so that its oscillations can be kept in phase with the day-night rhythm. To this end, a circadian clock must be able to respond to daily cues such as rhythmic changes in light and temperature. Third, the clock has some form of input compensation, such that the period is constant, even when the temperature or light intensities change for longer times. Importantly, these last two requirements seem to be at odds with each other. A clock that is easily entrained, because, for instance, the rates of the biochemical reaction that determine the period strongly depend on temperature, would seem to have a period that also depends on the temperature. This clock would be a bad predictor of time. As was shown in [146, 147], cyanobacteria with clock mutants having intrinsic periods ranging from 22 to 30 hours, grow significantly slower in a 12:12 light-dark cycle than wild-type cells. Therefore, input compensation is critically important to the function of a circadian clock. On the other hand, if a clock would be completely insensitive to any external change, it would trivially have a period that is insensitive to changes in the environment. However, such a clock would not be entrainable, and as a result of biochemical noise, it would inevitably run out of phase with the day-night rhythm. Clearly, entrainability is essential to keep the clock in phase with the day, which means that the clock needs to be sensitive to changes in the environment. What the properties of an oscillator should be to fulfill both conditions has been studied in different systems, both experimentally and theoretically [6, 52, 148–151].

We use the post-translational oscillator of the Kai circadian clock, found in the freshwater cyanobacterium *Synechococcus elongatus* PCC 7942, as a model system. It is well known that this circadian clock is sensitive to transient changes in its environment, and hence entrainable, yet robust to permanent changes in its input, thus showing input compensation [5, 53, 73, 152, 153]. It was shown in an seminal experiment in 2005, that the core oscillator can be reconstituted in-vitro, and consists of the three proteins KaiA, KaiB and KaiC, in solution with ATP [12]. KaiC is a phosphotransferase [116, 117], which, depending on its conformation, switches between phases of phosphorylation and dephosphorylation [15, 123]. KaiA is a nucleotide exchange factor, that facilitates exchange of ADP to ATP in the nucleotide binding pockets of KaiC [127], which enhances phosphorylation of KaiC (see chapter 4). KaiB counteracts the effect of KaiA, by binding to KaiC, and sequestering KaiA from solution [109, 111]. Remarkably, even in an in-vitro essay, the phase of the oscillator can be reset while keeping a robust circadian period, by changing the redox state [154, 155], temperature [12, 136, 156] or the ratio of ATP to ADP in the buffer [7, 26].

In this work, we study entrainability and input compensation under changes in the bulk ATP fraction, by comparing three different models of the post-translational Kai oscillator. We have chosen to investigate the influence of the ATP fraction, because experiments suggest that this is the most important mechanism for entrainment of the oscillator [30], and because its effect has been studied in great detail [7, 26, 157]. Specifically,
we will study the effect of the ATP fraction on the hexamer model by Van Zon et. al. [15], the extended monomer model, based on the original model by Rust et. al. [22, 26], and the new model introduced in this thesis (see chapter 4). In general, as shown in experiments, the effect of the ATP fraction on the oscillator is that the phosphorylation rate is roughly proportional to the ATP fraction, and the overall rate of dephosphorylation is unaffected [7]. We will study the entrainability by applying a 6 hour pulse, during which the ATP fraction is reduced from 100% to 40%, at different phases of the oscillation, and compare the maximally induced phase shift in each model. We analyze period stability in each model by running the models at different constant ATP fractions and observe how the period and other important quantities of the oscillations change. Lastly, simulations of our new model allow us to track each individual hexamer, and measure the timing between states as it proceeds through its cycle. In this way, to our knowledge for the first time, we can predict how individual KaiC hexamers respond to external cues.

Below we briefly describe the three models, and the mechanisms they employ to achieve input compensation and entrainability. We discuss three mechanisms of input compensation, two of which have been partly identified elsewhere, and one of which is novel. The first, present in all models, is related to the delay between the moment the KaiC front runners (hexamers that are more phosphorylated than the average) reach the top of the cycle, which is the point they no longer need KaiA to progress along the cycle, and the later time at which the front runners reach a state in which they sequester KaiA [26]. As the ATP fraction in the bulk is reduced, the rate of phosphorylation decreases, which means that it takes longer to reach the top of the cycle, thus extending the phosphorylation phase. However, the lower rate of phosphorylation also means that during the delay less hexamers make it to the top of the cycle. This decreases the number of KaiC molecules that can participate in sequestering KaiA, thus shortening the dephosphorylation phase, counteracting the longer phosphorylation phase. The second mechanism, present only in the Rust model, is related to the positive feedback in that model which results from the mutual inhibition between the sequestration of KaiA by serine-phosphorylated KaiC (the S state, see chapter 4), and KaiA stimulating the transition from serine-phosphorylated to doubly phosphorylated KaiC (the D state). The third mechanism, present in our new model, and first identified here, concerns the path individual hexamers take through phosphorylation state space: At lower ATP fraction, the individual hexamers move through a smaller phosphorylation cycle, which compensates for the lower rate at which they traverse this cycle. The generic idea of input compensation via a trade-off between the size of the cycle in state space and the speed at which it is traversed was presented by Hatakeyama and Kaneko [149]. Here we present a specific manifestation of this mechanism at the level of individual hexamers. This mechanism is absent in the Van Zon and Rust models, where the phosphorylation cycles of the hexamers or monomers through state space are independent of the ATP fraction.

As period robustness can trivially be achieved by making the oscillator completely insensitive to the ATP fraction, we next investigate the phase response curve for each model, which describes the induced phase shift of the clock upon a pulse of ADP [145]. We find that the models of Van Zon and Rust are, depending on their sensitivity to the ATP fraction, either strongly entrainable yet have a period that depends on the ATP fraction or have a very stable period but are not entrainable. In contrast, our new model
exhibits both input compensation and entrainability.

5.2. Theory
Here we give a description of the three models of the post-translational Kai circadian clock studied here, and how we included the sensitivity of the phosphorylation rates to the ATP fractions in the bulk. Furthermore, we explain which mechanisms for period stability are present in each model, to compensate for the dependence of the phosphorylation rates on the ATP fraction. Two of the three mechanisms that we describe below have been partly presented elsewhere [26], yet we will discuss here how they are implemented specifically in the respective models. For completeness, we give here also a qualitative description of the third, novel, mechanism, which is employed only by the new model; this mechanism is discussed in much more detail in the Results section.

5.2.1. Van Zon Model
The Van Zon model describes the phosphorylation cycle at the level of KaiC hexamers, and does not explicitly keep track of the KaiC monomers. A simplified scheme of the model is shown in Fig. 5.1A: A hexamer can be in the active conformational state, denoted by $C_i$, or in the inactive state, denoted by $\tilde{C}_i$, where $i$ denotes its phosphorylation level. In the presence of free KaiA, active KaiC (enclosed by the green box in Fig. 5.1A) is phosphorylated with a rate which depends on the KaiA concentration. When the hexamer has reached the fully phosphorylated state, $C_6$, it flips to the inactive conformation, $C_6 \to \tilde{C}_6$, where it immediately binds KaiB. The delay between full phosphorylation and KaiA sequestration, essential for synchronized oscillations, is set by the two dephosphorylation steps, $\tilde{C}_6 \to \tilde{C}_5 \to \tilde{C}_4$ (blue box). The complexes $\tilde{C}_4 - \tilde{C}_1$, have a very high affinity for KaiA, allowing them to sequester all free KaiA from the solution (red box). This sequestration of KaiA forces the front runners that have reached the bottom of the cycle ($C_0$) and are ready to be phosphorylated again, to wait, because KaiA is needed for phosphorylation. Sequestration of KaiA thus allows the laggards (the hexamers that are falling behind in the phosphorylation cycle) to catch up with the front runners, leading to the synchronization of the oscillations of the individual hexamers. Only when most KaiC has reached $C_0$, is KaiA released in solution, and can a new phosphorylation cycle start again.

We use a coarse grained description to model the effect of the ATP fraction, $\alpha_{ATP} = [ATP]/([ATP]+[ADP])$, where [ATP] and [ADP] are the ATP and ADP concentrations in the bulk, respectively, on the phosphorylation rates. Just like in the Rust model [7], we assume that, when KaiA is bound, the probability of having ATP instead of ADP bound, is

$$\beta_{ATP} = \frac{\alpha_{ATP}}{\alpha_{ATP} + K_{ATP/ADP} (1 - \alpha_{ATP})},$$

where $K_{ATP/ADP}$ is the relative dissociation constant for binding ATP over ADP. The effective phosphorylation rates become $k_{phos} = \beta_{ATP} k^0_{phos}$, where $k^0_{phos}$ is the phosphorylation rate at 100% ATP. Dephosphorylation rates are independent of $\alpha_{ATP}$.

The Van Zon model employs one mechanism for period stability, which is partly identified in [26]. The mechanism is a direct consequence of the temporal delay be-
5.2. Theory

between the moment a hexamer reaches the top of the cycle, i.e. the state $\hat{C}_6$ in which it no longer needs KaiA to progress along the cycle, and the time at which it reaches $\hat{C}_4$ and starts sequestering KaiA. Thus, in particular, there is a lag between the moment when enough KaiC hexamers to fully sequester KaiA have passed $\hat{C}_6$, and so are committed to the path towards sequestration, and the moment when full sequestration is actually reached. The number of additional hexamers that reach $\hat{C}_6$ during this delay is given by the duration of the delay multiplied by the phosphorylation speed. Importantly, while the duration of the delay is independent of the ATP fraction (since dephosphorylation from $\hat{C}_6$ till $\hat{C}_4$ is independent of the ATP fraction), the rate of phosphorylation decreases as the ATP fraction decreases. Consequently, the lower the ATP fraction, the smaller the number of hexamers that can reach the state $\hat{C}_6$ during the delay. The smaller number of $\hat{C}_6$, in turn, leads to a shorter time interval in which all KaiA stays sequestered. This shortens the dephosphorylation phase, which counteracts the longer phosphorylation phase, stabilizing the period.

Fig. 5.1B shows time traces for the KaiC phosphorylation level $p(t) = \sum_{i=1}^{6} i(C_i + \bar{C}_i)/(6\text{KaiC}_{\text{tot}})$ (dotted lines), and the fraction of inactive hexamers (solid lines), at $\alpha_{\text{ATP}} = 100\%$ and 50\%. The number of hexamers that can sequester KaiA increases with the amplitude of the inactive fraction, such that this amplitude sets the duration of the dephosphorylation phase. Indeed, as a clear signature of the stability mechanism, at 50\% ATP fraction, both the phosphorylation level and the fraction of inactive KaiC rise slower while having a lower amplitude compared to the oscillations at 100\% ATP fraction.

5.2.2. Rust model

Contrary to our new model and the Van Zon model, the Rust model describes the oscillations at the level of single monomers [7, 22, 26]. As shown in Fig. 5.1C, each monomer goes through the ordered phosphorylation cycle $U \rightarrow T \rightarrow D \rightarrow S \rightarrow U$. Only when monomers have reached the phosphorylation states $S$ and $D$, they can bind KaiB with a low rate, and form D-B and S-B, respectively. Importantly, only the S-B state sequesters KaiA, while KaiA impedes the occupation of the S-B state by enhancing the transition from S-B back to D-B. This mutual inhibition between KaiA and the S-B state creates a positive feedback loop for KaiA sequestration that is essential for oscillations. Initially, when D-B transforms into S-B, KaiA stimulates the reverse reaction. During this period of a quasi-equilibrium between the D-B and S-B states, the concentration of their sum rises, $[D\cdot B] + [S\cdot B]$, up to the point that $[S\cdot B]$ reaches a level where it sequesters all KaiA. At this moment, the positive feedback is broken, and the system rapidly switches to the dephosphorylation phase in which KaiA is fully sequestered for a long time. The positive feedback thus creates a sharp transition between the phase in which KaiA is free to simulate phosphorylation, and the phase in which all KaiA is sequestered.

To include the effect of the bulk ATP fraction, we use the same coarse grained description on the phosphorylation rates as in the original work: $k_{\text{phos}} = \beta_{\text{ATP}} k_{\text{phos}}^0$, where $\beta_{\text{ATP}}$ is defined in Eq. 5.1. As was shown in [26], the ATPase activity in the CI domain, which sets the rate of KaiB binding in this model, does not depend on the bulk ATP fraction.

The Rust model implements two methods for period stability, where the first is similar to that identified in the Van Zon model and described by Phong et al. [26]. The slow
KaiB binding step creates a temporal delay between, on the one hand, the phosphorylation states D and S, which can only be reached in the presence of free KaiA, and, on the other hand, the state S-B, which sequesters KaiA. This delay allows monomers to reach the D and S state through phosphorylation before all KaiA is sequestered. The number of monomers that can reach the KaiB bound states increases with the phosphorylation speed, set by $\alpha_{\text{ATP}}$, and will determine the duration of the period in which all KaiA is sequestered.

The previous mechanism assumes that the delay between reaching the D or S phosphorylated states and sequestering KaiA is constant, but the input compensation can be enhanced further if this delay gets shorter when ADP is added. More precisely, in order to sequester all KaiA, the concentration of S-B monomers has to fulfill $n_{\text{seq}}^{S}[S \cdot B]_{\min} \geq [\text{KaiA}_{\text{tot}}]$, where $n_{\text{seq}}^{S}$ is the number of KaiA monomers sequestered by a single S-B KaiC monomer and $[S \cdot B]_{\min}$ is the minimal concentration of S-B monomers to sequester all KaiA, $\text{KaiA}_{\text{tot}}$. Because the transitions between the states S-B and D-B are faster compared to the transitions from these states to the U and T, the S-B $\rightarrow$ D-B transitions are in quasi equilibrium. This means that $[D \cdot B]$ is related to $[S \cdot B]$ via the steady state relation, $[D \cdot B]/[S \cdot B] \approx k_{\text{SD}}(\alpha_{\text{ATP}})/k_{\text{DS}} = f(\alpha_{\text{ATP}})$, which depends on the effective (de)phosphorylation rates, $k_{\text{SD}}(\alpha_{\text{ATP}})$ and $k_{\text{DS}}$, respectively, and, importantly, on the bulk ATP fraction. Consequently, the amount of D-B required to have enough S-B to sequester all KaiA, is $D \cdot B \approx S \cdot B f(\alpha_{\text{ATP}})$, which is related to $\alpha_{\text{ATP}}$. Thus, at a lower bulk ATP fraction, the concentration of KaiC bound to KaiB necessary to sequester all KaiA, $[D \cdot B] + [S \cdot B]$, will be lower, which compensates for the slower formation of these complexes during the phosphorylation phase. Indeed, as shown in Fig. 5.1D, the concentration of KaiC-bound KaiB increases much slower at a 50% ATP fraction compared to 100%, but the concentration of KaiC-bound KaiB at the moment that all KaiA is sequestered, is also lower. Because fewer D phosphorylated monomers are required to sequester all KaiA, the subsequent sequestration time is smaller, shortening the period even more.

5.2.3. NEW MODEL

Our new model, explained in more detail in chapter 4, is again a hexamer model. This model, shown in Fig. 5.1E, explicitly describes the state of individual monomers, and in particular their serine and threonine phosphorylation sites. Each monomer in a hexamer is phosphorylated in a well defined order: First the threonine site is phosphorylated and then the serine site. Phosphorylation of the two sites has an antagonistic effect on the conformational state of the hexamer: The U and T states stabilize the active conformation and the D and S states stabilize the inactive conformation. Due to this antagonism, the relative stability of the conformations do not depend on the absolute number of monomers in a certain state, but rather on the difference between the number of phosphorylated threonine and serine sites [18]. Roughly, when more serine sites are phosphorylated than threonine sites, the hexamer will switch conformation. After flipping to the inactive state, the hexamer binds KaiB, but it can only sequester KaiA after 6 KaiB monomers are bound. This delay allows hexamers lagging behind to continue phosphorylation and reach the inactive state, which is an essential property of our model to generate robust oscillations.
The new model explicitly simulates the binding and unbinding of nucleotides, and the hydrolysis of ATP in the CII domain, where the ATP is used to phosphorylate the threonine and serine sites. The ATP fraction in the CII binding pocket is dependent on the bulk ATP fraction, because the binding of nucleotides is directly proportional to $\alpha_{\text{ATP}}$. Furthermore, the ATP fraction in the binding pocket depends on the hydrolysis rate of ATP, and the relative affinity for ATP and ADP. Importantly, both the effective phosphorylation and dephosphorylation rates depend on the ATP fraction of the binding pockets, because both events occur via phosphotransfer with the nucleotide. This means that a change in $\alpha_{\text{ATP}}$ has a much bigger effect on the (de)phosphorylation dynamics compared to models where dephosphorylation proceeds through a $\alpha_{\text{ATP}}$ independent phosphatase reaction.

Motivated in chapter 4, we choose the relative affinity of nucleotides for the binding pockets of the CII domain, $K_{\text{CII}}^{\text{ATP/ADP}} = 0.1$, much lower than what is used in the Rust model [7]. We have, however, also investigated the behavior of the other two models with a relative affinity that is similar to our model. In the results section below, we will compare the other models with the high and low relative affinity, with our new model.

The oscillator employs two mechanisms of period stability. First, due to the slow KaiB binding, there is a delay between flipping to the inactive conformation after phosphorylation, and KaiA sequestration. This creates a pool of hexamers in the inactive conformation that increases with the speed of phosphorylation, set by the ATP fraction. Again, at lower $\alpha_{\text{ATP}}$, fewer hexamers make it to the inactive state, such that the dephosphorylation phase is shorter, which counteracts the longer phosphorylation phase.

The second mechanism for period stability is related to the path individual hexamers traverse through phosphorylation state space before switching to the inactive conformation. A hexamer switches to the inactive state when the number of phosphorylated serine sites, $n_S$, exceeds the number of phosphorylated threonine sites, $n_T$. Due to the ordered phosphorylation of each monomer, the threonine sites are phosphorylated before the serine sites, such that a hexamer makes a wide arch in phosphorylation state space, $(n_T, n_S)$, before the diagonal, and the flipping criterion $n_S > n_T$, is reached. As the effective phosphorylation rates decrease for lower $\alpha_{\text{ATP}}$, the size of the arch decreases, as discussed in more detail below. This shorter path in state space counteracts the effect of a slower progression along the path (due to the slower phosphorylation), and creates another mechanism for input compensation, at the level of individual hexamers.

Note that the positive feedback loop on KaiA sequestration in the Rust model, due to the mutual repression between KaiA and the S-B state of KaiC, is not present in our model. In this feedback loop in the Rust model, KaiA stimulates the transition from the S to the D state, thereby preventing its own sequestration (because only the S-B state significantly sequesters KaiA). However, because in our model both the S and the D state stabilize the ADP bound state in the binding pocket of the CI domain, which stabilizes the inactive conformation and the subsequent KaiB binding (leading to KaiA sequestration), KaiA does not prevent its own sequestration by stimulating the S to D transition. Therefore, the mechanism of input compensation in the Rust model resulting from the positive feedback loop, does not apply to our model.
Figure 5.1: The different models of the Kai system employ different mechanisms of period stabilization. (A,B): Van Zon model; (C,D): Rust model (from [26]); (E,F): new model. Phosphorylation level (dotted lines, B, D, F) and fraction of KaiC in inactive state (solid lines, B, F) or in S.B + D.B (D, solid lines) at \( \alpha_{\text{ATP}} = 100\% \) (blue) and 50\% (orange). Shaded regions indicate the phase where all KaiA is sequestered, at \( \alpha_{\text{ATP}} = 100\% \) (gray) and 50\% (dark gray).

(continued next page)
**5.3. Results**

5.3.1. Dependence of oscillations on the ATP fraction reveals input compensation on the ensemble level

To find out how effective the mechanisms for period stability are in the three models, we run simulations of the models at constant bulk ATP fractions from 100% to 50%. For the Van Zon and Rust model, we consider two values of the relative dissociation constant for ATP versus ADP: 1) With equal affinity for ATP and ADP, $K_{\text{ATP/ADP}} = 1.0$ (solid lines in Fig. 5.1), as used and motivated in the original model by Rust [7], and 2) With a lower affinity for ADP, $K_{\text{ATP/ADP}} = 0.19$ (dashed lines), such that the effect on the ATP fraction in nucleotide binding pocket, $\beta_{\text{ATP}}$ (Eq. 5.1), as $\alpha_{\text{ATP}}$ decreases from 100% to 50%, is similar to the drop in our new model of about 15%.

Fig. 5.1A shows how the period varies with decreasing $\alpha_{\text{ATP}}$. Remarkably, the three models have a different response to lowering $\alpha_{\text{ATP}}$: Whereas in our new model the period is almost constant, in the Van Zon model it increases by 20%, while in the Rust model it decreases by 20%, as $\alpha_{\text{ATP}}$ decreases from 100% to 50%. This is reflected in the change in the amplitude of the phosphorylation levels, panel B, which decreases the strongest in the Rust model and the least in the Van Zon model, with decreasing $\alpha_{\text{ATP}}$. The results in panels A and B are consistent: given the period stabilization mechanism due to the delay, we expect that in all models fewer hexamers or monomers make it through the cycle as $\alpha_{\text{ATP}}$ decreases, such that the amplitude of the oscillation decreases, shortening the length of the dephosphorylation phase. This view is further supported in panels C and D:
Panel C shows the length of the phosphorylation phase, $\Delta t_{\text{phosphorylation}}$, defined as the time between a trough and the next peak in phosphorylation level. As expected, this time increases in all models as $\alpha_{\text{ATP}}$ decreases: due to the lowering of the phosphorylation rates, it takes more time to reach the required phosphorylation state to sequester enough KaiA. The length of the subsequent dephosphorylation phase, $\Delta t_{\text{dephosphorylation}}$, defined as the time between a peak and the next trough in the phosphorylation level, decreases in the new model and the Rust model, as a result of the stability mechanism. However, in the Van Zon model, the stability mechanism does not work as $\Delta t_{\text{dephosphorylation}}$ increases with decreasing $\alpha_{\text{ATP}}$. Because KaiC is unable to sequester all KaiA in the system during the dephosphorylation phase, the phosphorylation of active hexamers continues during this phase, which decreases the net dephosphorylation rate and extends its duration.

We point out that as the phosphorylation rates decrease, the number of hexamers and monomers that traverse a full cycle each period, and sequester KaiA, decreases. To show the effect of $\alpha_{\text{ATP}}$ on this fraction, we plot the total flux of monomers or hexamers that move through the cycle per period, described in more detail in section 5.5, for each model in Fig. 5.1E. Panel E shows a decrease in flux of around 15% for the Van Zon and our new model at $\alpha_{\text{ATP}} = 50\%$. The Rust model has a much larger decrease in flux of around 60%, which confirms the idea that input compensation is achieved in this model by letting fewer monomers participate in the cycle and sequester KaiA per period. Panel F gives the time interval per period when all KaiA is sequestered by KaiC, $\Delta t_{\text{KaiAsequestered}}$, as defined in section 5.5, which shows that in all models the time of full sequestration indeed shortens. Here, remarkably, the decreases upon lowering $\alpha_{\text{ATP}}$ is the strongest for the Van Zon model, even though the decrease in the fraction of hexamers that go through a cycle and sequester KaiA (panel E) is much less. This is probably related to the fact that in the Van Zon model a hexamer can only sequester 2 KaiA dimers, while in our model 6 dimers per hexamer and in the Rust model 2.5 dimers per monomer are sequestered. Therefore, the number of inactive hexamers required to sequester all KaiA, is much higher in the Van Zon model, compared to the other two models. Hence, a small change in the amplitude in the concentration of inactive hexamers, has a big effect on $\Delta t_{\text{KaiAsequestered}}$. Note that below $\alpha_{\text{ATP}}$ of 65%, KaiA is never fully sequestered in the Van Zon model, while oscillations persist.

Comparing results between different relative affinities, $K_{\text{ATP/ADP}}$, it is clear that both the Van Zon and the Rust model are much less affected by the bulk ATP fraction, when $K'_{\text{ATP/ADP}}$ is lower. For lower $K'_{\text{ATP/ADP}}$, the probability that KaiC is bound to ATP instead of ADP, is much less affected by changes in $\alpha_{\text{ATP}}$. This makes the phosphorylation rates, and hence the oscillations, less sensitive to $\alpha_{\text{ATP}}$. However, a lower sensitivity might hamper the entrainability of the clocks. Therefore, in the next section, we study how each model of the Kai oscillator responds to a transient lowering of the ATP fraction.

### 5.3.2. Van Zon and Rust Models Only Show Strong Entrainability at Equal Nucleotide Affinities

We expose the oscillator, running at a 100% ATP fraction, to a transient, six hour pulse of a 40% ATP fraction, starting at different times from the last trough in the phosphorylation fraction. To calculate the phase shift induced, we compare the time trace of the
5.3. RESULTS

**Figure 5.1:** Dependence of the new (blue solid lines), Van Zon (red) and the Rust (orange) models on the bulk ATP fraction, $\alpha_{ATP}$. All curves are normalized by their values at 100% ATP fraction, indicated by the horizontal dashed line. For the Van Zon and the Rust models, we study versions with equal sensitivity for ATP and ADP, $K_{ATP/ADP} = 1.0$ (solid lines), and a smaller sensitivity for ADP, $K_{ATP/ADP} = 0.19$ (dashed lines). (A) The ATP fraction has an opposite effect on the period in the Van Zon and our new model compared to the Rust model, (B) but the amplitude increases with increasing $\alpha_{ATP}$ in all models. (C) The time between a through and the next peak in the phosphorylation level, $\Delta t_{\text{phosphorylation}}$, decreases with $\alpha_{ATP}$ in all models, because the phosphorylation rates increase. (D) In both our new model and the Rust model, the time between a peak and the next trough, $\Delta t_{\text{dephosphorylation}}$, increases with $\alpha_{ATP}$, which compensates for the faster phosphorylation rates. In the Van Zon model however, the dephosphorylation time interval decreases with $\alpha_{ATP}$ such that the compensation for a stable period does not occur. (E) As the ATP fraction increases, the number of hexamers or monomers that go through a full cycle per period increases, such that the amount of KaiA that can be sequestered increases. (F) Therefore, the time interval per period that all KaiA in the system is sequestered by KaiC, becomes longer with increasing $\alpha_{ATP}$.

phosphorylation fraction two troughs after the onset of the pulse, with a control where no pulse is given.

Fig. 5.2, panels A, B and C, show phase response curves for our new model, the Van Zon model and the Rust model, respectively, showing the induced phase advances.
Comparing our new model, with $K_{\text{ATP/ADP}} = 0.1$, with the other two models with equal nucleotide affinities, $K_{\text{ATP/ADP}} = 1.0$ (solid lines), we see that the phase response curves are comparable: Both the maximally induced phase advance and delays are around 4 hours in all models, although the Rust model is capable of a particularly large phase advance of almost 10 hours. All models have a dead zone, where the pulse does not induce a phase change, starting about 12 hours from the trough, which is thought to be essential for entrainment [6]. However, when we reduce in the Van Zon and Rust models the relative affinity to $K_{\text{ATP/ADP}} = 0.19$ (dashed lines), the amplitude of the phase response curves become much smaller: In the Van Zon model the maximal phase advance and delay are reduced to around one hour. In the Rust model, the maximal phase delay is also reduced to about one hour, which is significantly smaller than that in the new model; the maximal phase advance is also strongly reduced, although it is reduced to a value that is only slightly lower than that in the new model. Comparing Fig. 5.2A-C with Fig. 5.1A shows that the new model gives the best trade-off between entrainability and input compensation: On the footing of equal phase response curves, Fig. 5.2A-C, the new model has the smallest change in the period upon changing $\alpha_{\text{ATP}}$, Fig. 5.1A.

We want to know how the phase advance and delay is achieved in each model. To this end, in panels D-I, we look at time traces of the phosphorylation level (solid black lines) and the fraction of sequestered KaiA (solid purple lines), when a pulse of ADP is given (shaded regions), where we set $K_{\text{ATP/ADP}} = 1.0$ for the Van Zon and Rust models. For comparison, the dashed lines show the control where no pulse is given. We choose the starting times of the pulses such that a maximum phase advance (panels D-F) or phase delay (G-I) is induced, as indicated by the arrows in the phase response curves, panels A-C.

When the pulse is given at the moment that leads to a maximum phase delay, then, in all three models, the ADP pulse only slows down the increase of the phosphorylation level. When the ATP level returns to 100%, the phosphorylation level reaches the same peak height as compared to a situation where no pulse is given. Therefore, the subsequent dephosphorylation phase, when all KaiA is sequestered, has the same length as when no pulse is given. The ADP pulse thus predominantly slows down phosphorylation.

When the pulse is given at the moment that leads to a maximum phase advance, then, in all three models, the ADP pulse causes the immediate start of the dephosphorylation phase. Therefore, the peak in the phosphorylation level is lower compared to the unperturbed case, and the number of hexamers or monomers that is able to sequester KaiA is also smaller. The subsequent dephosphorylation phase shortens, causing the phase advance. Only in the Rust model, the ADP pulse also initiates the immediate sequestration of KaiA, because, due to the sudden drop in phosphorylation rate from the S to the D state, KaiA is incapable anymore to prevent its own sequestration.

5.3.3. AS THE ATP FRACTION DECREASES, INDIVIDUAL HEXAMERS GO THROUGH A SMALLER CYCLE IN PHOSPHORYLATION STATE SPACE

Up to now, the effect of ATP on the phosphorylation level has only been studied at the level of the mean phosphorylation level, both experimentally and theoretically. We want to know, for our new model, the effect of the ATP fraction on the phosphorylation dy-
Figure 5.2: Sensitivity of the phase of the phosphorylation level, in our new model (left column), Van Zon model (middle column) and the Rust model (right column), to a 6 hour pulse of lowering of the ATP fraction to 40%. Panels A, B and C show the phase advance in hours, due to the pulse, starting at the time indicated on the x-axis, measured from the minimum in the phosphorylation level. The shaded region in panel A shows the standard deviation of 10 independent runs of the new stochastic model.

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Figure 5.2: (continuing from previous page) For the Van Zon and Rust models, we show phase response curves for a scenario with an equal relative affinity for ATP and ADP, $K_{ATP/ADP} = 1.0$ (solid line), and a version with a lower affinity for ADP, $K_{ATP/ADP} = 0.19$ (dashed line). Arrows indicate extrema in the phase response curves, for which we show the corresponding time traces in the panels with the adjacent label. Panels D,E and F show the effect of a pulse (shaded region) when the phase delay is the largest, on the phosphorylation level (solid black line) and the fraction sequestered KaiA (solid purple line). Dashed lines show the development in the case no pulse is given. Panels G,H and I show the effect of the pulse when the phase advance is the largest. Results for the new model were obtained using kinetic Monte Carlo, and ODE’s for the Van Zon and Rust models.

5.3. Microscopic cycles illuminate input compensation in individual hexamers

Given our observation that individual hexamers make smaller cycles in phosphorylation state space when $\alpha_{ATP}$ decreases, we wanted to know how this affects the timing for switching between the active and inactive conformation. This timing is an important factor in the period of the oscillation, because it determines when and how long KaiA is sequestered. To this end, we measure the time between two important events during a full cycle of a hexamer, illustrated in Fig. 5.4A: 1) The start of the phosphorylation cycle, when a hexamer is in the active state and, for the first time, either a threonine site or serine site is phosphorylated; 2) and half-way of the cycle, when the hexamer is in the inactive state and is bound to six KaiB monomers. For all hexamers, we track the first passage time from the start to reaching half-way, $\Delta t_{active}$, and from half-way to the start of a new cycle, $\Delta t_{inactive}$. The time of a full cycle is defined as $\Delta t_{cycle} = \Delta t_{active} + \Delta t_{inactive}$.

Note that the time in the active phase, $\Delta t_{active}$, not only includes the phosphorylation and the switching to the inactive state, but also includes the binding of KaiB monomers. We include the binding of KaiB in this state definition, because KaiC recrosses the dividing surface that separates the active from the inactive state many times, before it is
Figure 5.3: In the new model, at a lower bulk ATP fraction, individual hexamers make a smaller cycle through phosphorylation state space. We track the number of phosphorylated threonine sites, $n_T$, and serine sites, $n_S$, in each individual hexamer for one simulated hour at different phases of the oscillation. In panels A and G, the shaded regions indicate at which phase of the oscillation hexamers were probed and in which panel the result is shown.

(continued next page)
Figure 5.3: (continuing from previous page) Panels B-F ($\alpha_{\text{ATP}}=100\%$, left column) and H-L ($\alpha_{\text{ATP}}=50\%$, right column) show histograms of the probability of finding a hexamer in a certain phosphorylation state, indicated by the color bar to the right of the right column. Arrows are proportional to the flux through the state the arrow originates from. Comparing states near the peak of the phosphorylation level, panels D and J, hexamers at $\alpha_{\text{ATP}}=50\%$ go through a smaller cycle compared to the situation at 100% ATP. Specifically, the majority of hexamers go through $n_T=4$ or 5 at $\alpha_{\text{ATP}}=100\%$, while at 50% hexamers only reach $n_T=2$ or 3. Furthermore, the ensemble is less synchronized near the trough of the phosphorylation levels, comparing panels F and L. Results shown are averaged over 400 consecutive oscillation cycles.

Finally, hexamers are committed to the inactive state. Including the binding of KaiB into our criterion does not affect the results, because the rate of KaiB binding is independent of $\alpha_{\text{ATP}}$, and any change in $\Delta t_{\text{active}}$ due to a different $\alpha_{\text{ATP}}$ is therefore related to changes in phosphorylation rates.

In Fig. 5.4B we show histograms of individual cycle times, $\Delta t_{\text{cycle}}$, at 100% ATP (solid blue line) and 50% (dashed orange line). The distribution has maxima at multiples of the period of the oscillation, indicated by dashed vertical lines. Clearly, the cycle times of individual hexamers coincide with the period of the oscillation. Peaks at multiples of $\Delta t_{\text{cycle}}$ correspond to hexamers that do not bind 6 KaiB monomers during the first period, and hence have to wait for another round, or more, to make the full cycle. Since the histogram for 50% ATP has a fatter tail, hexamers are more likely to wait multiple periods before completing the cycle, showing that indeed fewer hexamers participate in an oscillation at lower ATP fractions. Fig. 5.4C shows the distributions of $\Delta t_{\text{active}}$, at 100% and 50% ATP. The distribution again has multiple peaks, mirroring those in the distribution of cycle times (panel B). This indicates that at lower $\alpha_{\text{ATP}}$, synchronization becomes impaired because fewer hexamers make it to the top of the cycle, where they have 6 KaiB bound and are committed to the inactive state.

The inset of Fig. 5.4C zooms in on the first peak of the distribution, emphasizing that even though the phosphorylation rates are different, the modes of the first passage time distribution are remarkably similar; the difference is only 1-2 hours. This paradox can be resolved by noting that the switch from the active to inactive state is determined by the difference between the number of phosphorylated serine and threonine sites, $n_S - n_T$, respectively. In our model, the phosphotransfer rates for the threonine site are much faster than for the serine site (see Table 4.1 and [22]). Therefore, compared to the slow phosphorylation of the serine sites, $n_T$ will quickly reach its steady-state level during the phosphorylation phase. The steady-state level of $n_T$ will thus set the number of serine sites that need to be phosphorylated before the hexamer can switch to the inactive state. The steady-state level of $n_T$ decreases as the ATP fraction of the buffer is reduced, because the rate of phosphorylation decreases and the rate of dephosphorylation increases (in the presence of KaiA) with lower $\alpha_{\text{ATP}}$, respectively. Consequently, as $\alpha_{\text{ATP}}$ is decreased, less serine sites need to be phosphorylated for the hexamer to switch conformation, which compensates for the lower rate of phosphorylation. This reasoning implies that the levels $n_S$ and $n_T$, at which a hexamer switches to the inactive state, also decreases. This can be seen in panel E and F, which show the distribution of phosphory-
lation states \((n_S, n_T)\), at the moment a hexamer flips from active to inactive, at \(\alpha_{\text{ATP}}=100\%\) and 50\%, respectively. Clearly, \(n_S\) and \(n_T\) tend to be lower at the moments of switching, when \(\alpha_{\text{ATP}}=50\%.\) This also suggests that at lower \(\alpha_{\text{ATP}}\), fewer monomers are double phosphorylated. Fig. 5.4G shows histograms of the phosphorylation states of the hexamers at the moment when all KaiA is sequestered in the system for the first time during a period. At \(\alpha_{\text{ATP}} = 50\%,\) 40\% of the hexamers have one or more monomers in the D state, while at \(\alpha_{\text{ATP}}=50\%,\) this fraction is reduced to 10\%.

Lastly, the distribution of inactive times, \(P(\Delta t_{\text{inactive}})\) in Fig. 5.4D, can be explained. The distribution exhibits a shoulder at \(\alpha_{\text{ATP}}=100\%,\) which is due to the fact that: 1) The number of hexamers that are in the inactive state is higher, reflected by the higher first peak in Fig. 5.4B, resulting in a longer time where all KaiA is sequestered. Therefore, the time hexamers have to wait before another round of phosphorylation starts increases, which is included in \(\Delta t_{\text{inactive}}\); 2) Hexamers start their inactive phase at a higher phosphorylation level, comparing panels E and F in Fig. 5.4, which results in a longer dephosphorylation phase.
Figure 5.4: Microscopic dynamics of the new model reveals input compensation at the level of individual hexamers. (A) For each hexamer we measure, 1) $\Delta t_{\text{active}}$: The time between the first phosphorylation event, and having six KaiB bound to the hexamer for the first time. 2) $\Delta t_{\text{inactive}}$: The time between having 6 KaiB bound and, after dephosphorylation and switching back to the active state, the first phosphorylation event. The time for completing a full cycle is: $\Delta t_{\text{cycle}} = \Delta t_{\text{active}} + \Delta t_{\text{inactive}}$.
Figure 5.4: (continuing from previous page) B, C and D show histograms of these time intervals, comparing situations with 100% ATP (blue solid lines) and 50% ATP in the bulk (orange dashed lines). (B) Distribution of times for completing a cycle. Peaks are at multiples of the oscillator’s period of 24.3 hrs, as indicated by the vertical dotted lines (period at 100% ATP). Peaks at $\Delta t_{\text{cycle}} > 24.3$hrs show hexamers that could not complete a full cycle during one period of the oscillation. (C) Distribution of times for phosphorylation and KaiB binding. Event though phosphorylation rates are lower at $\alpha_{\text{ATP}}=50\%$ as compared to 100%, the modes of their distributions are remarkably similar, as emphasized by the inset which zooms in on the first peak. (D) Distribution of times for dephosphorylation and waiting for a new round of phosphorylation. The bigger shoulder at $\alpha_{\text{ATP}}=100\%$ is a manifestation of the longer time during which all KaiA is sequestered at this ATP level. At the end of their cycle, hexamers have to wait longer before KaiA returns to solution and phosphorylation starts again. (E,F) Histograms of the number of phosphorylated threonine sites, $n_T$, and serine sites, $n_S$, in individual hexamers, at the moment when they switch to the inactive state, at $\alpha_{\text{ATP}}=100\%$ (E) and 50% (F). At higher $\alpha_{\text{ATP}}$, hexamers switch to the inactive state at higher phosphorylation levels. (G) Histograms of the number (different colors, defined on the right side) of U,T,D and S phosphorylated monomers inside a hexamer, at the moment when all KaiA is sequestered. At $\alpha_{\text{ATP}}=100\%$ more monomers are doubly phosphorylated.
5.4. DISCUSSION

All circadian clocks have to fulfill two seemingly conflicting requirements in order to be a good predictor of time: A robust circadian period under a wide range of external conditions, and entrainability such that it always moves in phase with the day-night cycle. As shown in recent experiments, the daily change in ATP fraction in the cyanobacterium *Synechococcus elongatus*, is an important cue for entrainment of its circadian Kai oscillator. We compared two canonical models of the post-translational oscillator, the hexamer model by Van Zon *et. al.* and the monomer model by Rust *et. al.*, with our new model, and studied how well they fulfill the robustness and entrainability criteria.

We find, in agreement with experiments [26], that the period in our new model is almost unaffected by the bulk ATP fraction, and that its hard to determine from the mean quantities related to the phosphorylation level what sets this period stability. Apart from the amplitude in the phosphorylation level, other quantities such as the length of the phosphorylation and dephosphorylation phase, do not change much with the bulk ATP fraction in our new model. The other two models show clear signatures of input compensation. The Van Zon model has too little input compensation, however, as shortening the dephosphorylation phase by sequestering less KaiA is unable to compensate slower phosphorylation. On the other hand, the Rust model has too much input compensation, since the period decreases with lower ATP. Lowering the phosphorylation rates has a too strong effect on the positive feedback loop regulating KaiA sequestration, shortening the period too much. The fact that our new model is so stable, is perhaps not so surprising, as the sensitivity to the ADP level was a factor 10 lower in our model compared to the other two.

We then checked the entrainability of the respective models, and found that the entrainability of our model is comparable to that of the other models, even though the relative affinity of ADP versus ATP is lower in our model. When we make the relative ADP/ATP sensitivity in the Van Zon and Rust models similar to that of our new model, the amplitudes of the phase response curves in these other models become very small, leading to poor entrainability. This showed that our new model, contrary to previous models, is capable of maintaining a robust circadian period, while at the same time being strongly entrainable.

To elucidate how our model achieves this combination of period robustness and good entrainability, we studied the phosphorylation cycle of the threonine and serine sites in individual hexamers, at 100% and 50% bulk ATP fractions. This showed that at 50% ATP, when effective phosphorylation rates are lower, individual hexamers go through a smaller phosphorylation cycle. Our analysis also revealed that the distribution of times for hexamers to complete a full cycle, peaks at multiples of the period, and that peaks at times higher than the period become more pronounced at lower ATP fraction, as fewer hexamers make it trough the full cycle each period. Remarkably, the time required to complete the first part of the cycle, between when phosphorylation starts and when 6 KaiB monomers are bound, seems to be little affected by the bulk ATP fraction. This leads to the question of how, at different effective phosphorylation rates, the timing of the conformational switch can be almost unaffected.

To address this question, we made histograms of the phosphorylation states of the hexamers when they switch to the inactive state. At lower phosphorylation rates, hex-
amers switch to the inactive state at a lower phosphorylation level, compensating the longer time they need to phosphorylate the sites. This is in marked contrast with previous models by Van Zon et al. and Rust et al., where the time required for individual units (be it hexamers or monomers) to complete a full cycle, can only increase with decreasing ATP fractions. In these models, period stability can only be achieved through the interaction between the units via KaiA sequestration [149], that is, via the delay between the moment where KaiC no longer needs KaiA to progress along the cycle and the point where KaiC sequesters KaiA: At lower $\alpha_{ATP}$, less KaiC makes it to the top of the cycle during the delay, making the dephosphorylation phase shorter, which can then counteract the longer phosphorylation phase [26].

The new model presented here exploits this mechanism too, yet also employs another one, which acts at the level of the individual hexamers. This mechanism utilizes the ordered phosphorylation of the threonine and serine site in the monomers, in combination with their antagonistic effect on the conformation of the hexamer. Previously, Lin et al. argued that this antagonism creates an ultra-sensitive switch which provides robustness against a varying KaiA concentration [18]. However, his antagonism also plays a key role in our mechanism of input compensation. The antagonism entails that the state at which the hexamer switches conformation does not depend on the absolute number of phosphorylated threonine and serine residues, but rather only on their relative amounts. When the ATP fraction is low, their are typically fewer serine and threonine sites phosphorylated (see Fig. 4.1 and [26]), but since the switch to the inactive conformation does not depend on the absolute number of phosphorylated sites, but on their relative amounts, hexamers switch conformation at a lower number of phosphorylated sites. The net result is that, although the cycle in state space is smaller at lower ATP fraction, the moment a hexamer switches is robust against changes in the ATP fraction. And, since the phosphorylation rates are sensitive to changes in the fraction, the hexamer still undergoes a phase shift, essential for entrainability. We thus propose a new function for the ordered phosphorylation cycle of the KaiC monomers: It allows the oscillator to combine period robustness with high entrainability.

5.5. METHODS

We use the Rust model described the SI of [26] and the Van Zon model in the SI of [15], both described with ordinary differential equations (ODE’s), propagated using the ND-Solve function of Mathematica 8 (Wolfram Research). Our new model, introduced in chapter 4, is propagated using the dedicated Monte Carlo algorithm described in the same chapter.

We want to compare the Van Zon and Rust models, using a relative affinity for ATP versus ADP, such that the change of the ATP fraction in the nucleotide binding pockets given that KaiA is bound, due to changes in $\alpha_{ATP}$, is similar to the change in our new model. In our new model, the steady state fraction of ATP in the CII binding pocket, $\beta_{ATP}^{CII}$, given that KaiA is always bound to CII, is given by (section 4.3.2)

$$\beta_{ATP}^{CII} = \frac{\alpha_{ATP} k_{CII-ADP}^{CII} k_{off,KaiA}^{CII}}{\alpha_{ATP} k_{off,KaiA}^{CII} + k_{hyd}^{CII} + (1 - \alpha_{ATP}) k_{off,KaiA}^{CII-ADP} k_{ATP/ADP}^{CII}}.$$ (5.2)
Here, $k_{\text{off,KaiA}}^{\text{CII-ADP}}$ and $k_{\text{hyd}}^{\text{CII}}$ are the dissociation rate of ADP when KaiA is bound and the hydrolysis rate of ATP, in the CII domain of KaiC, respectively. Using the parameters presented in Table 4.1, we find that $\beta_{\text{ATP}}^{\text{CII}}$ decreases with 15% as we lower $\alpha_{\text{ATP}}$ from 100% to 50% in Eq. 5.2. The ATP fraction in $\beta_{\text{ATP}}$, Eq. 5.1, has a similar scaling when we set $K_{\text{ATP/ADP}} = 0.19$.

5.5.1. Calculating mean quantities, Fig. 5.1 and Fig. 5.2

Results shown in Fig. 5.1 were taken after 10 oscillations, such that the system has reached steady state oscillations. For the new model, presented quantities are averages over 400 consecutive oscillations. The period is defined as the mean of the peak-to-peak time in the phosphorylation level.

To calculate the flux through a cycle, Fig. 5.1E, we require a reaction that has to take place at least once, in order for a hexamer or monomer to complete a full cycle. Here, a full cycle is defined as a series of states the hexamer or monomer has to go through in order to be able to sequester KaiA. For the Rust model, we calculate the flux between the U and T phosphorylation states, integrated over a period $P$:

$$Q_{\text{Rust}} = \frac{1}{P} \int_0^P (k_{\text{UT}}(t) U(t) - k_{\text{TU}}(t) T(t)) \, dt,$$  

where $k_{\text{UT}}$ and $k_{\text{TU}}$ are the time dependent (depend on the free KaiA concentration) rates for phosphorylation and dephosphorylation of the T state, and $U(t)$ and $T(t)$ are the concentrations of U and T phosphorylated monomers. For the Van Zon model, we calculate the flux between switching to the inactive state

$$Q_{\text{VanZon}} = \frac{1}{P} \int_0^P (k_{\text{fw}} C_6(t) - k_{\text{bw}} \tilde{C}_6(t)) \, dt,$$

where $k_{\text{fw}}$ and $k_{\text{bw}}$ are the rate constants for switching to the inactive or active state, respectively. In our new model we measure the flux by counting the number of hexamers that have six KaiB monomers bound at some point during the period, averaged over 400 oscillations.

The time interval in which all KaiA is sequestered, Fig. 5.1F, is defined as the time interval when more than 99% of all KaiA dimers are sequestered by KaiC.

To generate the phase response curves (PRC) shown in Fig. 5.2A-C, we applied a 6 hour step-wise decrease in the ATP fraction. For the ODE models, we derived the induced phase change by comparing the time of second trough in the phosphorylation level after the onset of the pulse, with the same trough of the control where no pulse is given. For our new model, which contains stochasticity, we fit a sinusoidal function to three oscillations in the time trace of the phosphorylation level, one oscillation after the pulse is given. We compare this with a fit to the phosphorylation level in a control simulation, where no pulse is given, and calculate the phase shift between the fits. To suppress the intrinsic number fluctuations present in the Monte Carlo simulations, we used a simulation volume of 6 cubic microns, which is three times as large as the original volume. Finally, to get the PRC in panel A, we averaged over 10 different trajectories for each pulse start. The shaded region in Fig. 5.2A indicates the standard deviation in the phase shift of these 10 runs.
5.5.2. Microscopic dynamics of the hexamer, Fig. 5.3 and Fig. 5.4

To get the snapshots of the distribution of hexamers in phosphorylation state space shown in Fig. 5.3, we first need to register the time of the troughs in the phosphorylation level, \( t_{tr}^i \), for a time trace containing over 400 oscillations. This allows us to calculate the trough-to-trough time, \( T_{tr}^i = t_{tr}^i - t_{tr}^{i-1} \), for each oscillation \( i \). Then we define time intervals of one hour for the whole trajectory, \((t_{tr}^i + \gamma T_{tr}^i, t_{tr}^{i+1} + \gamma T_{tr}^i + 1.0)\), where \( \gamma \in [0,1) \) sets the phase of the period at which the time intervals start. During these intervals we keep track of the number of phosphorylated threonine sites, \( n_T \), and serine sites, \( n_S \), in each hexamer. This allows us to calculate the occupancy of each phosphorylation state, \( P_{n_T,n_S} \), and the fluxes between these states, during the one hour time window. These quantities are described in more detail in section 4.5.3.
BIBLIOGRAPHY


