1. Introduction

Circadian rhythms (Bünning, 1963; Dunlap et al., 2003; Edmunds, 1988) play important roles in the adaptation of organisms to their environments. They act as physiological clocks and exhibit homeostasis of the circadian period against environmental variations such as in temperature, pH, or nutrients (Pittendrigh, 1993; Pittendrigh and Caldarola, 1973). The use of molecular genetic tools has helped to identify clock genes such as period (per) (Konopka and Benzer, 1971; Rosbash et al., 2003) and frequency (frq) (Feldman and Hoyle, 1973; Froehlich et al., 2003) in Drosophila and Neurospora, respectively.

A common element in the mechanisms of circadian rhythms is the presence of negative feedback loops (Dunlap, 1999). Recently, however, positive feedback loops have also been identified (Cyran et al., 2003; Lee et al., 2000), which points to the possibility that, similar...
to chemical oscillators (Franck, 1980; Higgins, 1967), positive and negative feedback loops are both important for the generation and stability of circadian rhythms.

Computational models (Goldbeter, 2002) have the potential to provide insights into the cellular processes, environmental influences (such as temperature, light, pH, nutritional conditions), and other yet unexplored aspects of the circadian oscillator. Such models are capable of making (quantitative) predictions which can be tested experimentally. In recent years a variety of reaction kinetic models have been developed for model organisms such as *Drosophila* (Hong and Tyson, 1997; Leloup and Goldbeter, 2000; Smolen et al., 2004; Ueda et al., 2001), *Neurospora* (Gonze et al., 2000; Ruoff et al., 1999a, b, 2001; Smolen et al., 2003), mammals (Forger and Peskin, 2003; Leloup and Goldbeter, 2003), plants (Johnsson et al., 1973; Lillo and Ruoff, 1984; Lutte, 2000; Neff et al., 1998), and other insects except *Drosophila* (Lewis, 1994). Among these the *Drosophila* circadian clock is most intensively studied: the basic mechanism involves the expression of the PERIOD (PER) and TIMELESS (TIM) proteins and the formation of a heterodimer (PER/TIM), which is then transported into the nucleus where it inhibits the transcription of PER and TIM by binding to their transcription factor dCLK.CYC, a heterodimer between dCLK and CYCLE (Lee et al., 1999). The CYC concentrations were found to be in excess over the dCLK concentrations suggesting that the activity of dCLK.CYC is determined by the amount of dCLK (Bae et al., 2000).

Recently, two additional feedback loops were identified as parts of the core circadian pacemaker in *Drosophila*. In one of the loops, VRILLE (VRI), a protein which is activated by dCLK.CYC was found to repress transcription of the *dClk* gene forming a negative feedback loop, while in another positive feedback loop the protein ‘PAR Domain Protein 1’ (PDP1) activates the transcription of *dClk*, which itself activates the transcription of *Pdp1* (Blau and Young, 1999; Glossop et al., 2003). Because of the presence of both positive (activatory) and negative (inhibitory) feedbacks which deviate from previously studied single negative feedback models, we were interested in investigating the possible roles of the positive and negative feedback loops in a model with respect to the generation and stabilization of circadian oscillations in *Drosophila*.

Here we show that a representation of the negative and positive feedback loops by (mostly) first-order processes suggests that the PER/TIM heterodimer with support from the Pdp1 mediated positive feedback loop, acts as an amplifier and stabilizer for the vri/Pdp1-based positive feedback oscillator, the interlocking-feedback loop model can easily account for *per* and *vri* gene dosage effects on the circadian period. While our work was in progress, results from a corresponding model appeared (Smolen et al., 2004). Although our calculations agree in many aspects with those of Smolen et al. (2004), for example in *per* and *vri* gene dosage effects on the period, a significant difference exists with respect to the role of the PER/TIM complex as an oscillation amplifier and the importance of the Pdp1-based positive feedback loop for the stabilization of the oscillations.

2. Computational method

2.1. The model

Analogous to the Goodwin oscillator (Goodwin, 1965; Ruoff et al., 1996), transcription and translation processes in our model are represented as first-order processes (Eqs. (1)–(12)), with exception of reactions (1) and (17) (Fig. 1). The representation of the (enzyme-catalysed) processes by first-order reactions complies with the view that many enzymes in vivo are present in low concentrations and work in the first-order range of their respective substrates (Dixon et al., 1979). Non-linear terms are included only for the activation of *dClk* transcription (positive feedback) by PDP1 and the inhibition of *dClk* transcription (negative feedback) by VRI (Fig. 1). The respective cooperativities (Eq. (1)) are described by numbers *m* (0 ≤ *m* ≤ 1) and *n* (1 ≤ *n* ≤ 6). For the sake of simplicity, *per* and *tim* are described as one variable (*per/tim*) and a distinction between the cytosolic and nuclear forms of PER/TIM have not been made. The active nuclear form of VRI (VRI*) inhibits the transcription of *dClk* while the active nuclear form of PDP1 (PDP1*) activates *dClk* transcription. Because CYC is always present at much higher concentrations than dCLK (Bae et al., 2000), the transcription factor dCLK.CYC is represented by a active nuclear form of dCLK, i.e. dCLKn*. The genes *vri, Pdp1,* and *per/tim* are activated by dCLKn*, while dCLKn* becomes inactive after binding to PER/TIM (Fig. 1). Because we consider in our model only nuclear proteins in their active forms (i.e. VRI*, PDP1*, and dCLKn*) no explicit mass balance between nuclear forms (which should include active and inactive species) and cytosolic forms is formulated. This is an analogous approach as taken earlier in the Goodwin model (Ruoff and Rensing, 1996). The model’s rate equations are as follows:

\[
\frac{d[dClk - mRNA]}{dt} = k_1[PDP1]_n^m \frac{K_d}{K_d + [VRI]_n^m} - k_3[dClk - mRNA],
\]

**(1)**

\[
\frac{d[dCLK_\text{n}*]}{dt} = k_2[dClk - mRNA] - k_23[dCLK_\text{n}].
\]

**(2)**
Fig. 1. Structure of the studied model. All reactions are first-order with exception of reactions (1) and (17). Reactions \( i \) (Table 2 and following tables) are those reactions with rate constant \( k_i \). Reaction intermediate numbers \( j \) are given as numbers in parenthesis. Left (the “core”) part can show oscillations without involvement of PER/TIM \((k_5 = 0 \text{ h}^{-1})\). However, oscillations increase considerably in stability and amplitudes when expression of PER/TIM by \( \text{dCLK}_n^* \) (the “amplifier”) is coupled to the core.

\[
\frac{d[\text{dCLK}_n^*]}{dt} = k_{22}[\text{dCLK}_n] - k_{10}[\text{dCLK}_n^*] \\
- k_{15}[\text{PER/TIM}] [\text{dCLK}_n^*] \\
+ k_{18}[\text{PER/TIM} \cdot \text{dCLK}_n^*],
\]

(3)

\[
\frac{d[vri - \text{mRNA}]}{dt} = k_{3}[\text{dCLK}_n^*] - k_{21}[vri - \text{mRNA}],
\]

(4)

\[
\frac{d[VRI_c]}{dt} = k_{20}[vri - \text{mRNA}] - k_{11}[VRI_c],
\]

(5)

\[
\frac{d[VRI_c^*]}{dt} = k_{4}[VRI_c] - k_{12}[VRI_c^*],
\]

(6)

\[
\frac{d[pdp1 - \text{mRNA}]}{dt} = k_{5}[\text{dCLK}_n^*] \\
- k_{25}[pdp1 - \text{mRNA}],
\]

(7)

\[
\frac{d[PDP1_c]}{dt} = k_{24}[pdp1 - \text{mRNA}] \\
- k_{13}[PDP1_c],
\]

(8)

\[
\frac{d[PDP1_c^*]}{dt} = k_{6}[PDP1_c] - k_{14}[PDP1_c^*],
\]

(9)

\[
\frac{d[\text{per/tim} - \text{mRNA}]}{dt} = k_{7}[\text{dCLK}_n^*] \\
- k_{15}[\text{per/tim} - \text{mRNA}],
\]

(10)

\[
\frac{d[\text{PER/TIM}]}{dt} = k_{8}[\text{per/tim} - \text{mRNA}] \\
- k_{16}[\text{PER/TIM}] \\
- k_{17}[\text{PER/TIM} \cdot \text{dCLK}_n^*] \\
+ k_{18}[\text{PER/TIM} \cdot \text{dCLK}_n^*],
\]

(11)

\[
\frac{d[\text{PER/TIM} \cdot \text{dCLK}_n^*]}{dt} = k_{17}[\text{PER/TIM}] [\text{dCLK}_n^*] \\
- (k_{18} + k_{19})[\text{PER/TIM} \cdot \text{dCLK}_n^*].
\]

(12)

Eqs. (1)–(12) were solved numerically using the FORTRAN subroutine LSODE (Radhakrishnan and Hindmarsh, 1993).

For some of the results described later we found it more convenient to assign numbers to the different reaction intermediates. This assignment is shown in Table 1 and Fig. 1.

2.2. Control coefficients

Period and amplitude control (sensitivity) coefficients are defined as \( C_i^P = \partial \ln P/\partial \ln k_i \) and \( C_i^A = \partial \ln A_i/\partial \ln k_i \), where \( P \) is the oscillator’s period, and \( A_j \) and \( k_i \) are the amplitude of intermediate \( j \) and the rate constant of process \( i \), respectively. The \( C_i^P \) and \( C_i^A \)'s are measures of the sensitivity of the oscillator’s period \( P \) or amplitude \( A_j \) upon changes of the rate constant \( k_i \). Positive or negative control coefficients indicate that the period/amplitude increases or decreases, respectively, with an increase in \( k_i \). To determine \( C_i^P \) or \( C_i^A \) numerically, we
first calculated the logarithm of the period/amplitude for different values of the rate constant \( k_i \) near a chosen reference point. In a final step \( CP_i \) or \( C_{Aj} \) were obtained as the linear regression slopes of the \( \ln P - \ln k_i \) or \( \ln Aj - \ln k_i \) relationships. A numerical check of the calculated control coefficients can be made according to the summation theorems (Heinrich and Schuster, 1996; Ruoff et al., 2003):

\[
\sum_i C_i^p = -1, \tag{13}
\]

\[
\sum_i C_i^{Aj} = 0. \tag{14}
\]

However, it should be noted that the individual control coefficients depend on the values of the rate constants for a chosen reference state.

3. Results

3.1. Pdp1-positive feedback and PER/TIM-mediated amplification

The model can generate oscillations with period lengths within the circadian range and with relative phases of reaction intermediates that are close to experimentally observed values (Fig. 2). As indicated in Fig. 1, the model can be divided into two components, the “core” and the “amplifier”. The core segment consists of the Pdp1 positive and vri negative feedback loops, which regulate the transcription of dClk, and are capable of generating sustained oscillations even in the absence of per \( (k_7 = 0, n = 5) \). A more or less arbitrarily chosen set of rate constants which shows sustained core oscillations is given in Table 2 (left columns). The \( C_i^{Aj} \) values (Table 3) indicate that Pdp1-mediated positive feedback (by increasing the values of \( k_5, k_{24} \) or \( k_6 \)) increases the amplitude of the dClk-mRNA core oscillations, while promoting the vri-mediated negative feedback decrease the dClk-mRNA amplitudes. Fig. 3a illustrates the amplification effect of the positive feedback for core oscillations \( (k_7 = 0 \text{ h}^{-1}, n = 5) \). At \( t = 200 \text{ h}, k_6 \) is increased from 1 to 100 \text{ h}^{-1}. As expected from the \( C_6^{Aj} \) value (Table 3), the amplitude

<table>
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<tr>
<th>Reaction intermediate number ( j )</th>
<th>Name</th>
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<tbody>
<tr>
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<td>dClk-mRNA</td>
</tr>
<tr>
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<td>dCLK(_c^*)</td>
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<tr>
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<td>VRI(_c^*)</td>
</tr>
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<td>VRI(_n^*)</td>
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<td>PDP1(_c^*)</td>
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<tr>
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<td>PDP1(_n^*)</td>
</tr>
<tr>
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<td>per/tim-mRNA</td>
</tr>
<tr>
<td>8</td>
<td>PER/TIM</td>
</tr>
<tr>
<td>9</td>
<td>PER/TIM dCLK(_c^*)</td>
</tr>
<tr>
<td>10</td>
<td>vri-mRNA</td>
</tr>
<tr>
<td>11</td>
<td>dCLK(_c)</td>
</tr>
<tr>
<td>12</td>
<td>Pdp1-mRNA</td>
</tr>
</tbody>
</table>

Table 1

Number assignment to reaction intermediates

Fig. 2. (a) Concentration oscillation profiles for dClk-mRNA, PDP1 ([PDP1\(_c^*\), [PDP1\(_n^*\)]), CLK ([dCLK\(_c\), [dCLK\(_n^*\)]), VRI ([VRI\(_c^*\), [VRI\(_n^*\)]) and PER/TIM of complete model (core + amplifier). Rate constant and initial concentration values are given in Table 2. However, \( k_1-k_5 \) were slightly increased (from 1.0 to 1.1 \text{ h}^{-1} \) to get a 24 h period length. (b) Scaled concentration profiles for the oscillations shown in Fig. 2a for dClk-mRNA, PDP1 and VRI. (c) Experimental concentration profiles of dClk (dClk-mRNA), PDP1 and VRI replotted from Cyran et al. (2003).
of the dClk-mRNA oscillations increase as \( k_6 \) (or any other rate constants which promotes the positive feedback loop) is increased.

In the complete model consisting of the core and the amplifying part, the dCLK\(_n^*\)-induced expression of PER/TIM couples with the core by binding to dCLK\(_n\) and thus decreasing its concentration. This removal of dCLK\(_n^*\) by PER/TIM results in a dramatic increase in amplification by the Pdp1-positive feedback loop. Table 4 shows that promoting the positive feedback (by increasing \( k_5, k_24 \) or \( k_6 \)) increases the amplitudes of all reaction intermediates. Fig. 3b shows the corresponding dClk-mRNA oscillations as in Fig. 3a for \( t \geq 200 \) h, but keeping \( k_6 \) constant to 100 h\(^{-1}\) for all \( t \). At \( t = 200 \) h \( k_7 \) is increased from 0 to 1, which leads to a large increase in the amplitude of dClk-mRNA. Fig. 3c compares the dClk-mRNA amplitudes for core and complete model oscillations as a function of \( k_6 \).

Surprisingly, the amplitude control coefficients associated with the negative feedback loop (i.e., \( C_{C_3}^i \), \( C_{C_4}^{i-1} \) and \( C_{C_4}^j \) have become positive! However, a closer inspection revealed that increasing \( k_3, k_20 \) or \( k_4 \) values lead to negative \( C_{C_3}^i \), \( C_{C_4}^{i-1} \) and \( C_{C_4}^j \) values and never show the amplifying effects which are due to increasing values of the rate constants within the positive feedback loop (data not shown). This behavior shows that the control coefficients may change sign/value depending upon the values of the rate constants.

Removal of CLK\(_n^*\) by PER/TIM with increased \( k_{17} \) values leads to an increase in the dClk-mRNA amplitude with saturation at high \( k_{17} \) values (Fig. 4), which explains the low control coefficients for \( k_{17} \) values used (Tables 2 and 4). These results clearly demonstrate that the reason for the dClk-mRNA amplitude increase is the removal of CLK\(_n^*\) by PER/TIM (Fig. 4) through a coupling with the Pdp1-mediated positive feedback loop (Fig. 3).

### Table 2

<table>
<thead>
<tr>
<th>Reaction ( i )</th>
<th>Core model(^a)</th>
<th>Complete model(^b)</th>
</tr>
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<tbody>
<tr>
<td>( C_i^p )</td>
<td>( C_i^p )</td>
<td></td>
</tr>
<tr>
<td>( k_i ) (h(^{-1}))</td>
<td>Value of ( k_i ) (h(^{-1}))</td>
<td>Value of ( k_i ) (h(^{-1}))</td>
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<td>1</td>
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<tr>
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<td>0.7</td>
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<td>0.04</td>
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<td>0.05</td>
</tr>
<tr>
<td>17(^e)</td>
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</tr>
<tr>
<td>18(^f)</td>
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<tr>
<td>25</td>
<td>0.036</td>
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\(^a\)Initial concentrations for core oscillations: \([dClk\text{-mRNA}] = 6.0470, [dCLK_n^*] = 0.40639, [VRI] = 0.80317, [VRI_n^*] = 1.1364, [PDP1_n^*] = 24.5861, [PDP1_n^*] = 161.956, [vri\text{-mRNA}] = 0.40448, [dCLK_n] = 4.2548, [Pdp1\text{-mRNA}] = 3.4214, K_d = 1.0, n = 5, m = 0.5. \(^b\)Initial concentrations for complete model oscillations: \([dClk\text{-mRNA}] = 1.172e + 1, [dCLK_n] = 6.815e – 7, [VRI] = 1.203e – 2, [VRI_n^*] = 3.839e – 2, [PDP1_n^*] = 1.973, [PDP1_n^*] = 12.64, [per/tim\text{-mRNA}] = 0.9686, [PER/TIM] = 2.106. [PER/TIM,dCLK_n^*] = 1.435e–6, [vri\text{-mRNA}] = 1.822e–3, [dCLK_n] = 14.35, [Pdp1\text{-mRNA}] = 0.2412, K_d = 1.0, n = 5, m = 0.5. \(^e\)Reaction (17) is a second-order process with dimension [concentration (a.u.)] \( ^{-1} \) h\(^{-1}\). \(^f\)\( k_{19} \) can vary over several orders of magnitude without significantly affecting the oscillator’s period.
Table 3  
$c_i^{\text{eq}}$ values for core oscillations


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<th>4</th>
<th>5</th>
<th>6</th>
<th>10</th>
<th>11</th>
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<th>$\sum_j c_i^{\text{eq}}$</th>
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<td>-0.300</td>
<td>-0.309</td>
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</table>

Index $i$ (columns) identifies the reactions, index $j$ (rows) identifies the reaction intermediates (see Fig. 1, Table 1). Rate constant values are given in Table 2.

3.2. PER/TIM-mediated stabilization of oscillations: importance of the positive and negative feedback loops

The coupling of PER/TIM to the core not only increase the amplitude of dClk-mRNA oscillations but also makes the oscillator much more robust with respect to variations in the VRI* cooperativity $n$ (Eq. (1)). Even for $n$-values as low as 1 the model generates sustained oscillations. Fig. 3d shows the behavior of the system as in Fig. 3b, but the value of $n$ was reduced from 5 to 1. Now the core is no longer able to show sustained oscillations, while the complete model still shows large amplitude oscillations. This suggests that the per gene not only plays a crucial role in regulating the oscillator’s amplitudes but is also important for the oscillator’s stability/robustness.

Although our simulations show that the Pdp1-mediated positive feedback loop plays an important role in terms of promoting and stabilizing the oscillations, both positive and negative feedback loops are necessary to generate oscillations, at least for the parameter values used in this study. The model was unable to generate oscillations when one of the rate constants within the positive (or negative) feedback loop was set to zero (or were very small), even for $n$ values as high as 11 (data not shown).

3.3. Effect of gene dosage and rate constants on period length

Smith and Konopka (1982) found that the period length of the Drosophila circadian rhythm is shortened when the gene dosage at the per locus is increased. While this behavior is not easily modeled with a single negative feedback oscillator (as indicated by the control coefficients for the Goodwin oscillator; see Ruoff and Rensing, 1996), the present model can readily account for per dosage effects as described in Table 2 and Fig. 5a. In Fig. 5a experimental per gene dosage data (Cote and Brody, 1986) are compared with data obtained from our model. Calculations show that the period length decreases with increasing $k_7$ values, which reflect increasing per gene dosage. In relating $k_7$ with per gene dosage, a power-law relationship between rate constant $k_7$ and the per (in our model per/tim) dosage is considered, i.e.,

$$k_7 = a \text{(dose of per/tim)}^b.$$  

Choosing $a = 6$ and $b = 0.131$ ($k_6 = 60 \text{h}^{-1}$) the calculated period length (open circles in Fig. 5a) match closely the experimental data (solid squares and cross). By increasing $k_6$, we found that the slope of the period length vs. $k_7$ relationship becomes less negative, which means that for different $k_6$ values the model still can describe the experimental per dosage data, but with different values of $a$ and $b$ (Eq. (15)).

Blau and Young (1999) observed that reducing the dosage of vri shortened the period of the flies’ activity rhythm. This finding is in agreement with the model, which predicts robust oscillations and an increase/decrease in the period when the transcription rate (gene dosage) of vri is increased/decreased (Table 2,
In accordance with the $C_T^i$ control coefficient (Table 2, complete model), the model also predicts that the period length should depend on $Pdp1$ gene dosage more or less identical to the $vri$ dosage (data not shown).

An important question is how circadian oscillations depend on the stabilities of key proteins. For example, from studies of the Goodwin oscillator it was predicted that an increased/decreased FRQ protein stability should increase/decrease *Neurospora*’s circadian period length, and should also alter temperature compensation of the clock (Ruoff et al., 1996, 1999b). Subsequent experiments showed that phosphorylation and kinase activities indeed play an important role in the stability of FRQ and in determining the period length of *Neurospora*’s circadian clock (Liu et al., 2000). Similarly, the Goodwin model was able to describe the altered periods in *Drosophila’s* per and per$^+$ clock mutants with their characteristic reciprocal behavior in temperature compensation (Konopka et al., 1989) in terms of altered stability of the PER protein and an altered PER–PER or PER–protein interaction (Ruoff et al., 1996). In particular, a decreased stability in PER protein predicted a shorter period (Ruoff et al., 1996), which appears to be related to the phosphorylation state of PER (Preuss et al., 2004; Rosbash et al., 2003). Also in the present model we found that an increase in the PER/TIM stability (i.e. a decrease in the rate constant $k_{16}$) results in an increased period length and a partial loss of the oscillator’s temperature compensation (Table 2, see also next chapter). As indicated by the $C_T^i$ coefficients, *Drosophila* mutants with increased...
Table 4

C_i values for oscillations of the complete model

<table>
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<th>i/j</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<th>10</th>
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<th>( \sum c_i^j )</th>
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<td>0.192</td>
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<td>0.218</td>
<td>1.346</td>
<td>0.769</td>
<td>1.163</td>
<td>1.056</td>
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<td>1.199</td>
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</table>

\[ \sum c_i^j = 0.001 \]

Index \( i \) (columns) identifies the 26 reactions, index \( j \) identifies the 12 reaction intermediates (Fig. 1, Table 1). Rate constant values are given in Table 2.

3.4. Influence of temperature and temperature compensation

Temperature is an important “Zeitgeber” for many circadian clocks (Rensing and Ruoff, 2002) while temperature compensation is an essential property of all circadian (and ultradian) clocks. Temperature compensation means that the oscillator’s period \( P \) is practically unchanged under different constant environmental temperatures, despite the fact that physiological component processes are generally quite dependent upon temperature (Ruoff et al., 2000). The condition for obtaining temperature compensation for a reaction kinetic oscillator is given by

\[
RT^2 \frac{d \ln P}{dT} = \sum_j \frac{\partial \ln P}{\partial \ln k_j} E_j + \sum_i \frac{\partial \ln P}{\partial \ln K_l} \Delta H^0_l = 0,
\]

(16)

where \( k_i \) are rate constants and \( K_l \) are equilibrium constants of any rapid equilibrium which might have been established in the system. The temperature dependence of the rate constants are usually described by the Arrhenius equation (Laidler and Meiser, 1995)

\[
k_j = A_j \exp(-E_j/RT),
\]

(17)

![Fig. 4. The dClk-mRNA amplitude shows saturation at high values of k17, explaining the low C_i^j values.](Image)
where $E_j$ is the activation energy of process $j$, and $A_j$ is the pre-exponential factor. $R$ and $T$ are the gas constant and temperature (in Kelvin), respectively. The activation energy and the pre-exponential factor in Eq. (17) vary generally little with temperature and are therefore often considered (as here) as temperature independent. In the case where rapid-equilibrium constants $K_i$ are included in the rate equations, the temperature-dependence of $K_i$ can be described in an analogous way as the Arrhenius equation, i.e. by substituting the activation energy by the pre-exponential factor.

It should be noted that there is, in principle, an infinite number of activation energy $(E_i)$ combinations which for a given set of $C_i^p$ values satisfies Eq. (18) and lead to temperature compensation. Because activation energies are always positive, Eq. (18) requires (in addition to Eq. (13)) that some of the $C_i^p$’s are also positive. Although Eq. (13) opens the possibility that all $C_i^p$’s could be negative (which would not allow any form of temperature compensation by Eq. (18)), so far, in all reaction kinetic oscillator models investigated, both positive and negative $C_i^p$’s have been found.

Small changes in the rate constants $k_1$–$k_3$ (Table 2) from 1.0 to 1.1 h$^{-1}$ result in oscillations with a period close to 24 h (Fig. 2a). This set of rate constants has been used as a starting point to investigate temperature compensation in the model. In order to apply Eq. (18), we have to first define a reference temperature $T_{ref}$, for which the chosen set of rate constants designated as $\{k_{chosen}\}$ applies. In the next step, the $A_j$ values are determined as $A_j = k_j^{chosen} \exp(E_j/RT_{ref})$ and substituted into Eq. (17), from which the rate constants can be calculated for a desired temperature $T$.

Before investigating temperature compensation, we were interested in studying the temperature dependence of the period under a situation where all activation energies assume the same value. Curve I in Fig. 6a (gray diamonds) shows how the period length of the oscillations shown in Fig. 2a decreases with increasing temperature when all the activation energies are set to 30 kJ/mol. The $T_{ref}$ was set to 25 °C; at this temperature the oscillator shows, as expected, a period of about 24 h (Fig. 2a). In the light of Eq. (13), the period decreases with increasing temperature because all activation energies are considered to be equal $(E_i = 30 \text{kJ/mol})$ leading to $\sum_i C_i^p E_i = -30 \text{kJ/mol}$. In fact, an Arrhenius plot, i.e. plotting the inverse of the period vs. $1/T$ (Laidler and Meiser, 1995), would have a slope $(-E_a/R)$ with an overall activation energy $E_a$ of 30 kJ/mol (data not shown). Such a strong decrease of the period with increasing temperature is often experimentally observed in chemical oscillators, for example in the Belousov–Zhabotinsky reaction (Nagy et al., 1996; Ruoff, 1995). It reflects the situation that activation energies of the underlying component processes in chemical oscillators are randomly distributed (and not subject to evolutionary mutation and selection mechanisms as in biological clocks) which gives $dP/dT$ (according to Van’t Hoff’s rule) a large negative value. Interestingly, the condition of temperature compensation in chemical oscillators have recently been experimentally studied (Kóvacs and Rábai, 2002; Rábai and Hanazaki, 1999).
and the results are in close accordance with the principle of opposing reactions as described by Eq. (18).

In search for activation energy combinations which result in temperature compensation over a larger temperature range, we used a “stochastic fitting method”. In this method we started with the set of rate constant values shown in Table 5 (column per\(^+\)) defined at 11 °C, where all activation energies are initially given a value of 30 kJ/mol. During an iterative procedure a randomly chosen activation energy value (up to a maximum of 60 kJ/mol) is then assigned to a randomly chosen rate constant (reaction). Rate constants (by applying Eq. (17)) and periods then are calculated for different temperatures \(T_i\) with a given temperature interval. The calculated periods \(P_{calc}(T_i)\) are compared with a pre-defined target function \(P_{target}(T_i)\). The deviation \(DEV\) between the calculated and pre-defined period values

\[
DEV = \sqrt{\frac{1}{N} \sum_{i=1}^{N} (P_{calc}(T_i) - P_{target}(T_i))^2}
\]  

(19)

is evaluated by Eq. (19), where \(N\) is the number of temperature points within the temperature interval. Once a randomly selected rate constant gives a lower \(DEV\) compared with previously calculated \(DEV\) values, the previous activation energy for this rate constant is replaced by the new one, and is updated again when a new random selection leads to an even lower \(DEV\) value. Curve 2 (Fig. 6) shows an example for an activation energy combination (Table 5) leading to temperature compensated oscillations when \(P_{target}(T_i) = 24\) h (for all \(T_i\)), exhibiting \(per^+\) behavior. Curves 3 and 4 show the period length as a function of temperature when activation energies and rate constants in the \(per/\)tim expression pathways of the temperature compensated set (curve 1) are changed (Table 5, grayed entries, resembling \(per^L\) and \(per^S\) behavior, respectively. The experimental data on the period lengths as a function of temperature for the \(per^+, per^S\), and \(per^L\) mutant flies are shown as solid points (Konopka et al., 1989). The model predicts (Table 5, outlined) that the \(per^S\) mutation (Yu et al., 1987) is associated with an increased turnover and increased temperature sensitivity (increased \(E_{16}\)) of PER/TIM. For the \(per^L\) temperature response behavior, the model suggests (Table 5, grayed entries in right columns) that several processes in the PER/TIM expression pathway might be affected by the \(per^L\) mutation (Huang et al., 1995), but the largest change appears to be due to a reduction in the stability of the PER/TIM.dCLK\(_d\) complex (decrease of \(k_{17}\)) accompanied by an increase in its temperature sensitivity (increase of \(E_{17}\)). The calculated \(\sum E_i\) values for \(per^+, per^S\) and \(per^L\) parametrizations are also shown in Table 5. Similar predictions for the \(per^L\) and \(per^S\) temperature behaviors have earlier been made using the Goodwin oscillator (Ruoff et al., 1996). Fig. 6b shows...
the PER/TIM oscillations of \( \text{per}^+, \text{per}^S, \) and \( \text{per}^L \) parametrizations at 25°C (298 K). Fig. 6c shows that the PER/TIM amplitude of the temperature compensated \( \text{per}^S \) parametrization increases with increasing temperature, which is in agreement with the amplitude model (Lakin-Thomas et al., 1991). In the amplitude model temperature compensation is ensured by an increase in the amplitude of oscillation: although the velocity of the oscillator’s trajectory in phase space increases with increase in temperature, an increase in amplitude compensates for the increased velocity in phase space such that the period remains unaltered. Although the predicted amplitude effects (Lakin-Thomas et al., 1991) have been observed in other temperature compensated reaction kinetic oscillators (Ruoff, 1992; Ruoff et al., 2003), exceptions have been found theoretically as well as experimentally (Leloup and Goldbeter, 1999; Rensing and Ruoff, 2002; Ruoff et al., 2003).

### 3.5. Light/dark (L/D) entrainment and temperature pulse PRCs

Although periods of circadian rhythms are affected little by different constant temperature levels, their phases are sensitive to sudden temperature variations (steps or pulses) which lead to phase shifts. In *Drosophila* studies to estimate phase shift involve maintaining flies in L/D cycles (or in continuous light) before temperature pulses/steps are applied at different phases under constant darkness (DD) (Chandrashekar, 1974; Zimmerman et al., 1968). A plot of the resulting phase shifts between unperturbed and perturbed rhythms as a function of the phase of perturbation defines a phase response curve (PRC). We have calculated the temperature pulse PRC of our model according to a protocol by Zimmerman et al. (1968). First, the rhythm is entrained to a L/D regime at 20°C (or 28°C) and then 12h temperature pulses from 20 to...
28°C (or from 28 to 20°C) were applied at different phases of the rhythm in DD. Before we present the modeling of the temperature PRCs we briefly refer to the corresponding effects of light. It has been shown experimentally that light increases the degradation of PER/TIM (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Young et al., 1996; Zeng et al., 1996), a feature which has been used to model light responses and entrainment to L/D cycles (Klarsfeld et al., 2003; Leloup and Goldbeter, 1998; Smolen et al., 2004; Tyson et al., 1999). To model entrainment to L/D cycles we have (either at 20 or 28°C) increased the degradation rate constant of PER/TIM (k₁₆) during the light phase and restored its original dark value during the dark phase. The extent of increased PER/TIM degradation during light conditions were arbitrary chosen. Fig. 7 shows that the phase of PER/TIM changes relative to the L/D cycles is dependent upon the magnitude of PER/TIM removal during the light phase (T = 25°C).

In order to model the temperature pulse PRCs we first imposed an L/D cycle at 20 or 28°C (other conditions were the same as those described in Fig. 7b) and then used 12h temperature pulses, either a high temperature pulse (HTP, 20→28°C) or a low temperature pulse (LTP, 28→20°C), respectively, covering the subsequent dark phase after the LD→DD transition (which defines circadian time 12; ct 12) for three cycles (72h in DD). Surprisingly, the oscillator showed entrainment to L/D cycles only within a small window near its natural period, which is the reason for the 11.25:11.25 L/D entrainment scheme shown in Fig. 7. Figs. 8a, b show the results of the calculated HTP and LTP PRCs which match closely the experimental PRCs (Chandrashekar-an, 1974; Zimmerman et al., 1968). The model is able to describe the characteristic behaviors of the HTP and LTP PRCs.

4. Discussion

4.1. Phase behavior of the Model

The aim of the present study was to investigate the roles of PER/TIM in connection with the Pdpl mediated positive and vri mediated negative feedback loops. In the present model we have deliberately taken (most of) the component processes as first-order reactions, in order to keep the model as simple as possible, and to also allow a comparison with previous results obtained with the Goodwin oscillator. The study of Kurosawa and coworkers on a single negative feedback oscillator showed that by introducing Michaelis–Menten kinetic terms within the model, the
oscillations and their robustness may be enhanced (Kurosawa and Iwasa, 2002; Kurosawa et al., 2002), which will probably also be the case in the present model. In fact, in many of the recent models of the Drosophila or mammalian circadian clocks (Goldbeter, 2002; Gonze et al., 2000, 2003; Leloup and Goldbeter, 1998, 2000, 2003; Smolen et al., 2001), Michaelis–Menten type kinetics have been included. However, whether Michaelis–Menten kinetic terms are essential in circadian time keeping mechanisms is yet to be understood.

Although the relative phases of dClk-mRNA, and PDP1 and VRI proteins match experimental findings (Figs. 2b, c), there are also some significant discrepancies between model and experiment. For example in the first part of the cycle where experiment (Fig. 2c) shows a low and slowly increasing PDP1 concentration, the calculations show that PDP1 values rapidly decrease and reach a steep minimum (Fig. 2b). Further, the experimental data demonstrate (Cyran et al., 2003; Glossop et al., 2003) that VRI is in antiphase compared to dClk-mRNA, while calculations suggest that the VRI maximum occurs slightly before the dClk-mRNA minimum (Figs. 2b, c). These phase behavior properties of the model were quite rigid indicating that certain phase determining aspects between PDP1, VRI and dClk might still be missing.

4.2. Influence of PDP1-positive feedback on properties on the oscillation

The observation that the expression of per/tim acts as part of an amplifier, with the Pdp1-positive feedback loop as the source of the oscillator’s amplification and stabilization (Fig. 3) assigns a new putative function for PER/TIM and the Pdp1-positive feedback loop to the Drosophila circadian clock. This result is in contrast to the findings that in oscillator models consisting of positive and negative feedback loops with time delays, the positive feedback was not found crucial for the oscillations (Smolen et al., 2001, 2002, 2004). This discrepancy may be due to the fact that delay terms alone can generate oscillations. Consider the following sequence of first-order reactions

\[ X_0 \rightarrow X_1 \rightarrow X_2 \rightarrow \cdots X_i \rightarrow \cdots X_N, \]  

(S1)

where the appearance of \( X_N \) is delayed by a certain time interval \( \tau \) compared with the disappearance of \( X_0 \). In reaction kinetics it is well established that process S1 will not be able to show sustained oscillations (Higgins, 1967). However, by representing the disappearance/appearance of \( X_0, X_N \) as a process of the form

\[ X_0 \rightarrow X_N \]  

(S2)
described by the delay equations

\[
\frac{dX_N}{dt}(t) = kX_0(t-\tau),
\]  

(20a)

\[
\frac{dX_0}{dt}(t) = -kX_0(t-\tau)
\]  

(20b)

and the mass balance

\[
\frac{dX_N}{dt} + \frac{dX_0}{dt} = 0
\]  

(20c)

oscillations in \( X_0 \) and \( X_N \) can be observed when \( \tau \) is suitably chosen (for example for \( \tau = \pi/2 \) time units the solution becomes oscillatory \( X_0(t) = \sin(kt) \) or \( X_0(t) = \cos(kt) \), opposite to what is expected from the non-oscillatory behavior of process S1. This suggests that it may not be unproblematic to approximate/simplify chemical rate equations by delay equations. Furthermore, the time discontinuity in the system imposed by Eqs. (20) leads to a concentration discontinuity. As \( X_0 \) is not defined for \( t < t_0 \) (because then \( X_0(t_0) \) would not be an initial value) one has to assume that the
time discontinuity of Eq. (20) applies for $t \geq t_0 + \tau$. By assuming this, $X_0(t)$ (Eq. 20b) becomes periodically discontinuous (with a period of $\tau$), leading to oscillations in $X_0$. While differential equations with delay terms are used in modeling circadian rhythms (Johnsson and Karlsson, 1972; Johnsson et al., 1973; Lewis, 1994), their application in reaction kinetic models (Epstein, 1990) appears to be unclear and implications on the importance of the positive feedback loops (Smolen et al., 2002, 2004) may be blurred by potential artefacts that may be generated in (chemically unrealistic) delay equations. In summary, the issue related to the usage of delay equations leading to possible artefacts needs a more careful analysis. In a personal communication Dr. P. Smolen informed us that in the Smolen et al. (2004) model circadian oscillations are preserved when the time delay (with or without positive feedback) is removed.

4.3. Effect of gene dosages

With a single negative feedback model, such as the Goodwin oscillator (Goodwin, 1965) the effect of per dosage was difficult to model, because all synthesis reactions within the negative feedback loop have positive $C_i^p$ values (Ruoff and Rensing, 1996). With the coupling of the per/tim expression to the Pdp1 and vri feedback loops (the "amplifier", Fig. 1), the effect of per dosage can be modeled by assuming a power-law relationship between the rate constant of transcription ($k_7$) and the per/tim dosage (Eq. (15), Fig. 3a). Transcription/translation involves many proteins and processes (Alberts et al., 2002) and Eq. (15) is an empirical relationship between the gene dosage and the assignment of a single rate constant to those processes. In chemical kinetics such empirical power law relationships (reaction orders) are often used when the molecular mechanism for a process is unknown (Laidler and Meiser, 1995).

4.4. Temperature compensation, PER/TIM and PER/ TIM dCLK CYC stabilities

Temperature compensation and other homeostatic regulation of the circadian period (Pittendrigh and Caldarola, 1973; Ruoff et al., 2000) are important clock properties. Temperature compensation is observed although many, if not all, of the underlying component processes are considered to have $Q_{10}$ values of about 2–3 ("Van’t Hoff’s rule"). To obtain temperature compensation for a reaction kinetic oscillator model, some of the $C_i^p$’s need to be positive such that Eq. (18) is fulfilled and positive and negative contributions balance each other within a certain temperature range. The possibility that temperature compensation may be understood in terms of such opposing reactions was already suggested in the late 1950 (Hastings and Sweeney, 1957), and is considered to be a latent property found in any reaction kinetic oscillator (Ruoff, 1992). In the temperature compensated set describing per (Table 5), we found that some of the stochastically selected $E_i$ values were quite low (for example $E_{16}$ for the PER/TIM degradation). This may suggest that for some of the processes (such as the PER/TIM degradation) additional compensation mechanisms may come into play to ensure such a low activation energy. Additional mechanisms may be the “instantaneous compensation”, which has been observed for Michaelis–Menten type kinetics or diffusion controlled processes (Andjus et al., 2002; Ruoff et al., 2000). In instantaneous temperature compensation, the $K_M$ and $V_{\text{max}}$ values of an enzyme-catalysed process increase both with temperature such that the overall reaction becomes independent of temperature (Andjus et al., 2002). In diffusion controlled processes the reactions are so fast that the transport of reactants by diffusion becomes the rate limiting step. In the latter case reactions depend little on temperature, but viscosity changes of the reaction medium (cytoplasm) due to temperature variations have normally the largest impact on diffusion controlled reactions. One may even speculate whether the cell may have evolved mechanisms which can keep the viscosity of the cytosol constant at different temperatures to ensure that reaction rates of diffusion controlled processes become independent of temperature. However, it should also be noted that compared to the cellular dimensions, diffusion of (clock) proteins is a fairly rapid process, hence diffusion is generally not a rate limiting step in the generation of circadian rhythms (Winfree, 2000).

As already predicted in an earlier study on the Goodwin oscillator (Ruoff et al., 1996) the temperature response behavior of perS mutants can be obtained by decreasing the PER protein stability (i.e. by increasing $k_{16}$) and making the degradation of PER/TIM more temperature sensitive by increased $E_{16}$ values (Table 5). We are not aware of any experiment, which has measured the turnover of PERS compared to PER+.

In describing the perL mutant, the largest change needed in our model compared to per+ is a reduction of $k_{17}$, which suggests that the binding between PER/TIM and dCLK (Bae et al., 2000; Lee et al., 1999) should be weakened in perL mutants (Table 5). It was previously shown that perL contains a point mutation within its PAS domain, which seems to be of importance for the PER/TIM–dCLK interaction and the formation of PER dimers. Alternatively, the PAS domain of PER can interact intramolecularly with another domain within PER. Both intra- and intermolecular interactions have been suggested to be of importance for Drosophila’s temperature compensation (Huang et al., 1995).
4.5. Influence of light and temperature pulse PRCs

In order to model temperature pulse PRCs we used the temperature compensated \(per^+\) parametrization (Table 5) and, as in the Zimmerman et al. (1968) experiments, first entrained the oscillator to light dark cycles at 20 or 28 °C, before 12 h dark HTTs or LTPs (20→28 °C or 28→20 °C) were applied (Fig. 8). However, to our surprise, the oscillator entrained to L/D cycles only within a very small window near its natural period. The reason for such “rigidity” of the oscillator towards L/D cycles seems to be that the influence of light is represented by only one reaction, i.e. by an increased degradation of PER/TIM. On the other hand, when analysing temperature entrainment of the temperature compensated oscillator, the oscillator seems to entrain more easily and to a wider range of T-cycles (data not shown). We wish to return to the question which factors influence/enhance the entrainment of a biological oscillator by L/D or temperature cycles in a later study.

However, when entrainment by L/D cycles does occur in the model, the phase of PER/TIM is dependent upon the rate of PER/TIM degradation during the light phase (Fig. 7). It was recently shown (Bao et al., 2001) that under 12:12 L/D cycles the phase of PER is delayed in \(per^S\) mutants relative to PER in \(per^+\) flies. The results described in Fig. 7 indicate that a delayed appearance of PER as in \(per^a\) (Fig. 7c) may be due to a diminished light-induced degradation of PER (Fig. 7d). Because the model suggests that PER degradation is already increased in darkness compared to \(per^+\) degradation (Table 5), light may have a smaller effect on PER degradation than on \(per^+\) degradation, and therefore could lead to a delay in the appearance of the PER peak in \(per^S\). Furthermore, the reduced maximum level of PER in \(per^S\) compared to \(per^+\) can be accounted for by the generally increased degradation of PER in \(per^S\) flies both under light or dark conditions (Fig. 6b).

The calculated PRCs of temperature pulse perturbations (Fig. 8) closely match the classical experiments by Zimmerman et al. (1968) and Chandrashekaran (1974). The reason why there is a slightly better agreement between experimental and simulated PRCs for the LTP case (28→20°C, Fig. 8b) is not clear. However, additional calculations showed that altered temperature levels during the prior L/D cycles may lead to a better match between experimental and simulated data (data not shown). Another aspect, which we found interesting and that might be worth investigating in a later study is how a temperature PRC may vary due to changes in activation energies, while keeping temperature compensation intact.

4.6. Oscillations in PER-less mutants

As seen from the results in Fig. 3, the interlocking-feedback loop model offers the possibility of “core” self sustained oscillations even in the absence of \(per/tim\). However, even under such conditions \(dClk\) should be still an essential oscillator element. In a related model of the mammalian circadian clock which was lacking PER protein oscillations could still be observed (Leloup and Goldbeter, 2003, 2004).

There have been several reports indicating oscillations may occur in fly strains that lack functional \(per\) (Dowse and Ringo, 1987; Helfrich and Engelmann, 1987; Helfrich-Förster, 2001; Yoshii et al., 2002). Experimental data often show a lengthening of the period for \(per^0\) flies that are still rhythmic. This is also observed in our model (data not shown) and is consistent with the model’s description of \(per\) dosage effects (Fig. 5a).

By using a time series technique, Dowse and Ringo (1987) analysed the signal-to-noise ratio (SNR) of locomotor activities in \(per^+, per^S, per^L\) and \(per^0\) strains. They found that ultradian rhythm components are present in \(per^0\) flies, and that the SNR increased significantly in the presence of \(per\), which indicates that PER indeed acts as an amplifier (Fig. 3a, b). Helfrich and Engelmann (1987) found that the range of L/D entrainment in \(per^0\) flies is significantly narrowed compared to \(per^+\) flies, and that \(per^0\) flies are capable of generating endogeneous oscillations. They concluded that the \(per\) gene product is necessary as a normal output of the clock controlling locomotor activity, but that \(per\) is not concerned with the central clock structure. Helfrich-Förster (2001) found evidence for a two oscillator system (described as morning and evening oscillators) in which the morning oscillator is assumed to track light. Two reports recently showed that the morning and evening locomotor behavior of *Drosophila* is controlled by \(per\) by using coupled but different clock neurons (Grima et al., 2004; Stoleru et al., 2004).

By applying periodic temperature cycles on a variety of mutants, Yoshii et al. (2002) found evidence that \(per^L\) flies still have a weak oscillatory mechanism, which seem to require \(dClk\) and cyc. Our results that the model is poorly entrained by L/D cycles may indicate that part of the circadian light tracking system is incomplete in the model and that the “core” may contain additional light mediating properties and feedback loops which would enhance the sensitivity of the model to L/D cycles. These aspects will be considered in a future study on an extended variant of the interlocking-feedback loop model.

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References


