KURZE ORIGINALMITTEILUNGEN

Naturwissenschaften 83, 514–517 (1996)

Circadian Rhythms and Protein Turnover: The Effect of Temperature on the Period Lengths of Clock Mutants Simulated by the Goodwin Oscillator

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Recent reports that the circadian clock in both *Drosophila* and *Neurospora* consists of a negative feedback loop between clock gene (*per, frq*) activity and amount of clock protein make the Goodwin oscillator a timely model for circadian rhythms. This model is characterized by a negative feedback loop in the clock gene expression with synthesis/degradation reactions associated with each of the intermediates in the loop. The model predicts that predominantly degradation processes of clock mRNA or clock protein control the circadian period and temperature compensation. Assuming a turnover homeostasis of the clock protein, the model explains temperature effects on period length in *per* and *frq* mutants.

Recent results obtained in *Drosophila* [1] and *Neurospora* [2] show that the circadian clock consists basically of a negative feedback loop. This makes the Goodwin model [3, 4] (Fig. 1) and related oscillators [5] useful models for kinetic studies of circadian rhythms. The Goodwin oscillator shows many properties also observed in circadian clocks, such as temperature compensation [6, 7], phase response curves for temperature steps and pulses, and entrainment by temperature cycles [8]. Therefore we investigated whether the Goodwin model is also able to describe the behavior of period length mutants of *Neurospora* and *Drosophila*, which are additionally defective in temperature compensation. We found, in agreement with experimental results in *Drosophila* [9] but contrary to the predictions by the Goldbeter model [5], that higher turnover of the clock protein leads to shorter period lengths and lower turnover to longer period lengths. Assuming such a difference in degradation rates for different *Neurospora* clock mutants with long and short period lenghts, it is possible to simulate the effect of temperature on the period length of these mutants. In regard to *Drosophila*, the Goodwin oscillator was extended by a temperature-dependent equilibrium between two forms of the PER proein (Y and Y′) corresponding to an oligomeric form of PER (Y) and a monomeric form (Y′) [10]. In PER^L increasing temperature shifts the equilibrium to the Y′ side, while Y and Y′ degrade more slowly than the corresponding forms of PER^+. For PER^S the Y–Y′ equilibrium is the same as for PER^+ (wild type), but PER^S is degraded faster than PER^+ [9].

In the calculations we used a three-dimensional version of the Goodwin model [8], in which the variables X, Y, and Z represent mRNA, clock protein, and a transcriptional repressor, respectively. In addition, an equilibrium between Y (oligomer) and Y′ (monomer) (Y⇄Y′) was added to simulate inter- and intramolecular interactions in the *Drosophila* clock protein complex (Fig. 1). The differential equations generated by the model were solved numerically with LSODE (Livermore solver for ordinary differential equations [11]). The rate constants in the model were chosen to make computational results comparable to experimental periods. However, this choice of parameters resulted in (slightly) damped oscillations. Because many circadian oscillations in constant conditions are damped, the stability of the oscillations appear to be of less importance. As shown previously [8], temperature compensation is in fact independent of the stability of the oscillations.

The large exponent in the inhibition term $k_1/(1+Z^9)$ (Fig. 1) is often criticized as biochemically unrealistic. However, we decided to keep the number of kinetic variables as low as possible. To obtain more realistic values of the exponent the number of species in the negative feedback loop must be increased [12]. This appears

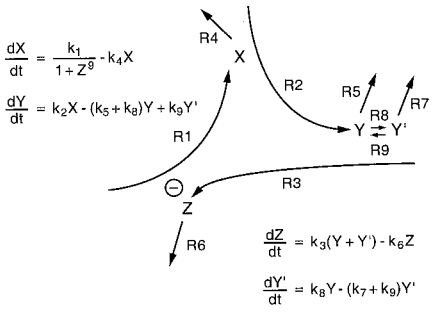

Fig. 1. The Goodwin model with rate equations used in the simulations. The (dimensionless) rate constant values k_i refer to reactions Ri. The negative sign in the loop indicates the repression of X (mRNA) by the transcriptional inhibitor Z. Y is the clock protein. Y′ is a particular conformational state of the clock protein used in the *Drosophila* calculations

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Table 1. Effect of loop rate constant values k_1, k_2, k_3 and turnover rate constant values k_4, k_5, k_6 on period length

k_1	Period[a]	k_2	Period[b]	k_3	Period[c]
0.1	23.3	0.1	23.1	0.1	23.1
1.0	23.4	1.0	23.4	1.0	23.4
10.0	23.6	10.0	23.6	10.0	23.6
100.0	23.6	100.0	24.0	100.0	24.0
1000.0	23.9	1000.0	24.6	1000.0	24.6
k_4	Period[d]	k_5	Period[e]	k_6	Period[f]
0.05	39.3	0.05	39.0	0.05	29.1
0.15	26.2	0.15	26.2	0.15	21.0
0.3	20.0	0.3	20.0	0.3	16.3
0.45	17.7	0.45	17.7	0.45	13.8
0.6	16.6	0.6	16.4	0.6	12.7

[a] $k_2=k_3=1.0$; $k_4=k_5=0.2$; $k_6=0.1$; $k_7=k_8=k_9=0.0$
[b] $k_1=k_3=1.0$; $k_4=k_5=0.2$; $k_6=0.1$; $k_7=k_8=k_9=0.0$
[c] $k_1=k_2=1.0$; $k_4=k_5=0.2$; $k_6=0.1$; $k_7=k_8=k_9=0.0$
[d] $k_1=k_2=k_3=1.0$; $k_5=0.2$; $k_6=0.1$; $k_7=k_8=k_9=0.0$
[e] $k_1=k_2=k_3=1.0$; $k_4=0.2$; $k_6=0.1$; $k_7=k_8=k_9=0.0$
[f] $k_1=k_2=k_3=1.0$; $k_4=k_5=0.2$; $k_7=k_8=k_9=0.0$

The oscillations reported here are slightly damped. The damping increases at the border values of the rate constants, but this has no effect on the reported temperature compensation (see [8] or on the conclusions derived in this paper

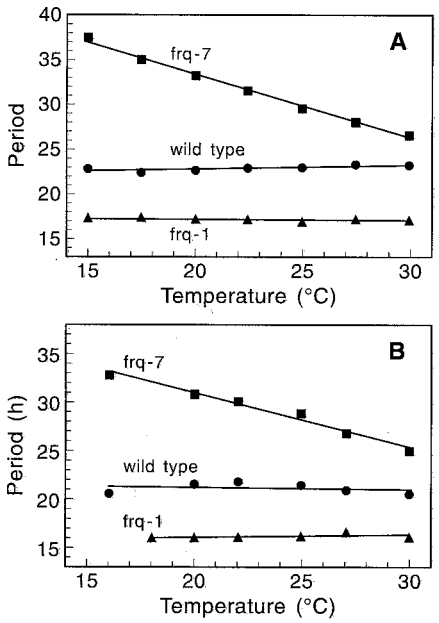

Fig. 2. A) Calculated temperature behavior of *Neurospora crassa frq* mutants. E_i is the activation energy of process Ri. The numerical parameters are: *wildtype:* $E_1=2.36\times10^4$ J/mol, $E_2=9.26\times10^4$ J/mol, $E_3=6.25\times10^4$ J/mol, $E_4=7.63\times10^2$ J/mol, $E_5=1.92\times10^2$ J/mol, $E_6=2.02\times10^2$ J/mol. At 25°C: $k_1=k_2=k_3=1.0$, $k_4=k_5=0.2$, $k_6=0.1$, $k_7=k_8=k_9=0.0$. *frq-1:* $k_5=0.5$ (25°C), $E_5=1.92\times10^2$ J/mol; other parameters are as for *wild type. frq-7:* $k_5=0.1$ (25°C), $E_5=5.0\times10^4$ J/mol. The other parameters are as for *wild type.* B) Experimental results replotted from the work of Gardner and Feldman [18]

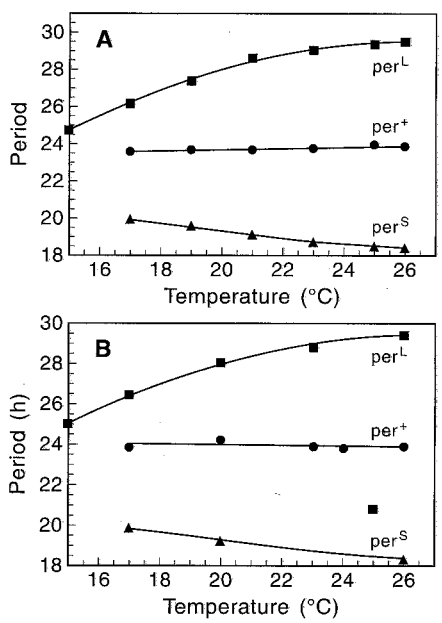

Fig. 3. A) Calculated temperature behavior of *Drosophila melanogaster per* mutants. E_i is the activation energy of process Ri. The numerical parameters are: *per*$^+$: $E_1=2.36\times10^4$ J/mol, $E_2=9.26\times10^4$ J/mol, $E_3=6.25\times10^4$ J/mol, $E_4=7.63\times10^2$ J/mol, $E_5=1.92\times10^2$ J/mol, $E_6=2.02\times10^2$ J/mol, $E_7=E_8=E_9=1.0\times10^2$ J/mol. At 25°C: $k_1=k_2=k_3=1.0$, $k_4=0.15$, $k_5=0.25$, $k_6=0.1$, $k_7=0.25$, $k_8=k_9=1.0$. *per*^{S}: $k_5=k_7=0.4$ (25°C), $E_5=5\times10^4$ J/mol, other parameters as for *per*$^+$. *per*^{L}: $k_5=0.3$, $k_7=0.15$ (25°C), $E_5=2.5\times10^4$ J/mol, $E_8=1.0\times10^5$ J/mol, other parameters as for *per*$^+$. B) Experimental results replotted from the work of Konopka et al. [25]

to be the case in view of recent results (see below).

A feature of the Goodwin model is that the rate constants of the anabolic loop reactions (R1–R2–R3) can be changed by several orders of magnitude without any significant effect on the period (Table 1). This remarkable "intrinsic homeostasis" – if also present in reality – may be of particular relevance for the mechanism: the period is not controlled by the transcription or translation rates but rather by the degradation reactions of the nucleic acid or protein species. Here we show that the temperature dependence of the period lengths of *Neurospora frq* and *Drosophila per* mutants can be explained by temperature effects on clock protein turnover. The influence of temperature is introduced by means of the Arrhenius equation:

$$k_i = A_i \exp(-E_i/RT)$$

where E_i is the activation energy and T is the temperature in K (for a more detailed discussion about the use of the Arrhenius equation see [8]).

It is well known that protein metabolism affects the period length of the circadian clock. Thus, continuous treatment with translational inhibitors lengthened the period in various organisms [13–16], a behavior which we wanted to test also in *Neurospora crassa.* Table 2 shows that continuous cycloheximide (CHX) treatments lengthen the conidiation rhythm with increasing CHX concentrations. The lengthening of the rhythm by about 3 h, however, is considerably smaller than the effect on the growth rate (~70% reduction). Most interestingly, the long period mutant of *Neurospora, frq*^{7}, is relatively insensitive to CHX treatments, i.e., the clock of *frq*^{7} continues to run relatively unimpeded even in the virtual absence of protein synthesis [17].

The dynamic properties of the Goodwin model can provide an explanation of these results: although protein synthesis inhibitors decrease the rate of processes R2, and R3, *the period is more affected by the degradation processes R4, R5 and/or R6.* Consequently the temperature effect on the period lengths of clock mutants in *Neurospora* and *Drosophila* can be simulated by changing the temperature effect on these turnover reactions.

Table 2. *Neurospora crassa bd* period length as a function of cycloheximide (CHX) concentration

CHX, μM[a]	Growth, cm/24 h	Period, h
0	3.6±0.2	20.0±0.5
0.3	1.7±0.2	21.7±0.5
0.5	1.1±0.2	23.4±0.5
1.0	No growth	

[a] Concentrations in race tube gels, consisting of 0.4% saccharose, 1.0% agar in 1×Vogel's salt [26, 30]

Figure 2 shows the calculated temperature effects on short-(frq^1), wild-type (frq^+), and long-period (frq^7) mutants of *Neurospora* in comparison to the experimental results [18, 19]. While in these calculations the period is considered to be controlled by the protein turnover reaction R5 (Fig. 1), it should be noted that a control of period length could equally well take place at the mRNA level by reaction R4 (compare the influence of k_4 and k_5 on period length, Table 1). In the latter case, short-period mutants should have shorter *frq* mRNA half-lives than long-period mutants (Table 1) [20]. At the protein level the candidate for the turnover control is the clock protein FRQ, which has been shown to contain PEST sequences [21] typical of proteins with higher turnover rates.

If the assumption is correct that mainly clock protein turnover is controlling the circadian period (Fig. 2), FRQ^7 should be more resistant to proteolytic degradation. A higher stability of FRQ^7 towards proteases would also explain the relative insensitivity of this mutant towards CHX [17], because CHX has been shown drastically to inhibit not only protein synthesis but also protease activity [22] and thus probably FRQ turnover. CHX should then lengthen the period as was observed experimentally in the *bd* mutant (Table 2). Despite the assumed higher stability of FRQ^7, experiments have indicated a greater temperature sensitivity of the frq^7 period length than that of other *frq* mutants. If the rate of clock protein degradation defines whether the period is long (low degradation rate) or short (high degradation rate), temperature sensitivity of the rate of clock protein degradation will result in a temperature sensitivity of the period. A possible explanation for this temperature sensitivity of FRQ^7 is that the PEST site [21] of FRQ which apparently represents a signal for rapid proteolysis [23] is exposed differently at different temperatures. This temperature-dependent exposure may be due to stronger conformational changes in FRQ^7 than in FRQ^+ and thus lead to an increase in FRQ turnover with increasing temperature. In *Drosophila* a temperature-dependent conformational change of PER has recently been found [10].

Compared to per^+ and per^S, the *Drosophila* per^L mutant showed increasing intramolecular interaction between the PAS and C domains of PER at increasing temperatures [10]. Huang et al. [10] and Price [24] have speculated that the increased PAS-C domain interaction may be a reason for the loss of temperature compensation in per^L. In fact, by adding a temperature-dependent interaction to the model (process $Y \rightleftarrows Y'$) the temperature behavior of per^L can easily be simulated. It should be noted, however, that in these calculations *Y as well as Y'* are allowed to form the transcriptional repressor (Z). If the repressor can be generated only by Y, agreement with experimental results becomes rather poor, i.e., the period increases exponentially with increasing temperature. Figure 3 shows the results of our calculations compared with experimental results [25]. While PER^S and PER^+ differ only marginally in their intramolecular binding [10, 24], the Goodwin model predicts different turnover rates for PER^+, PER^S, and PER^L, i.e., $PER^S > PER^+ > PER^L$.

In fact, for *Drosophila* there is experimental evidence that a higher degradation rate of PER is correlated with shorter period lenghts [9]. Recent results demonstrate that another protein, TIM, (for "timeless" [26, 27]) binds to PER and thus serves as an important regulatory factor of PER stability. This PER TIM complex appears to be necessary in the cytoplasma for proper PER phosphorylation and nuclear entry and in the nucleus for PER phosphorylation and eventual degradation [29]. Thus, transcriptional inhibition of *per* ist a rather complex process, providing several intermediate conformational states which may be incorporated in the model.

For *Neurospora* the Goodwin oscillator predicts an analogous control of the period by means of clock protein degradation, but interpretations are so far based only on studies with protein synthesis inhibitors (Table 2 [13–17]).

We thank Stuart Brody, Van Gooch, Christopher D. Thron, and Michael Vicker for critically reading the manuscript and providing valuable comments. P.R. thanks Stavanger College for financial support of a sabbatical stay at the University of Bremen.

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