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The peptide PIN changes the timing of transitory burst activation of timer-ATPase TIME in accordance with diapause development in eggs of the silkworm, *Bombyx mori*

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Abstract

TIME is an ATPase that measures a time interval by exhibiting transitory burst activation in eggs of the silkworm, *Bombyx mori* L. PIN is a peptide that regulates the time measurement of TIME. To address the mode of action of PIN, interactions between TIME and PIN were investigated. First, TIME was mixed with PIN for various periods (days) at 25 °C. The longer TIME was mixed with PIN, the later the transitory burst activation of TIME ATPase activity occurred, while no such delay occurred at 5 °C. Second, the capacity of PIN to bind with TIME was measured at the two temperatures by fluorescence polarization. The binding interaction was much tighter (nearly 1000 times) at 25 °C than that at 4 °C. Because the log EC_{50} (in nM) at 4 °C was about 7, PIN must dissociate from TIME at low temperatures at the physiological concentration of TIME in eggs. Thus, TIME appears to be restructured into a time-measuring conformation by PIN at the high temperatures of summer, whereas the TIME–PIN complex would dissociate at the low temperatures of winter. This dissociation acts as the preliminary cue for the ATPase activity burst of TIME, which in turn causes the completion of diapause development and initiates new developmental programs.
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1. Introduction

Although simple thermal quiescence of an insect ends when favorable conditions recur, diapause does not cease immediately in response to favorable environmental changes; in fact, warm conditions, such as summer temperatures, may maintain a state of diapause. The termination of diapause requires, or is at least accelerated by, exposure to cold for a minimum fixed duration (Kai and Nishi, 1976; Denlinger et al., 2001; Denlinger, 2002; Masaki, 2002). One of the most important factors in the termination of diapause appears to be its timing and synchronization with seasonal conditions. Despite their importance, the mechanisms underlying diapause termination by cold remain unclear. In previous studies, we examined the role of an esterase in terminating diapause in response to cold in eggs of the silkworm *Bombyx mori* L. (Kai and Nishi, 1976; Kai et al., 1981, 1984, 1987). Recently, the esterase was shown to be an ATPase that may measure the duration of cold, thus acting as a diapause-duration timer (Kai et al., 1995b, 1996, 2004; Tani et al., 2001; Kai, 2002). We referred to the enzyme as TIME after the initials of time-interval measuring enzyme (Tani et al., 2001), and have focused our attention on the time measurement of TIME in a direction to address the

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mechanism of diapause termination by cold temperature.

When TIME is purified from eggs and chilled at 5 °C in test tubes, a transitory burst of ATPase activity occurs within a definite period. The period required to activate the enzyme in vitro is equivalent to that observed in vivo and coincides with the chilling period required for diapause termination, shortly before completion of diapause development (Kai et al., 1995b; Ti et al., 2004). In *B. mori* eggs, diapause development is a strict stage that requires cold temperatures for the termination of diapause (Kai et al., 1995a; Ti et al., 2004). In addition, winter cold exerts its effect only on diapause development in the eggs (Ti et al., 2004). Therefore, the TIME ATPase activity shortly before the completion of diapause development is activated by cold temperatures. The mechanism by which cold activates TIME to measure the time-interval may help to explain in biochemical terms this insect's adaptation to its seasonal environments. In the present study, we investigate how cold temperatures initiate and control the length of diapause development (and ultimately the termination of diapause) by examining the interval-timer activation of TIME.

In a previous study, we suggested that the duration of diapause development can be controlled by the conformation of TIME (Ti et al., 2004), that is, the timer mechanism may be built into the TIME protein structure (Kai et al., 1995b). The conformation of TIME may be regulated by the peptide PIN, which coexists with TIME in *B. mori* eggs (Isobe et al., 1995; Kai et al., 1996, 1999; Tani et al., 2001, 2002). PIN binds with TIME, forming an equimolar complex (Tani et al., 2001), and at 5 °C PIN holds the timer, possibly by inhibiting the conformational change of TIME (Kai et al., 1996, 1999). The equilibrium constant K_D for the TIME-PIN interaction was relatively high, at about 460 nM at room temperature (Tani et al., 2001); the 10^{-7} M range of the constant suggests that an association–dissociation conversion is involved in the regulatory mechanism. Thus, the interactions between TIME and PIN may play a central role in the timer system that controls the duration of diapause development.

Although previous studies have shown that the interaction between TIME and PIN is important in the timer system, many questions remain to be answered. For instance, TIME, a single protein that affects the length of diapause development, is active at different times under different egg conditions. The chilling period required for completion of *B. mori* diapause depends upon the age of the eggs (review by Takami, 1969), and a study by Ti et al. (2004) showed that the duration of diapause development was longer when chilling was delayed after oviposition. The timing of TIME ATPase activity also changes with the length of time before egg chilling; in in vitro studies, the later that TIME was extracted from eggs after oviposition, the later the ATPase activity appeared in the test tubes (Ti et al., 2004). How does PIN control the timing of TIME's ATPase activity? One possible explanation is that PIN may not only hold the timer at 5 °C but may actively structure the TIME molecules into a time-measuring conformation at 25 °C during the period before chilling. No previous studies have shown such activity by a peptide. In the present study, therefore, we examine interactions between TIME and PIN at both 5 and 25 °C in a direction to address the mechanism of diapause in the synchronization with seasonal condition.

2. Materials and methods

2.1. Eggs and preparation of TIME for ATPase activity assay

Diapausing eggs of the C108 strain of *B. mori* were used. The eggs were prepared according to the method of Ti et al. (2004). Eggs laid within 1 h of each other were collected at 25 °C to obtain synchronous egg batches. Two days after oviposition, batches of the eggs (usually 5 g) were washed with cold acetone, and TIME and PIN (in the following Section 2.3.) were prepared from those eggs.

Unless otherwise noted, all preparation procedures for TIME and PIN were conducted in a cold room (4–5 °C) or in an ice-water bath. Protein concentrations were measured by a bicinchoninic acid assay (Pierce, Rockford, IL, USA) for both TIME and PIN preparation.

The TIME preparation procedures are described in detail in Kai et al. (1995b, 1999), Tani et al. (2001) and Ti et al. (2004). The procedures included the production of acetone powder of eggs, heat treatment at 85 °C, preparation with 80% saturated ammonium sulfate, and gel filtration through a Sephadex G-25 (Pharmacia Biotech AB, Uppsala, Sweden) column. Because the column used did not produce simple gel-filtration chromatography but rather affinity chromatography (Kai et al., 1995b), TIME was eluted later than the void volume fraction. The collected TIME fractions were cleared of contaminating PIN (Kai et al., 1996) by means of Centricon-10 centrifugation (Amicon, Lexington, KY, USA). PIN removal was accomplished by repeated dilution and filtration using HEPES buffer (25.0 mM HEPES, 12.5 mM Trizma base, 50.0 mM NaCl, 20.0 mM KCl, 1.0 mM EDTA, pH 6.8) according to the method of Kai et al. (1999). All ultrafiltration spins were run at 5 °C and less than 1000*g* (2500 rpm). The TIME thus obtained was then assayed for ATPase activity.

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