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# Distinctive Structural and Kinetic Properties of an Unusual Juvenile Hormone-Hydrolyzing Esterase

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**The insect juvenile hormone specific esterases (JHEs), related to acetylcholinesterases but exhibiting substrate specificity for juvenile hormone (JH), are essential enzymes for normal insect development, making them attractive targets for biorationally designed, environmentally safe pesticides. We examine here a new enzyme, JHER, related to, but yet structurally, biochemically, and kinetically distinct from, the classical JHE. Both classical JHE and baculovirus-expressed JHER hydrolyze JH show disproportionately higher catalytic rates at higher substrate concentrations (in contrast to substrate inhibition reported for acetylcholinesterase) and are similarly inhibited by an organophosphate. However, JHER, which possesses an unusual cysteine residue at +1 to the catalytic serine, is less sensitive to trifluoromethyl ketone transition state analogs designed around the structure of JH. We propose a model in which JHER is expressed just prior to metamorphosis for hydrolysis of a JH-like substrate with hydrophobic backbone, a proximal ester, and a terminal expoxide or related substitution.** 

The specialized insect juvenile hormone (JH) esterase is a pivotal regulator of hormonally-driven pathways of insect development (1, 2). Although most closely related to acetylcholinesterases, the JH esterases (JHE) are so specific for the substrate epoxymethylfarnesoate (juvenile hormone) that improper enzyme activity can result in derangement of normal metamorphic development (3, 4).

The central importance of this enzyme to normal insect development has generated much analysis of its catalytic properties and the architecture of its active site, with an objective of developing selective biorationally designed pesticides that inhibit its activity (5). The 'JH-specific' esterases identified and cloned to date all conserve residues hypothesized to constitute a ser-his-glu 'catalytic triad' (6–9). Recent homology-based modeling of a JH specific esterase built according to *Torpedo californica* acetylcholinesterase crystal structure has generated a single active site paradigm of the configuration of JH specific esterase, upon which design of selective JH esterase inhibitors may be premised (10). However, some studies have identified multiple catalytic forms of JH specific esterase (11–13).

We previously reported the developmental expression during metamorphosis of a JH specific esterase from *Trichoplusia ni* (14–17). We have also reported the expression during metamorphosis of a second esterase gene in *T. ni* that is more closely related to the 'classical' JH specific esterase than to any other esterase. Yet, it is distinctive in possessing an unusual cysteine residue immediately adjacent to the catalytic serine, instead of the conventional smaller alanine residue (8). This finding raised the question of whether there exists in this and perhaps other insects two related, but functionally different esterases expressed at metamorphosis, each with possible multiple catalytic sites or constants, each of which in turn may have a distinct role in the necessary catabolism of JH-like structures for proper insect development. We report here the production of this JH esterase-related (JHER) enzyme in a baculovirus system and the distinction of its structural, catalytic and biochemical properties from those of 'classical' JH specific esterase.

## MATERIALS AND METHODS

*Cells and viruses.* The *Spodoptera frugiperda* cell line (SF9) (18) was maintained in TC-100 medium (Sigma) supplemented with 10% fetal bovine serum free medium, SF 900II SFM (Gibco BRL) at 27°C. The wild-type AcNPV viral DNA was purchased from Invitrogen. The transfer vector, pVL1393 and the modified viral DNA (BaculoGold) for cotransfection were purchased from Pharmingen.

*Construction of recombinant viruses containing JHER gene.* An SpeI/NotI fragment containing the 1634 bp was subcloned into the

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corresponding JHER cDNAsites of pBluescript SK(+). The JHER cDNA was then excised from the latter construct as a BamHI/NotI fragment that was cloned into the corresponding sites of pVL1393 (Fig. 1). The constructed plasmid was transformed into *E. coli* HB101 cells and positive colonies confirmed by restriction analysis. The purified DNA of one positive clone, pVL1393-JHER1, and linearized AcNPV DNA, were used for cotransfection of SF9 cells with Lipofectin (Gibco BRL).

Homologous recombination between identical sequences of the transfer vector and viral DNA is expected to yield only recombinant viable viruses. After 5 days of cotransfection, the supernatant of the cotransfection plate was collected and recombinant viruses were cloned by end-point dilution. After 3 cycles of end-point dilution, a high titer stock of the recombinant viruses was obtained by amplification.

*Analysis of the recombinant protein synthesized in SF9 cells.* SF9 cells were infected with the recombinant or wild-type AcNPVs at a multiplicity of 5 PFU/cell in a spinner flask with $4.5 \times 10^7$ cell/100 ml medium and incubated at 27°C. The suspension-cultured media were collected daily postinfection, counted for cell number, and tested for JH esterase activity. The culture supernatant of the cells on the 6th day of infection with the recombinant virus was concentrated with a Centriprep-10 (Amicon) (10 kDa exclusion).

The unconcentrated or concentrated culture supernatant or cell extracts were subjected to native-PAGE with CBB staining, or $\alpha$-naphthyl acetate ($\alpha$NA) esterase activity staining, of the gel. At high substrate concentrations JH specific esterase activity against $\alpha$NA can be detected in native gels (19). Recombinant JHER (rJHER) partially purified with Centriprep was subjected to isoelectric focusing (IEF). After electrofocusing the gel was sliced for measurement of pH and JHE activity. Each slice from two adjacent lanes was placed individually into 500 ml of phosphate buffer, pH 7.4 for assay of JHE activity or into distilled water for pH-determination. After elution overnight at 4°C, each eluted solution was measured respectively for pH or JHE activity.

*JHE assay.* The JHE assay was performed according to the partition method of Hammock and Sparks (20). Unlabeled JH I was obtained from Sigma and unlabelled JH III from Sigma, while C10-[$^3$H] labeled JH III was purchased from NEN. The unlabelled hormone in each assay was JH I, except where noted otherwise. Each incubation volume contained either diluted blood of day 4, last stadium larvae of the lepidopteran insect *Trichoplusia ni* (there is no JHER transcript on that day, by the Northern blot analysis (8)) or partially purified supernatant of the recombinant virus-infected cell culture. After a 10 min pre-incubation, the JH substrate was added in 1 $\mu$l ethanol. The inhibitors used (Fig. 1) were TFT (1,1,1-trifluorotetradeca-2-one) made according to the method of Hammock and Wing (21); EPPAT (*O*-ethyl *S*-phenyl phosphoramidothiolate; (22) a gift from Shell Chem. Co.); OTFP (3-octylthio-1,1,1-trifluoropropan-2 one) and OTFP-sulfone were the kind gift of Dr. M. Roe.

*Amino acid sequence analysis.* After purification by Centriprep and native-PAGE, rJHER was applied on SDS–PAGE and blotted on a PVDF membrane using 0.01% M CAPS, pH 11 with 10% methanol as a blotting buffer. After transfer, the membrane was stained with 0.1% CBB in 50% methanol and destained with 50% methanol and 10% acetic acid. The rJHER band was excised, and automated Edman degradation for amino acid sequence was performed by the Yale University Keck Foundation.

## RESULTS

### *Characterization of rJHER*

After the inoculation of the recombinant viruses aliquots of cell extracts and culture supernatant for each group possessed high JHE activity after the second day, until the number of recombinant virus-infected cells decreased at the end of the incubation on day 7. There was little or no activity detected in either the cell supernatants or cell homogenates of wild-type infected or uninfected cells. Staining of rJHER loaded onto nondenaturing PAGE, using $\alpha$NA as substrate, detected a band unique to the recombinant-virus infected cells, as compared to mock-infected or uninfected cells. This recombinant esterase was preparatively subjected to native PAGE, excised from the native gel, and fractionated by SDS–PAGE. The recombinant protein migrated under SDS–PAGE conditions with an $M_r$ of ca. 66 kDa (Fig. 2A), as predicted by its encoded sequence (8). Wide range isoelectric focusing of rJHER resolved two closely focusing electrophoretic forms, with pI 3.9 and 4.4 (Fig. 2B), which is more acidic than that observed for most classical JH-specific esterases produced from insect cells (23).

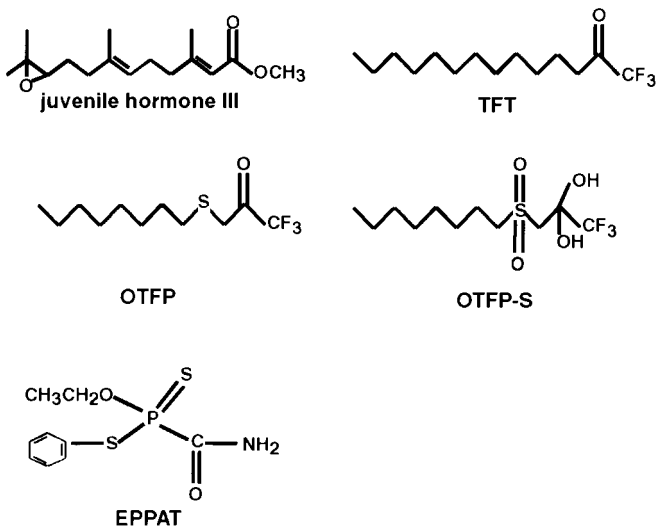


**FIG. 1.** Panel structure of the natural juvenile hormone III substrate and of inhibitors used in this study.

Previous analytical treatment of the open reading frame of JHER did not identity a definitive signal peptide splice site, leading to uncertainty as to whether JHER is a secreted protein (8). In the present study, we detected a significant amount of rJHER activity in the cell culture media, the N-terminus sequence. The sequence obtained, M-L-W-P-N, begins at position 21 of the encoded protein sequence (8). Thus, the occurrence of extracellular rJHER appears not to be artifactual due to cell lysis, but rather due to processing of a 20-amino-acid signal peptide as rJHER is secreted from the insect cells. We have found in preliminary studies that the genomic coding sequence of the signal peptide of both JH specific esterase and JHER is interrupted in a similar position by a large intron.

### *Kinetic Properties Toward JH Substrate*

The partially purified recombinant enzyme was kinetically analyzed with respective to the potential nat-

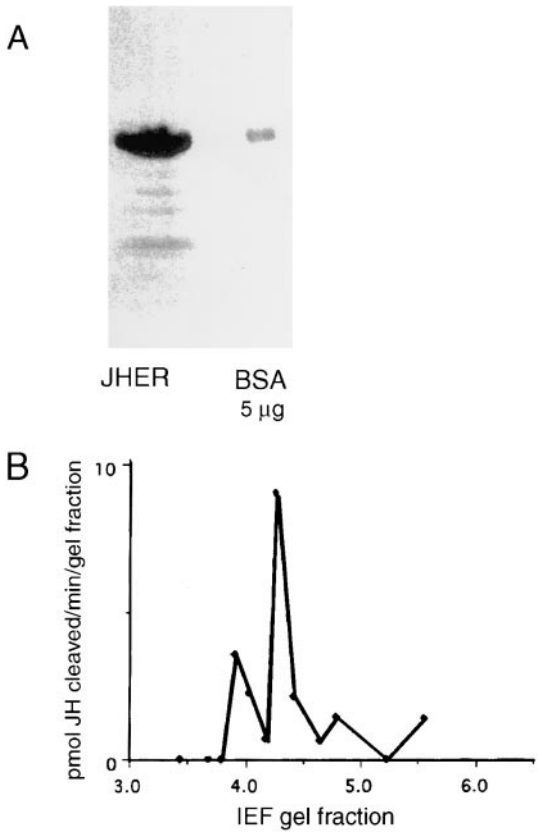


**FIG. 2.** (A) SDS–PAGE of recombinant JHER expressed in insect SF9 cells. (B) Isoelectric focusing analysis of recombinant JHER, focusing as two primary electrophoretic forms.

ural substrate, juvenile hormone. Two different $K_m$s were observed for this substrate, one of $2 \times 10^{-7}$ and the other $2 \times 10^{-6}$ M (Fig. 3B). In comparison, JH specific esterase activity in diluted insect serum showed a $K_m$ of $8 \times 10^{-8}$ M and a second, higher $K_m$ of and $3 \times 10^{-7}$ M (Fig. 3A), similar to that previously reported for the JH specific esterase of this species (11). Thus, the rJHER exhibits distinct catalytic activity toward juvenile hormone, but with a slightly higher $K_m$ than JH specific esterase exhibits toward that substrate.

The differences between the catalytic sites of rJHER and JH specific esterase were further explored with several inhibitors. Trifluoromethyl ketones have been a central area of research in the development of specific inhibitors of JH esterase, insofar as they mimic the transition state of JH during hydrolysis by JH specific esterase (24). We assessed three such transition state analogs, all of which possess both the terminal trifluoromethyl ketone or similar structure as well as distal conformation resembling juvenile hormone (TFT, OTFP, and OTFPS, Fig. 1). The JH specific esterase was very sensitive to all three, with $I_{50}$s in the low nanomolar range for each inhibitor (Figs. 3D–3F). In comparison, rJHER was at least three orders of magnitude less sensitive to TFT and OTFPS, and 1–2 orders of magnitude less sensitive to OTFP (Figs. 3D–3F). In contrast, the more general organophosphate inhibitor, *O*-ethyl, *S*-phenyl, phosphoramidothioate

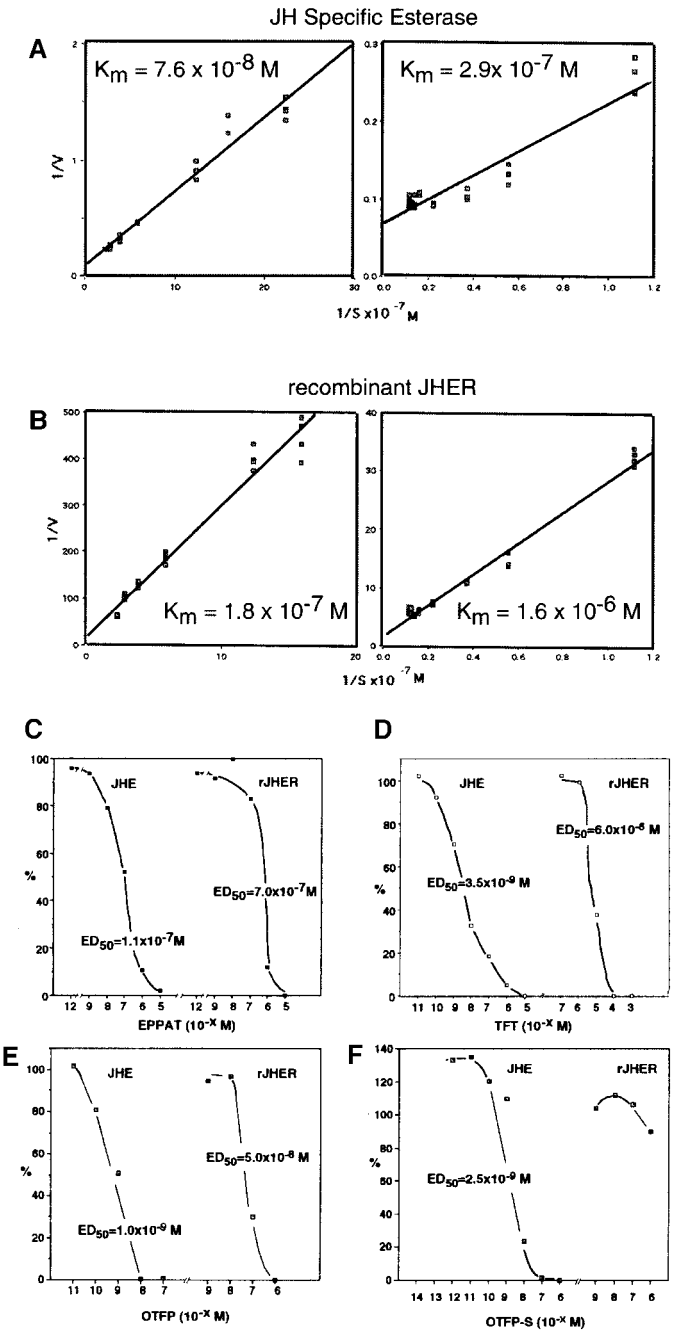


**FIG. 3.** Kinetic analysis of JH specific esterases and JHER activity. Lineweaver-Burke plots of kinetic activity toward JH by JH specific esterase (A) and by recombinant JHER (B). In both panels, the figure on the left is the analysis obtained at lower substrate concentrations and that on the right for higher substrate concentrations. For each enzyme, disproportionately greater velocity was observed at higher substrate concentrations, leading to the kinetic appearance of two different $K_m$s for each enzyme. C–F show the inhibition kinetics of the respective four inhibitors toward JH specific esterase (JHE) and recombinant JHER (rJHER).

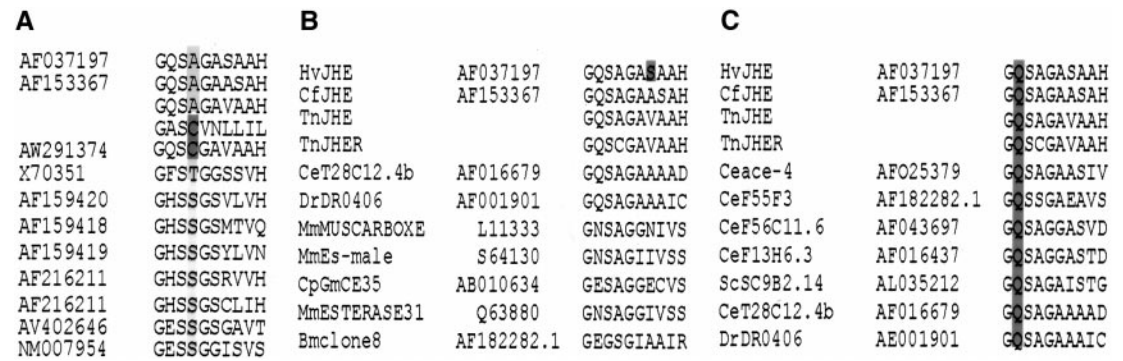


**FIG. 4.** Comparison of features around the catalytic serine motif in various esterases. For each panel, the left column is the name of the respective enzyme preceded by the first letter of the genus and species names of the respective organism. The middle column is the GenBank database Accession Number, and the right column is the respective catalytic serine motif with particular residues of interest indicated. A indicates variation in the position +1 to the catalytic serine, B concerns a color-indicated, proposed 'second serine' and C concerns the residue at position −1 to the catalytic serine.

(EPPAT, Fig. 1), yielded the more similar $I_{50}$s of 10 nM and 70 nM for JHE and rJHER, respectively (Fig. 3C).

### *Structural Features of JHER*

We inspected a number of structural features of the substrate binding sites of the JH specific esterases and JHER that may reflect the varying substrate/inhibitor specificities that each exhibited here. The proposed serine-glutamic acid-histidine residues of the catalytic triad. Also, each of the three JH specific esterases cloned thus far possesses a glutamine residue immediately N-terminal to the classical catalytic serine, and JHER also conserves that residue (Fig. 4). However, a marked difference in the immediate vicinity of the catalytic site is that while the three classical JH specific esterases, and many other esterases, possess an alanine immediately C-terminal to the classical catalytic serine, JHER possess a cysteine. Such a differential large side chain residue in the immediate vicinity of the catalytic serine likely influences or contributes to the local architecture that is conformed to bind the natural substrate(s). There is precedent for the presence of large side chain residues at that position, such as threonine and serine (Fig. 4), and even a cysteine has been reported from at least one other enzyme at that position (Fig. 4).

A fourth catalytic residue, a 'second serine' has been proposed to complete the formation of a 'catalytic tetrad' in esterases (25). This proposed second serine participating in catalysis is in position +4 relative to the classical catalytic serine in the JH specific esterase from *H. virescens* (6), is at position +5 in a second JH specific esterase (7) and in a third JH specific esterase there is no second serine at all (8) (Fig. 4). This second serine residues is also missing from a number of other reported esterases (Fig. 4). Thus, it is difficult at this time to postulate a role for this serine in the higher substrate specificity of JH specific esterases for JH as compared to the JHER enzyme, although, when present, that second serine may participate in the catalytic process *per se.*

More distally to the catalytic serine, homology modeling of the *H. virescens* JH specific esterase identified a number of residues (F338, P295, F127, and I449) as lining the region of the active site gorge where the terpenoid backbone of the natural JH substrate is putatively docked (13). JHER conserves the hydrophobicity of each these residues as F368, P325, I157, and I449, respectively. In addition, a very distal Y77 was been proposed to orient the terminal epoxide group of the natural JH substrate of JH specific esterases (13), and that residues is preserved as Y109 of JHER. However, a difference in structure of respective natural substrates for the two enzymes may be reflected in that another hydrophobic binding site residue modeled to be near the substrate terpenoid backbone in JH specific esterase (V75), is instead a glutamic acid residue in JHER (E107).

## DISCUSSION

Analyses of classical 'JH specific esterases' have shown that these esterases are very specific for juvenile hormones or closely related structures, and exhibit $K_m$ values toward juvenile hormones in the range of 10 to 100 nM (10, 11, 26–29). A homology model for the JH specific esterase from *H. virescens* has been built on the crystal structure for *Torpedo* acetylcholinesterase. These crystal structures and homology modeling revealed that the catalytic site of JH specific esterase is at the deep end of a hydrophobic 'gorge' that is formed by the juxaposition of two protein domains (13). Vertebrate acetylcholinesterase and butyrlcholinesterase exhibit complex kinetic properties on account of the effect of substrate binding to a peripheral binding site at the surface of the protein. In acetylcholinesterase,

binding of natural substrate to the peripheral site results in a 'substrate inhibition' effect, while binding of substrate to the peripheral site of butyrlcholinesterase causes a 'substrate activation' that yields increased substrate turnover. Both homology modeling and kinetic analysis of *Drosophila* acetylcholinesterase suggest it also exhibits the cooperative double activation-inhibition pattern arising from a peripheral site (30, 31).

Still unresolved is whether the insect JH specific esterases also possess a peripheral substrate binding site. Some JH specific esterases have exhibited a disproportionately higher hydrolysis of substrate at higher substrate concentrations. These kinetic data have previously been fit to a model of two catalytic activities with two $K_m$s, about an order of magnitude apart (10–12). Using affinity-purified JH esterase and additional analysis with transition state inhibitors, Abdel *et al.* (24) concluded that this kinetic behavior of the enzyme was due to two catalytic sites on the enzyme with different $K_m$ values about an order of magnitude apart and not due to substrate activation. However, the subsequent cloning of several JH esterase cDNAs and the homology modeling of a JH specific esterase did not reveal two catalytic sites on the enzyme (6–8, 13). In the present study, we have also detected a similar complex kinetic behavior of a disproportionately higher substrate hydrolysis at higher substrate concentrations, just as occurs with some classical JH specific esterases. Should the kinetic behavior of either the JH esterase related proteins, or the classical JH esterases, involve a peripheral substrate binding site, it will offer new opportunity for the design of selective inhibitors as environmentally safe alternatives in insect control, as has the peripheral site of acetylcholinesterase attracted therapeutic interest (32, 33).

Our data from several inhibitors with different mechanisms of inhibition provide functional evidence for potentially exploitable differences in architecture of the substrate binding sites of JH specific esterases vs. JHER-type enzymes. The trifluoremethyl ketone transition state analog TMTFA binds to the acetylcholinesterase active site in the same directional orientation as the natural substrate (34). Because the backbone structure of the transition state analogs used here in large part mimics juvenile hormone, it is likely that the trifluoromethyl ketones used in the present study also orient in JH specific esterase in the same directional orientation as the natural JH substrate. This inference is supported by that the transition state analogs used here have such high affinity for the active site of JH specific esterases (35, 36). Indeed, we determined here that the affinity of the transition state analogs TFT, OTFP and OTFP-S toward JH specific esterase of *Trichoplusia ni* is uniformly very high, $I_{50}$ of $1–3 \times 10^{-9}$ M, which is an affinity similar to that exhibited by these inhibitors toward a number of other insect JH specific esterases (19, 37, 38). However, the binding properties of the active site for JHER were distinguished from classical JH specific esterase by several parameters. First, the transition-state analog inhibitors had markedly less affinity for JHER than for JHE. Second, there were large differences affinities among these inhibitors for JHER itself (more than two orders of magnitude difference). In particular, the active site of JHER appears particularly sensitive to the nature of the moiety that is beta to the carbonyl carbon of the transition state analogs.

In a second line of functional evidence concerning the JHER active site, biochemical and crystal structure studies have shown that organophosphate inhibitors form essentially irreversible covalent complexes with the active serine of vertebrate acetylcholinesterases (39), in contrast to the slow, tight binding, but reversible inhibition of the transition state analogs. Stopped flow analysis has confirmed that organophosphate inhibitors bind to the same catalytic site of insect acetylcholinesterase to which the natural substrate binds (40). EPPAT, an organophosphate inhibitor of cholinesterase is also able to inhibit JH specific esterases (36). In contrast to the results with the transition state analogs, the organophosphate inhibitor EPPAT exhibited much less discrimination between the active sites of the JH specific esterase vs JHER.

Collectively, we hypothesize from these data that a natural substrate of JHER possesses hydrophobic backbone with a proximal ester and possibly a terminal epoxide or related substitution. Endocrinologically, the mechanisms through which JH controls Arthropod development are still being vigorously pursued, including identification of new JH structures (41), of nuclear receptors that can bind various JHs (42), and of specialized routes of targeting JH esterases for degradation (43). JH specific esterases are expressed at two stages of metamorphic development of insects, at one stage under neurohormonal control just before commitment to metamorphosis, and in the second stage under direct induction by JH just after metamorphic commitment (44). In contrast, JHER is expressed only at the same time as the precommitment peak in JH specific esterase activity. It has been demonstrated that immediately after metamorphic commitment, the JH producing glands, the corpora allata, change in the nature of the JH they produce, such as increased production of JH acid and decreased production of the natural JH ester (45). It may be that a change in the structure of the JH produced by the corpora allata, or a change in the anabolic conversion of JH/JH acid, immediately after metamorphic commitment is accompanied by a loss of expression of JHER at that time.

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