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Transition State Analogs as Ligands for Affinity Purification of Juvenile Hormone Esterase

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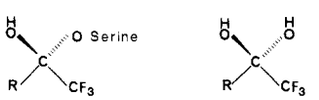
Insect juvenile hormones are metabolized in numerous species of caterpillars by low abundance, highly specific esterases. Because of their role in regulating and possibly disrupting juvenile hormone titer and thus insect metamorphosis, they are of interest to developmental biologists as well as scientists interested in selective insect control. However, the enzymes have defied attempts to purify and characterize them. Juvenile hormone esterase activity can be inhibited by a variety of 3-substituted 1,1,1-trifluoropropanone sulfides. These apparent transition state analogs were used as ligands and eluting agents to purify juvenile hormone esterase from four insect species from 500-fold to over 1000-fold in high yield. After elution from the affinity column, the enzymes were radiolabeled with paraoxon and analyzed by electrophoresis, and the results demonstrate a high degree of purity. Transition state analogs may be useful for the affinity purification of other enzymes.

IN HOLOMETABOLOUS INSECTS A PRECIPITOUS decline in the titer of juvenile hormone (see structure in Fig. 1) initiates a series of developmental changes resulting in metamorphosis to a pupal and then to an adult stage. In the lepidopterous species studied, it appears that this reduction in hormone titer is due to a decreased rate of biosynthesis as well as a dramatic increase in highly active and specific enzymes that hydrolyze the methyl ester of juvenile hormone (*1*). There has been interest in purification of these regulatory enzymes for a number of years as probes for the further understanding of insect metamorphosis and as a target for molecular approaches to the control of destructive insect pests. Since the maximum concentration of juvenile hormone esterase in the blood is in the low micromolar range (*2*), and since insect blood is difficult to obtain in large amounts, the isolated enzyme was never obtained in sufficient amounts for characterization. No attempts have been made to purify the enzyme from the key pests in the *Heliothis* complex or from strains of the silk moth *Bombyx mori*, which is widely used by developmental biologists.

The development of powerful and selective inhibitors for juvenile hormone esterase has proved useful for investigating both the biochemistry and the biological role of the enzyme. For instance, selective inhibition of juvenile hormone esterase results in maintenance of juvenile hormone titers and the production of giant larvae (*3*). Among these inhibitors have been trifluoromethyl ketones (*4, 5*), some of which exhibit slow tight-binding kinetics and react almost stoichiometrically with the target enzyme (*2, 6*).

The inhibitory mechanism proposed for these compounds is based on Pauling's idea (*7*) that a compound which even vaguely mimics a transition state should bind tightly to the enzyme. As increasingly powerful enzyme inhibitors such as transition state mimics are developed, observations of time-dependent, yet reversible, inhibition kinetics are becoming more common (*8*). Both esters and ketones are similar in terms of many physicochemical properties, including reactivity toward nucleophiles. Substitution of some of the hydrogen atoms α to the carbonyl with fluorine enhances the electrophile reactivity of carbonyls with a putative serine at the catalytic site of esterases. This substitution should not jeopardize the affinity for the enzyme through steric hindrance, since both the hydrogen and fluorine atoms have very similar steric properties (molar refractivities of 1.03 and 0.92, respectively) (*9*). Thus, it is likely that these trifluoroketones act as "transition state analogs" with juvenile hormone esterase.

Figure 1A shows the inhibition of crude juvenile hormone esterase by one such transition state analog, OTFP (3-octylthio-1,1,1-trifluoropropan-2-one). This compound, which has a molar refractivity very similar to that of juvenile hormone, is the most potent inhibitor yet reported for the enzyme from the cabbage looper, *Trichoplusia ni*, with a dissociation constant of $1.2 \times 10^{-10} M$ (*2*). The less selective phosphorylating agent paraoxon also is a powerful inhibitor of juvenile hormone esterase. The irreversible inhibition caused by paraoxon (Fig. 1B) supports the hypothesis that juvenile hormone esterase is a serine esterase and that the trifluoropropanones bind to the enzyme by a structure similar to the hydrated state of the trifluoropropanone in aqueous solution.



The smooth inhibition curves in most species support the hypothesis that similar catalytic sites are responsible for most of the juvenile hormone hydrolysis in the hemolymph; however, the broken line in the case of *Manduca sexta* indicates that at least two distinct catalytic sites are involved (Fig. 1).

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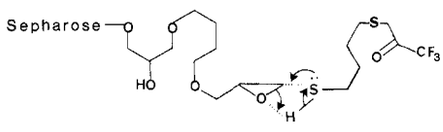
Table 1. Affinity purification of juvenile hormone esterase (JHE).

Source of data*	Hemolymph JHE				Affinity-purified JHE			Recovery (%)	Purification
	Volume (ml)	Protein (mg/ml)	Specific activity	Total activity	Total protein (mg)	Specific activity	Total activity		
				Manduca sexta					
Affinity column	19.0	20.1	0.73	279	0.390	702	273	98	962
Batching technique	23.5	17.3	1.75	713	0.422	1020	432	65	585
				Bombyx mori					
Batching technique	15.0	59.3	0.73	649	0.32	814	260	50	1120
				Heliothis virescens					
Affinity column	16.0	65.1	1.79	1860	0.48	2010	965	52	1120
				Heliothis zea					
Affinity column	20.0	31.7	5.21	3310	0.46	4001	1840	56	768

*Data were obtained from an affinity column or from a batching technique for gel loading in which the gel was swirled with diluted plasma and then collected by centrifugation and washed as described. The batching technique was used originally with *B. mori* since columns always clogged when used with plasma from this species. However, the batching procedure was used for both *B. mori* and *M. sexta* to indicate that binding to the affinity gel follows a time-dependent pattern which, in addition to very slow elution, indicates a slow tight-binding mechanism. Specific activity is expressed as nanomoles of substrate converted per minute per milligram of protein, whereas total activity is expressed as nanomoles per minute.

Since trifluoropropanones are such potent inhibitors of juvenile hormone esterase, it was thought that they might be effective ligands for the affinity purification of the enzyme. To this end a variety of ligands were made, with the most successful resulting from the reaction of bromotrifluoroacetone with butane dithiol to give 3-[(4′-mercapto)butylthio]-1,1,1-trifluoropropan-2-one. This ligand was reacted, in turn, with epoxy-activated Sepharose (*10*).



When 20 ml of insect blood were passed through 50 μl of this gel there was no detectable protein loss in the effluent, yet over 99 percent of the juvenile hormone esterase activity disappeared. Columns of similar hydrophobicity, structure, or both, yet lacking the trifluoroketone, failed to retain the esterase. Since the column effluent did not inhibit juvenile hormone esterase activity, one could conclude that the enzyme was bound to the affinity gel.

Elution of active enzyme proved difficult. Since the enzyme is remarkably stable to a variety of agents, extreme gradients of *p*H, ionic strength, substrate, and ionic and nonionic surfactants were used in unsuccessful attempts to remove the enzyme. Even when exceptionally low column loadings were used, very small amounts of enzyme were recovered when several of the most powerful trifluoropropanone inhibitors were used as eluting agents.

The answer to this dilemma came from recalling that these compounds showed slow, tight-binding inhibition kinetics (*2, 6*), a commonly observed characteristic of the inhibition caused by transition state analogs and other powerful inhibitors (*11*). Thus, after the affinity gel was loaded with enzymatically active insect blood, either by a column or batch procedure, it was extensively washed. Then, 1 ml of a $1 \times 10^{-3}M$ solution of OTFP was added to 100 μl of loaded gel in a small column and periodically shaken for several hours at 4°C to allow the enzyme to approach an equilibrium between inhibitor molecules in solution and on the column. The solution was eluted and the process repeated until no further protein was recovered from the column. The combined eluted fractions were dialyzed extensively to remove the OTFP and recover enzyme activity monitored as described in Fig. 1.

As shown in Table 1, this process has now been applied to juvenile hormone esterase activity from a variety of species of economic and biological interest. The very high yields obtained make it possible to carry out biochemical experiments on the purified en-

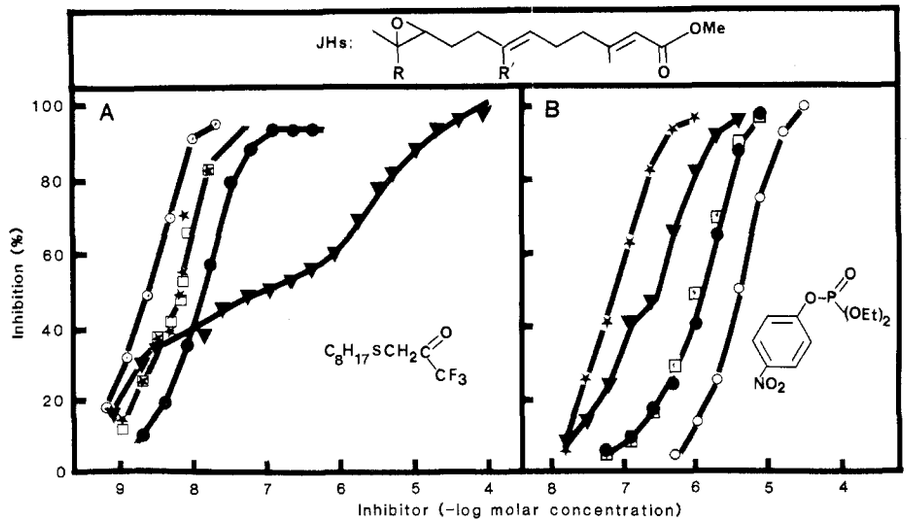


Fig. 1. Relation between log molar concentration of 3-octylthio-1,1,1-trifluoropropan-2-one (OTFP) (A) or *O,O*-diethyl *O*-(*p*-nitrophenyl)phosphate (paraoxon) (B) and percent inhibition of juvenile hormone (JH) esterase activity in the hemolymph of *M. sexta* (▼), *B. mori* (●), *H. virescens* (□), *H. zea* (★), and *T. ni* (○). For routine analysis JH III at a final concentration of $5 \times 10^{-6}M$ was used in the presence of $1 \times 10^{-8}M$ [10-^{3}H]JH III. To 100 μl of the enzyme solution in phosphate buffer, *p*H 7.4, 1 μl of ethanol or ethanol-inhibitor solution was added and incubated for 10 minutes at 30°C before the addition of the substrate. The residual juvenile hormone esterase activity was assayed by the partition assay (*3*) for an incubation time (5 to 15 minutes) that yielded linear rates of hydrolysis. Insets include the structures of the major known insect juvenile hormones (JH I, R = R′ = C_2H_5; JH II, R = C_2H_5, R′ = CH_3; JH III, R = R′ = CH_3), OTFP (A), and paraoxon (B).

zyme. Of biological importance, these high yields indicate that we are purifying the major rather than a minor but stable component of the juvenile hormone esterase activity in crude blood. The active, purified enzymes were compared to the crude juvenile hormone esterase activities in blood and their kinetic specificities were found to be the same with the three juvenile hormone substrates and a variety of inhibitors of different compound classes. The yields also indicate that most of the catalytic activity of the enzyme was retained during purification. In fact the specific activities reported here are orders of magnitude higher than those reported from classical purifications (*12*). The purification factors and recoveries reported in Table 1 indicate that this is among the most efficient affinity purification procedures reported (*10, 13*).

Juvenile hormone esterase is such a minor protein that it is not visible in hemolymph as a discrete band by electrophoresis or isoelectric focusing. When analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), the affinity-purified enzyme appears as a single major band for *Manduca sexta* and *Heliothis virescens* and two bands for *Bombyx mori* and *Heliothis zea* (Fig. 2). The apparent molecular weights are distinct but in the neighborhood of 60K for the four species. In contrast, juvenile hormone esterases purified from three separate strains of *B. mori* appeared identical when subjected to SDS-PAGE or analytical isoelectric focusing. When the purified enzymes were exposed to [^{3}H]ethyl paraoxon and examined by fluorography, discrete bands corresponding exactly to those observed with Coomassie blue were seen on SDS-PAGE with each species. Analysis of gel slices by liquid scintillation counting revealed one major peak in each case (Fig. 2). When active and inhibited purified enzymes were further examined by analytical isoelectric focusing, protein bands, catalytic activity, and the radiolabeled enzyme showed identical *p*I's.

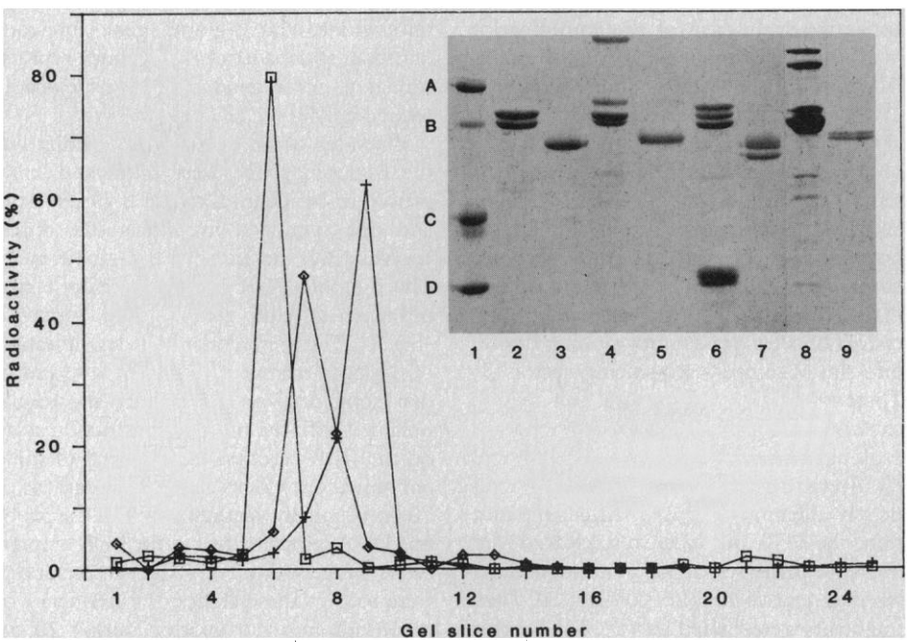


Fig. 2. Radioactivity in slices of gel following SDS-PAGE of juvenile hormone esterase affinity-purified from *M. sexta* (□), *B. mori* (+), and *H. virescens* (◇). Inset shows an SDS-PAGE gel with Coomassie blue staining of standard proteins (lane 1: phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; and carbonic anhydrase, 31,000) and crude and purified enzymes from *M. sexta* (lanes 2 and 3), *H. virescens* (lanes 4 and 5), *B. mori* (lanes 6 and 7), and *H. zea* (lanes 8 and 9). Note that the maximum radioactivity was recovered from the band corresponding to the purified enzyme detected with Coomassie blue. Exposure of the gel to x-ray film also indicated a single band in the case of *M. sexta* and *H. virescens* and two bands with *B. mori*, and in each case the x-ray band corresponded to the protein bands shown in the inset above. For these studies, 1 m*M* paraoxon containing 1 μCi of ^{3}H was incubated with 10 μg of purified enzyme or crude hemolymph for 1 hour at 30°C. The excess paraoxon was removed with 20% (v/v) of a charcoal solution (5% Norite 5G with 0.5% Dextran T70, 0.0015*M* EDTA, and 0.003*M* sodium azide in 0.01*M* tris buffer at *p*H 7.3). The samples were then analyzed by SDS-PAGE using a 12.5% separating gel and 4% stacking gel (typical data shown above) or by analytical isoelectric focusing (*p*H 3 to 11 and 4 to 6.5). No radioactivity was detected on the gels when a similar amount of crude protein was treated with [^{3}H]paraoxon as above. Analytical isoelectric focusing yielded a single major band for *H. virescens* and three extremely minor bands, three clear bands for *B. mori*, and two for *M. sexta*. In each case all of the protein bands that were detected with Coomassie blue also were labeled with paraoxon and had catalytic activity. Antibody to the juvenile hormone esterase purified from *M. sexta* detected a single band on SDS-PAGE of enzymatically active crude blood and pure enzyme, but no cross-reactivity was observed with juvenile hormone esterase from the other species studied when analyzed by Western blot.

This affinity method will make it possible to investigate the regulation and role of juvenile hormone esterase at both the biochemical and the molecular level. For instance, it has been used to demonstrate that the juvenile hormone esterase activity that appears in the hemolymph of pre- and postwandering larval stages of *M. sexta* results from proteins that are indistinguishable by SDS-PAGE. However, this affinity procedure has many other promising applications. When the substituents on trifluoropropanones are varied, one obtains potent inhibitors of a variety of biologically important esterases. These include acetylcholinesterases from several sources, human brain neurotoxic esterase, and the carboxylesterases degrade pesticides such as the pyrethroids and malathion and drugs such as clofibrate in rodent and primate liver. Preliminary studies showed differential sensitivity of the above enzymes to the trifluoropropanone sulfides (*4, 5, 14*). Thus, it is likely that affinity purification procedures based on trifluoropropanone sulfides can be applied directly to these toxicologically and pharmacologically important enzymes. Since transition state analogs are now known for all classes of enzymes (*15*), it is likely that transition state or metastable analogs will be more widely used as ligands or eluting agents for affinity chromatography (*13, 16*).

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Direct Cloning and Sequence Analysis of Enzymatically Amplified Genomic Sequences

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A method is described for directly cloning enzymatically amplified segments of genomic DNA into an M13 vector for sequence analysis. A 110–base pair fragment of the human β-globin gene and a 242-base pair fragment of the human leukocyte antigen DQα locus were amplified by the polymerase chain reaction method, a procedure based on repeated cycles of denaturation, primer annealing, and extension by DNA polymerase I. Oligonucleotide primers with restriction endonuclease sites added to their 5′ ends were used to facilitate the cloning of the amplified DNA. The analysis of cloned products allowed the quantitative evaluation of the amplification method's specificity and fidelity. Given the low frequency of sequence errors observed, this approach promises to be a rapid method for obtaining reliable genomic sequences from nanogram amounts of DNA.

UNDERSTANDING THE MOLECULAR basis of genetic disease or of complex genetic polymorphisms, such as those in the human leukocyte antigen (HLA) region, requires detailed nucleotide sequence information from a variety of individuals to localize relevant variations. Currently, the analysis of each allelic variant requires a substantial effort in library construction, screening, mapping, subcloning, and sequencing. We report here a method for the enzymatic amplification of specific segments of genomic DNA and their direct cloning into M13 vectors for sequence analysis, using modifications of the polymerase chain reaction (PCR) (*1, 2*). This in vitro amplification procedure is based on repeated cycles of denaturation, oligonucleotide primer annealing, and primer extension by the Klenow fragment of DNA polymerase, and results in an exponential increase in copies of the region flanked by the primers. This method greatly reduces the number of cloned DNA fragments to be screened, circumvents the need for full genomic libraries, and may allow cloning from nanogram quantities of genomic DNA. In addition, the cloning and sequencing of PCR-amplified DNA is a powerful analytical tool for the study of the specificity and fidelity of this newly developed technique.

To develop this technique for genomic sequence determination and to analyze the individual products of PCR amplification, we chose the oligonucleotide primers and probes previously described for the diagnosis of sickle cell anemia (*2*). These primers amplify a 110-bp segment of the human β-hemoglobin gene containing the Hb-S mutation (Fig. 1). They were modified near their 5′ ends (Fig. 1) to produce convenient restriction sites (linkers) for cloning directly into the M13mp10 sequencing vector (*3*). These modifications did not affect the efficiency of PCR amplification of the specific β-globin segment (Fig. 2B), even though the overall pattern of PCR-amplified products is different (Fig. 2A). After amplification, the PCR products were cleaved with the appropriate restriction enzymes and dialyzed to remove inhibitors of ligation. These fragments were ligated into the M13 vector, transformed into the JM103 host, and plated out, and the resulting plaques were screened by hybridization with a labeled oligonucleotide probe to detect the β-globin clones. The plaques were also screened with the labeled PCR oligonucleotide primers to identify all of the clones containing amplified DNA. Individual clones were then sequenced directly by using the dideoxy primer-extension method (*4*).

The incorporation of different restriction sites at the termini of the amplified product allows digestion of the vector with two restriction enzymes, thereby reducing the background of blue plaques from vectors without inserts to less than 11% (Table 1). More than 80% of the clones contained DNA inserts with the PCR primer sequences but only about 1% of the clones hybridized to the internal β-globin probe. These nonglobin fragments presumably represent amplifications of other segments of the genome. This observation is consistent with the gel and Southern blot analysis of the PCR-amplified DNA from a β-globin deletion mutant and a normal cell line (Fig. 2). The similarity of the observed gel profiles reveals that most of the amplified genomic DNA fragments arise from nonglobin templates. Sequence analysis of two of these nontarget clones showed that the segments between the PCR primer sequences were unrelated to the β-globin gene and contained an abundance of dinucleotide repeats, similar to some genomic intergenic spacer sequences (*5*).

When ten of the clones that hybridized to the β-globin probe were sequenced, nine proved to be identical to the β-globin gene and one contained five nucleotide differences but was identical to the δ-globin gene. The β-globin PCR primers each have two mismatches with the δ-globin sequence (Fig. 1). The preferential PCR amplification of β-globin relative to δ-globin observed in our clonal analysis agrees with the results obtained with the oligomer restriction assay on the PCR reaction (*2, 6*). Each of these ten sequenced clones contains a segment of 70 bp originally synthesized from the genomic DNA template during the PCR amplification process. Since no sequence alterations were seen in these clones, the frequency of nucleotide misincorporation during 20 cycles of PCR amplification is less than 1 in 700.

To analyze the molecular basis of genetic polymorphism and disease susceptibility in the HLA class II loci, this approach has been extended to the amplification and cloning of a 242-bp fragment from the second exon of the HLA DQα locus, which exhibits localized allelic variability. In this case the primers, whose sequence is based on conserved regions of this exon, contain 5′-terminal restriction sites with no homology to the DQα sequence (Fig. 1). The specificity of amplification achieved with these primers is greater than that achieved with the β-globin

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