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Aedes aegypti juvenile hormone acid methyl transferase, the ultimate enzyme in the biosynthetic pathway of juvenile hormone III, exhibits substrate control

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ABSTRACT

We report on the cloning, sequencing, characterization, 3D modeling and docking of *Aedes aegypti* juvenile hormone acid methyl transferase (*Aea*JHAMT), the enzyme that converts juvenile hormone acid (JHA) into juvenile hormone (JH). Purified recombinant *Aea*JHAMT was extensively characterized for enzymatic activity and the Michaelis Menten kinetic parameters K_m, V_{max}, k_{cat} (turn over number) and k_{cat}/K_m (catalytic efficiency) using JHA and its analogues as substrates. *Aea*JHAMT methylates JHA III 5-fold faster than farnesoic acid (FA). Significant differences in lower methyl transferase (MT) activities towards the cis/trans/trans, cis/trans/cis and the trans/cis/cis isomers of JHA I (1.32, 4.71 and 156-fold, respectively) indicate that substrate chirality is important for proper alignment at the catalytic cavity and for efficient methyl transfer by S-adenosyl methionine (SAM). Our 3D model shows a potential binding site below the main catalytic cavity for JHA analogues causing conformational change and steric hindrance in the transfer of the methyl group to JHA III. These, *in silico*, observations were corroborated by, *in vitro*, studies showing that several JHA analogues are potent inhibitors of *Aea*JHAMT. *In vitro*, and *in vivo* studies using [^{3}H-methyl]SAM show that the enzyme is present and active throughout the adult life stage of *A. aegypti*. Tissue specific expressions of the JHAMT gene of *A. aegypti* (*jmtA*) transcript during the life cycle of *A. aegypti* show that *Aea*JHAMT is a constitutive enzyme and *jmtA* transcript is expressed in the corpora allata (CA), and the ovary before and after the blood meal. These results indicate that JH III can be synthesized from JHA III by the mosquito ovary, suggesting that ovarian JH III may play an important physiological role in ovarian development and reproduction. Incubating *Aea*JHAMT with highly pure synthetic substrates indicates that JHA III is the enzyme's preferred substrate, suggesting that *Aea*JHAMT is the ultimate enzyme in the biosynthetic pathway of JH III.

1. Introduction

Aedes aegypti, the yellow fever mosquito, is an anautogenous mosquito transmitting dengue, chikungunya and yellow fever viruses in the tropics and subtropics, causing economical and public health hardship (Womack, 1993) highlighting the need for an efficient and sustainable control strategy. Juvenile hormone (JH) III is found in most insect orders, including mosquitoes. Its biosynthetic pathway starts after isopentenyl diphosphate (IPP) is produced by the mevalonate pathway, a common pathway used by bacteria, archaea, fungi, plants and animals to synthesize terpenoids (Goodman and Granger, 2005; Klowden, 2002; McMurry and Begley, 2005). Following phosphorylation and

Abbreviations: ACN, acetonitrile; *Aea*JHAMT, *Aedes aegypti* juvenile hormone acid methyl transferase; βME, β-mercaptoethanol; CA, corpora allata; *Cb*SAMT, salicylic acid carboxyl methyltransferase from *Clarkia breweri*; EDTA, ethylenediaminetetraacetic acid; EST, expressed sequence tags; FA, farnesoic acid; FA-o-MT, farnesoic acid o-methyl transferase; FPP, farnesyl diphosphate; HCA, hydrophobic cluster analysis; HF, homo farnesoate; IPP, isopentenyl diphosphate; IPTG, isopropyl β-D-1-thiogalactopyranoside; JH, juvenile hormone; JHA, juvenile hormone acid; *jmtA*, JHAMT gene of *A. aegypti*; MF, methyl farnesoate; MT, methyl transferase; PMSF, phenylmethanesulphonylfluoride; RBS, rapid biphasic separation; RT, reverse transcription; SAM, S-adenosyl methionine; SAM-MT, S-adenosyl methionine-dependent methyl transferase; TAE, tris-acetate-ethylenediaminetetraacetic acid; TIGR, the institute of genomic research.

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decarboxylation, IPP is converted into farnesyl diphosphate (FPP) and at this point the biosynthetic pathway of JH is insect specific and represents an important interdiction point for insect control. FPP is dephosphorylated forming farnesol, which is dehydrogenated and converted into the aldehyde form, farnesal. Further reduction of farnesal by dehydrogenation forms FA which can be converted into JH III by two different pathways: FA can be first epoxidized into JHA III and then methylated into JH III by a SAM-dependent methyltransferase (SAM-MT), or FA can be first converted by a SAM-MT into methyl farnesoate (MF) and then epoxidized into JH III (Goodman and Granger, 2005). Because JH III plays an important role in many physiological events in mosquitoes, its synthesis has been extensively studied (Borovsky et al., 1992, 1985; Li et al., 2003a; Readio et al., 1988; Shapiro et al., 1986). *In vivo* studies show that after female adult eclosion JH III titer increases, reaching a maximum of 22 fmol/h/female at 6 days, whereas *in vitro* studies show that JH III titer after adult eclosion first drops and then increases to 12 fmol/h/CA at 4–6 days after emergence. JH III biosynthesis sharply increases immediately after the blood meal and then starts to decline 4–10 h later, reaching a minimum at 24 h. The synthesis increases again at that time to prepare the ovaries and fat body for a second gonadotropic cycle (Borovsky et al., 1992). Because of the importance of JH III in different physiological aspects of insects, such as larval development (Wigglesworth, 1970) and vitellogenesis (Borovsky, 1984; Gwadz and Spielman, 1973), the JH III titer in the hemolymph is closely regulated. Several groups have reported on the characterization of JHAMT orthologs from *A. aegypti* (Mayoral et al., 2009), *Samia cynthia ricini* (Sheng et al., 2008), *Bombyx mori* (Shinoda and Itoyama, 2003), *Drosophila melanogaster* (Niwa et al., 2008), *Schistocerca gregaria* (Marchal et al., 2011) and *Apis mellifera ligustica* (Li et al., 2013). Borovsky and coworkers sequenced a full length transcript of *A. aegypti* JHAMT (*Aea*JHAMT) in 2006 (GenBank accession number DQ409061) and the sequence was re-confirmed by Mayoral et al. (2009).

Lack of extensive kinetic studies of JHAMT by several groups that initially characterized the enzyme in mosquitoes, lepidopterans and *Drosophila* (Mayoral et al., 2009; Shinoda and Itoyama, 2003; Niwa et al., 2008) prompted us to reinvestigate and extensively characterize *Aea*JHAMT, using 3D modeling and in depth kinetics studies, a sensitive radio assay, synthetic JHA analogues and its isomers. We also studied *jmtA* and its transcript during different developmental stages, showing that the CA and the ovaries synthesize JH III from JHA III. Our results suggest that *Aea*JHAMT is the ultimate enzyme in the biosynthetic pathway of JH III with a possibility that MF is synthesized by an alternative pathway using FA o-methyltransferase (FA-o-MT) (GenBank accession number XM_001658212).

2. Materials and methods

2.1. Chemicals

Homo FA, JH I (cis/trans/cis), JH I (trans/cis/cis), JH I (cis/trans/trans) and JH I bisepoxide were provided by Professor K. Sláma (Czech Republic, Prague). JH III bisepoxide and FA were provided by Professor G. Prestwich (University of Utah). JH III and SAM were purchased from Sigma (St. Louis, MO) and [^{3}H]JH III and [methyl-^{3}H]SAM were purchased from Perkin-Elmer (Waltham, MA). All substrates used were 90–100% pure as shown by C_{18} RP-HPLC.

2.2. Experimental insects

A. aegypti larvae were reared at 27 °C with a light:dark (16:8 h) cycle on brewer's yeast and lactalbumin (1:1). Newly emerged adults were fed sucrose (10% solution) or chicken blood. Female mosquitoes were maintained on water 24 h prior to blood feeding and were used at different intervals after adult emergence.

2.3. RNA extraction and purification

Total RNA was extracted from mosquito tissues with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

2.4. JmtA cDNA cloning and sequencing

A short sequence of *jmtA* (458 nt) was discovered in the *A. aegypti* expressed sequence tag (EST) libraries of the institute of genomic research (TIGR) by blasting the library with the *D. melanogaster* JHAMT sequence. *JmtA* specific primers DB 932 and DB 933 were synthesized (Table 1 and Fig. 1A). An RT-PCR reaction (20 μL) containing 4 μL 25 mM $MgCl_2$, 2 μL 10 × PCR buffer (Applied Biosystems, Foster City, CA), 6 μL sterile distilled water, 4 μL dNTP (10 mM each of dATP, dTTP, dCTP, and dGTP), 1 μL RNase inhibitor (20 U), 1 μL MMLV reverse transcriptase (50 U) was prepared containing 1 μL reverse primer (DB 933) (15 μM) and *A. aegypti* total RNA (1 μg). Reverse transcription (RT) was performed in a thermal cycler (Applied Biosystems) at 24 °C for 10 min, followed by 42 °C for 60 min, 52 °C for 30 min, 99 °C for 5 min, and 5 °C for 5 min. After RT, 3 μL 10 × Buffer, 25.5 μL sterile distilled water, 2.5 U AmpliTaq DNA polymerase (Applied Biosystems) and 15 μM of the forward primer DB 932 were added to the reaction mixture. PCR was carried out as follows: denaturation for 3 min at 95 °C (1 cycle), annealing for 4 min at 48 °C, extension for 40 min at 60 °C (1 cycle each), denaturation at 95 °C for 30 s, annealing for 30 s at 48 °C and extension for 2 min at 60 °C (40 cycles) with a final extension for 15 min at 60 °C. Following PCR, the dsDNA was separated by gel electrophoresis on 2% agarose gel in Tris–acetate–EDTA (TAE) buffer (pH 7.8) containing ethidium bromide at 100 v for 60 min. A DNA band (447 bp), visualized under UV light, was cut from the gel, eluted with QIAquick gel extraction kit (Qiagen, Germantown, MD) and cloned into TOPOpCR2.1 according to manufacturer instructions (Invitrogen, Carlsbad, CA). INVαF' *E. coli* cells were transformed, plasmids were purified with a QIAprep Spin Miniprep Kit (Qiagen), sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed at the University of Florida DNA sequencing core (http://langsat.biotech.ufl.edu/). The sequencing shows that 447 bp of the dsDNA obtained by RT-PCR from *A. aegypti* is identical with the major part of the EST-sequence released by TIGR. For rapid amplification of the 3′ cDNA end, a reverse primer (DB 265) with a dT_{17} adapter was synthesized (Table 1 and Fig. 1A) and used to reverse transcribe *A. aegypti* RNA which was then amplified in the presence of DB 932. After RT-PCR, a band

Table 1
PCR primers for cloning *jmtA* and Northern blot analyses.

Oligo name	Sequence (5′–3′)	*tm* (°C)
DB932	GCGTTTTCCAACATTTATAATCTT	49
DB933	CTTCACTGCATAAACCACCACAAGTAG	58
DB265	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTT	58
DB972	ATGAACAAACCTAATCTTTATCACCGA	54
DB954	GGATAGCTGATCATAAATGTCGAA	52
DB1015	AAAAAAG′GATCCTATGAACAAACCTAATCTTTATCACCGA	61
DB985	AAAAAAGC′GGCCGCCTACTTCACTGCATAAACCAC	66
DB1087	GAACATCCGGTGTTGTTGACGGAA	57
DB1088	ACGATTTCGCTTTCGGCGGTGGTA	59

Underlined: restriction sites for *Bam*HI (DB1015) and *Not*I (DB985).