NADP$^+$-dependent farnesol dehydrogenase, a *corpora allata* enzyme involved in juvenile hormone synthesis

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The synthesis of juvenile hormone (JH) is an attractive target for control of insect pests and vectors of disease, but the minute size of the corpora allata (CA), the glands that synthesize JH, has made it difficult to identify important biosynthetic enzymes by classical biochemical approaches. Here, we report identification and characterization of an insect farnesol dehydrogenase (*Aa*SDR-1) that oxidizes farnesol into farnesal, a precursor of JH, in the CA. *Aa*SDR-1 was isolated as an EST in a library of the corpora allata-corpora cardiaca of the mosquito *Aedes aegypti*. The 245-amino acid protein presents the typical short-chain dehydrogenase (SDR) Rossmann-fold motif for nucleotide binding. This feature, together with other conserved sequence motifs, place *Aa*SDR-1 into the "classical" NADP$^+$-dependent cP2 SDR subfamily. The gene is part of a group of highly conserved paralogs that cluster together in the mosquito genome; similar clusters of orthologs were found in other insect species. *Aa*SDR-1 acts as a homodimer and efficiently oxidizes C_{10} to C_{15} isoprenoid and aliphatic alcohols, showing the highest affinity for the conversion of farnesol into farnesal. Farnesol dehydrogenase activity was not detected in the CA of newly emerged mosquitoes but significant activity was detected 24 h later. Real time PCR experiments revealed that *Aa*SDR-1 mRNA levels were very low in the inactive CA of the newly emerged female, but increased >30-fold 24 h later during the peak of JH synthesis. These results suggest that oxidation of farnesol might be a rate-limiting step in JH III synthesis in adult mosquitoes.

mosquito | *Aedes* | farnesal | insect

Juvenile hormone III (JH) is the key hormone regulating metamorphosis and previtellogenic ovarian development in mosquitoes (1). JH is synthesized and secreted from the corpora allata (CA), a pair of endocrine glands with neural connections to the brain (2). The biosynthetic pathway of JHs is divided conventionally into two steps, the early steps and the late steps (3). The early steps of JH III biosynthesis follow the mevalonate pathway, with the formation of five-carbon (5C) isoprenoid units from acetate via mevalonic acid, with the sequential head to tail condensation of three 5C units to form farnesyl pyrophosphate (FPP) (4). In the late steps, FPP is hydrolyzed by a pyrophosphatase to farnesol (5), then oxidized successively to farnesal and farnesoic acid (FA) by an alcohol dehydrogenase and an aldehyde dehydrogenase, respectively (3). The last two steps diverge depending on the insect order. In Lepidoptera, a C-10,11 epoxidation by a P450 monooxygenase converts the FA to the epoxy acid (JH acid or JHA), that is subsequently methylated by an *O*-(SAM) dependent methyltransferase (JHAMT) to form the methyl ester. In Orthoptera and Dictyoptera epoxidation follows methylation (3, 6) and this is also the case in mosquitoes (2, 7). The conservation of the mevalonate pathway between vertebrates and insects has made possible the molecular cloning of the enzymes involved in the early steps of JH biosynthesis (8), but this option is not available for the later, JH-specific steps. Using molecular approaches, the last two enzymes of the pathway, the P450 epoxidase and JHAMT were recently characterized (9, 10).

The two short-chain dehydrogenases involved in the conversion of farnesol to farnesal and farnesal to FA have not been identified yet in insects. The first study of farnesol oxidation was performed using CA homogenates of the adult female sphinx moth, *Manduca sexta*, reporting that farnesol was sequentially converted to farnesal and farnesoic acid by farnesol and farnesal dehydrogenases, and that only farnesoic acid formation was significantly enhanced by the addition of nicotinamide adenine nucleotide (NAD$^+$) (11). Additional studies using CA homogenates of *M. sexta* larvae suggested that oxidation of farnesol was catalyzed by a metal- or flavin-alcohol oxidase rather than a dehydrogenase (12, 13). Here, we molecularly and functionally characterize a NADP$^+$-dependent farnesol-dehydrogenase (*Aa*SDR-1) that is expressed in the CA of an insect and transforms farnesol into farnesal. In addition, we describe the developmental stage-dependent expression of this gene in correlation to JH synthesis in the CA of the mosquito *Aedes aegypti*. These studies suggest that *Aa*SDR-1 is a rate-limiting enzyme in JH synthesis during reproductive development in mosquitoes.

APPLIED BIOLOGICAL SCIENCES

Results

Farnesol-Dehydrogenase Sequence from an *A. aegypti* CA EST Collection. Sequencing of an *A. aegypti* CA + CC (corpora cardiaca) cDNA library revealed one EST encoding a full length ORF for a 245-amino acid short chain dehydrogenase that could be responsible for the oxidation of farnesol into farnesal (14). In addition, an EST encoding a segment of an ortholog of this gene was isolated from a CA + CC cDNA library of the cockroach *Diploptera punctata* (GenBank acc: DV017939) (14). Screening the *A. aegypti* genome using this EST revealed an almost identical nonannotated sequence (*Aa*SDR-1) and 4 additional sequences with a high degree of similarity (72-83%) (*Aa*SDR-2 to *Aa*SDR-5) (Fig. 1). *Aa*SDR-1 is clustered in the same contig with two of the closest paralogs (*Aa*SDR-2 and *Aa*SDR-4). Analysis of the five *Aa*SDRs protein sequences showed the presence of a typical short-chain dehydrogenase fold composed of a central twisted parallel β-sheet consisting of seven β-strands, which are flanked by three α-helices on each side (Fig. S1). The five *Aa*SDRs have several conserved sequence motifs that place them in the "Classical" SDR family and the subfamily cP2, for example the pattern of three glycine residues that is distinctive of the nucleotide-binding region (TGxxxGhG), as well as motifs that have structural roles or are part of the active site (15) (Fig. 1). SDR orthologs were found in other species of insects and a phylogram of the phylogenetic relationship of these sequences was generated (Fig. 2). The closest sequences corresponded to those of other mosquito species. There are two clusters of orthologs in *Culex pipiens*, one containing six paralogs and one containing four paralogs, these clusters clearly originated by a duplication-inversion

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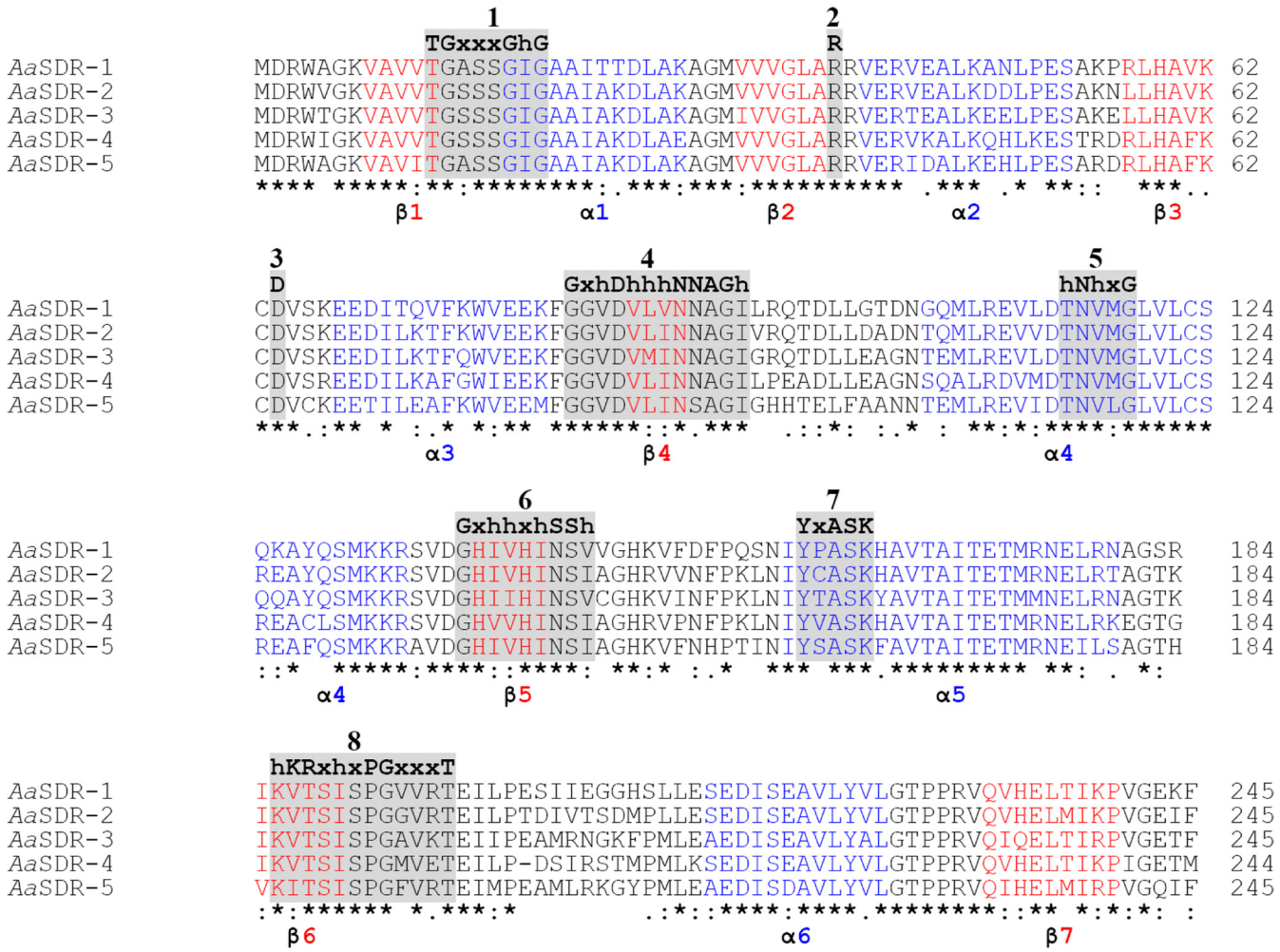

Fig. 1. Multiple alignments of the *Aa*SDRs amino acid sequences. α-helices and β-sheets are colored blue and red respectively. Conserved sequence motifs are highlighted by shaded boxes and numbers. Suggested functions for the motifs as described by Persson et al. (15) are: 1. Structural role in coenzyme binding region, 2. Subfamily cP2 key residue, 3. Adenine ring binding of coenzyme, and 4. Structural role in stabilizing central β-sheet; 5, 6, and 7 are parts of the active site, and 8 also has a structural role. In the motifs, ''h'' denotes a hydrophobic residue and ''x'' any residue.

event. Members of these two clusters have between 67 and 72% similarities with *Aa*SDR-1. There are six orthologs in *Anopheles gambiae* that have between 61–67% similarity with *Aa*SDR-1 and five of them cluster together in the chromosome region 2L. *Drosophila melanogaster* and *Tribolium castaneum* (Coleoptera) also have similar clusters (Fig. 2). Three ortholog sequences were found on *Acyrthosiphon pisum* (Hemiptera) and single orthologs were found in *Pediculus humanus* (Anoplura), *Apis mellifera* and *Nasonia vitripennis* (Hymenoptera). All of these insect SDRs also belong to the cP2 subfamily. Screening the *Bombyx mori* (Lepidoptera) genome using the *Aa*SDR-1 sequence revealed a single ortholog sequence with 40% similarity that has conserved sequence motifs that place it in the "Classical" SDR family and the subfamily cD1e (15). An additional *Aa*SDR cP2 (*Aa*SDR-6, 14% similarity with *Aa*SDR-1) expressed in the CA of *A. aegypti* adult female was included in the analysis as an outgroup.

Functional Characterization of *Aa*SDR-1. Recombinant *Aa*SDR-1 (r*Aa*SDR-1) was overexpressed and purified to homogeneity by one-step cobalt affinity chromatography with a yield of approximately 12 mg pure recombinant protein per liter of bacterial culture (Fig. 3*A*). The recombinant protein has a calculated molecular mass of ≈29 kDa. The native state of the recombinant protein was estimated using gel-filtration chromatography to be approximately 60 kDa, therefore the enzyme is a homodimer. *Farnesol* dehydrogenase activity of the purified protein was confirmed using a nitro blue tetrazolium (NBT)-based assay (Fig. 3*A*).

Purified r*Aa*SDR-1 oxidized farnesol (FOH) to farnesal in the presence of $NADP^+$ (Fig. 3*B*) with a specific activity of 9.7 ± 0.4 mol per min/mol enzyme for (*E*,*E*)-FOH and 9.0 ± 0.4 mol per min/mol enzyme for (*Z*,*Z*)-FOH. $NADP^+$ was absolutely required for the activity, and NAD^+ or FAD would not substitute. The highest enzymatic activities were detected for (*E*,*E*)-FOH, (*Z*,*Z*)-FOH, geraniol, nerol, and 2-decanol (Table 1). The enzyme also oxidized geranylgeraniol, citronellol, and octanol with low efficiency. No activity was detected for butanol, ethanol, or glycerol, or for farnesal or farnesoic acid. Unequivocal identification of oxidation products was achieved by accurate mass measurements using Direct Analysis in Real Time (DART)-Time-of-Flight (TOF) mass spectrometry (Fig. S2). The K_m values for (*E*,*E*)-FOH and (*Z*,*Z*)-FOH were approximately 90 μM (Table 1). Enzyme activity increased with increasing buffer alkalinity and optimum pH was between 10 and 11 (Fig. S3).

Tissue-Specific and Developmental Expression of *Aa*SDR-1 mRNA. Real time PCR was used to analyze the transcript tissue specificity. In adult females the highest levels of *Aa*SDR-1 mRNA were detected in the midgut and brain (Fig. 4); relatively high levels were

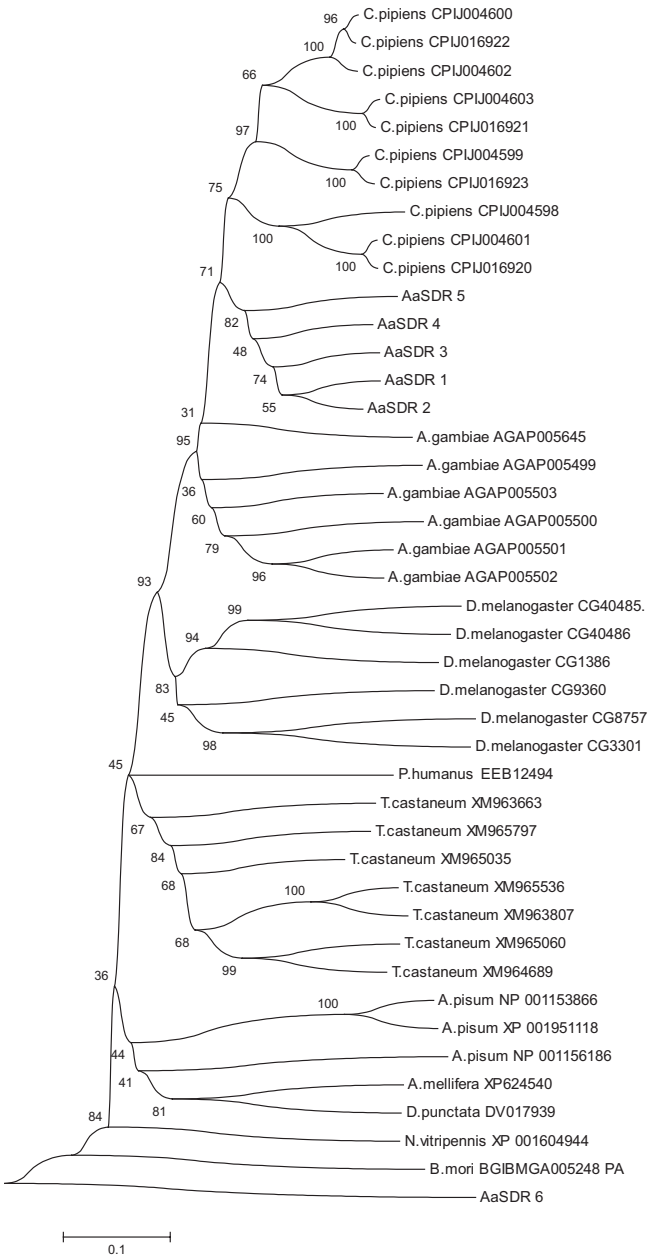

Fig. 2. Phylogenetic analysis of the SDRs of different species of insects based on the primary amino acid sequences. Sequences are labeled with the species names and accession numbers. All sequences are cP2 classic SDRs except for *B. mori*, which is a member of the cD1e classic SDR subfamily. Accession numbers for *Aa*SDRs: *Aa*SDR-1: GQ344797, *Aa*SDR-2: GQ344798, *Aa*SDR-3: AAEL007669, *Aa*SDR-4: GQ344799, *Aa*SDR-5: AAEL001461, and *Aa*SDR-6: AAEL002901.

also present in the abdominal and thoracic ganglia. Considerable levels were detected in CA, Malpighian tubules, and fat body. The mRNA levels in the ovaries were very low. In adult males substantial levels were detected in the testis (Fig. 4 *Inset*).

We could not detect *Aa*SDR-1 transcripts in pupae. When *Aa*SDR-1 transcript levels were analyzed in CA of sugar-fed females, the highest levels were observed on day 1 after adult eclosion (Fig. 5), at the time of maximum biosynthetic activity of the CA. Messenger levels significantly decreased 24 h after blood feeding, when JH synthesis is low, to increase again 48 h after a blood meal, just before the reactivation of JH synthesis 3 days after blood feeding (Fig. 5).

Farnesol Dehydrogenase Activity in Isolated CA. The activity of farnesol dehydrogenase in mosquito CA was determined using an in vitro colorimetric assay. Glands dissected from females 24 h after adult eclosion were strongly stained in the presence of farnesol, while CA dissected from newly emerged females had only a weak staining (Fig. 6). Staining of midguts and Malpighian tubules is described in the *SI Text* (Fig. S4).

Discussion

***Aa*SDR-1 Is a Classical cP2 SDR Expressed in the CA of Insects.** One EST was found in a CA library of *A. aegypti* encoding *Aa*SDR-1 and its ortholog was also present in a CA library of the cockroach *D. punctata* (14). Short-chain dehydrogenases/reductases constitute a large family of NAD(P)(H)-dependent oxidoreductases, sharing sequence motifs and displaying similar mechanisms (16). SDR enzymes play critical roles in lipid, amino acid, carbohydrate, cofactor, hormone, and xenobiotic metabolism. Sequence similarities are low, and the most conserved feature is an α/β folding pattern with a central β sheet flanked by two to three α-helices from each side, thus forming a classical Rossmann-fold motif for nucleotide binding (16). The common mechanism of action is an underlying hydride and proton transfer involving nicotinamide and a tyrosine residue active site, whereas substrate specificity is determined by a variable C-terminal segment (16).

A search of the genome of *A. aegypti* revealed 80 proteins containing SDR domains; among them were *Aa*SDR-1 and four additional paralogs with a high degree of sequence similarity (72-83%). Two of these genes (*Aa*SDR-4 and -5) were not expressed in the CA of the adult mosquito; of the additional three (*Aa*SDR-1, -2 and -3), only *Aa*SDR-1 was significantly expressed in the CA at the peak of JH synthesis, suggesting that this gene encodes the SDR responsible for the oxidation of farnesol to farnesal.

All of the critical conserved sequence motifs that characterize the "Classical" SDR family members are very well conserved in *Aa*SDR-1, as well as the four additional paralogs (*Aa*SDR-2 to *Aa*SDR-5). In addition, the residues that characterize them as members of the cP2 $NADP^+$-dependent SDRs are also conserved (15). It is remarkable that similar clusters of cP2 SDRs were also found in other insects, and the paralogs within each cluster always had higher sequence similarity with each other when compared with ortholog SDRs from other insect species. This implies that these SDR gene duplications and diversifications occurred independently and frequently during insect evolution, and suggest a physiological benefit is associated with these redundancies.

***Aa*SDR-1 Efficiently Oxidizes Farnesol to Farnesal.** Our enzymatic assays demonstrated that r*Aa*SDR-1 efficiently oxidizes C_{10} to C_{15} isoprenoid and aliphatic compounds, although it had the highest affinity for farnesol. Similar farnesol dehydrogenases that oxidize farnesol, as well as C_{10} to C_{15} isoprenoid and aliphatic compounds have been described in sweet potato (17) and human liver (18).

SDRs are often present as a group of related enzymes with overlapping tissue specificity and substrate promiscuity (16); for that reason the enzymatic activity of *rAa*SDR-1 was compared with those of two additional cP2 $NADP^+$-dependent *Aa*SDRs expressed in the CA of the adult mosquito. The closely related r*Aa*SDR-2 exhibited substrate specificity, pH optimum, and catalytic properties similar to those described for *Aa*SDR-1. To the contrary, r*Aa*SDR-6 had no activity on farnesol; confirming that not all cP2 *Aa*SDRs expressed in the CA are able to oxidize farnesol.

Little is known about farnesol oxidation in insect CA; the only previous studies were performed using *M. sexta* CA homogenates. Gland homogenates from adult female moths were found to have some substrate specificity for the 2E isomers of farnesol and geraniol, and enzymatic activity was not stimulated by the addition of NAD^+ or $NADP^+$ (11). On the other hand, farnesol oxidation by CA homogenates from *M. sexta* larvae was described as mediated

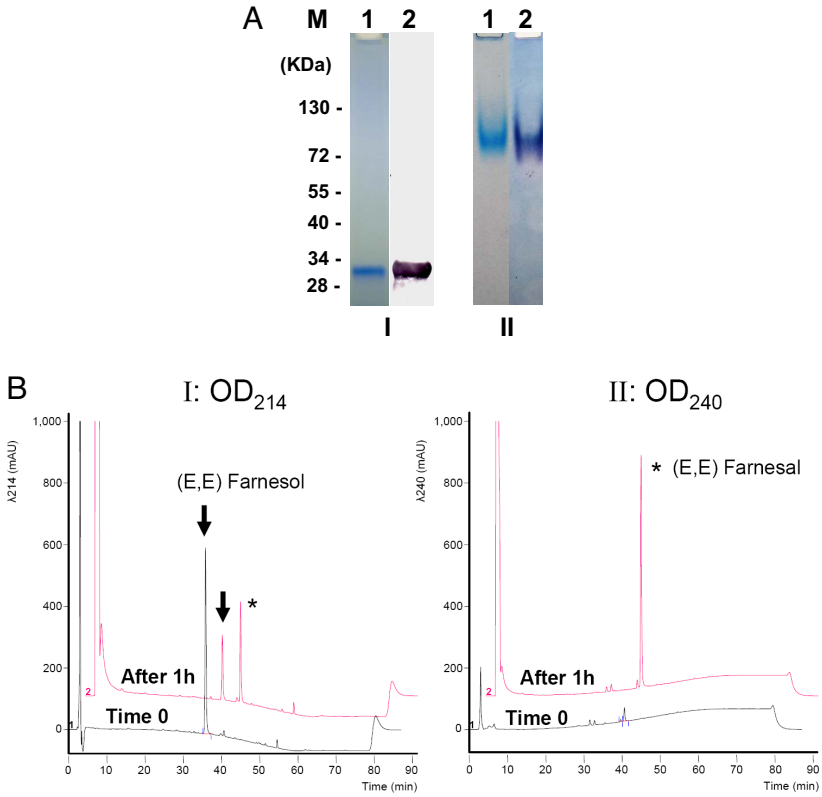

Fig. 3. Purification and enzymatic activity of recombinant *Aa*SDR-1. (*A*) PAGE analysis of recombinant *Aa*SDR-1. (*I*) SDS/PAGE analysis of r*Aa*SDR-1 after purification on a cobalt column. (*M*) Markers 1. *Aa*SDR-1 stained with Coomassie blue 2. *Aa*SDR-1 recognized by an anti-His tag antibody. (II) Native PAGE showing r*Aa*SDR-1. 1. r*Aa*SDR-1 stained with Coomassie blue. 2. Native gel incubated with farnesol and stained with NBT as described in *Materials and Methods*. (*B*) RP-HPLC analysis of *Aa*SDR-1 enzymatic activity. HPLC separation of metabolites produced when r*Aa*SDR-1 was incubated with $NADP^+$ and (*E* and *E*)-farnesol. (*I*) OD_{214} absorbance profile of metabolites. (II) OD_{240} absorbance profile of metabolites. Time 0: the reaction was stopped immediately after adding the enzyme. After 1 h: the reaction was stopped after 1 h of incubation at 30 °C. The arrows indicate the (*E* and *E*)-farnesol. The asterisks indicate the (*E* and *E*)-farnesal.

by a metal and/or flavin dependent alcohol oxidase that also metabolized geraniol and geranylgeraniol (12, 13). The discrepancies between *M. sexta* results and our data on cofactor dependence, isomer specificity and optimal pH are difficult to analyze because we used pure protein instead of CA crude extracts. Tissue extracts could have endogenous cofactors, inhibitors, substrates, or more than one SDR that could modify the activity measured. It is also possible that moths present a different mechanism for farnesol oxidation; it is compelling that while all of the SDR orthologs identified in several insect orders were cP2 $NADP^+$ dependent, the only ortholog that was found in the Lepidoptera *B. mori* belongs to the NAD^+-dependent cD1e subfamily.

Farnesol Homeostasis Determines *Aa*SDR-1 Tissue Specificity. It has been often stated that the enzymes of the last steps of the JH pathway would be CA specific and most likely encoded by single genes. This seems to be true for the methylation of JHA (9) and epoxidation of MF (10), two metabolic reactions that are exclusive for JH biosynthesis, and should be stereospecific to generate the natural enantiomers of JH. In fact, there is a single copy of the JHAMT and P450 epoxidase genes in the genome of *A. aegypti*, and the highest transcript levels of the two enzymes are found in the CA (19).

In contrast, farnesol homoeostasis is vital for cells in many insect

Table 1. Enzymatic activity of the recombinant *Aa*SDR-1 for different substrates

Substrate	C	K_M	V_{max}
(*E*,*E*) Farnesol	15	91 ± 27	29.1 ± 0.7
(*Z*,*Z*) Farnesol	15	97 ± 3	37.7 ± 1.4
2-Decanol	10	184 ± 6	112.3 ± 3.5
Geraniol (*E*)	10	208 ± 26	80.9 ± 0.4
Nerol (*Z*)	10	109 ± 4	48.8 ± 2.9
Citronellol	10	133 ± 17	3.3 ± 0.2
Octanol	8	195	4.9

C, number of the carbons of the substrates. K_M is expressed in μM (mean ± SD) and V_{max} is expressed in nM/L*s (mean ± SD).

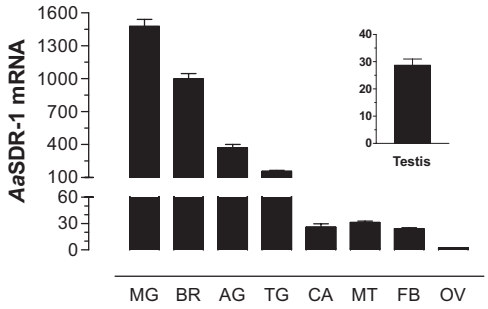

Fig. 4. Tissue specific expression of *Aa*SDR-1 mRNA. All tissues were dissected from 3-day-old sugar-fed females, except for testis dissected from 3-day-old sugar-fed males. MG: midgut; BR: brain; AG: abdominal ganglia; TG: thoracic ganglia; CA: corpora allata; MT: Malpighian tubules; FB: fat body, and OV: ovaries. The insert shows the male testis. *Aa*SDR-1 mRNA is expressed as copy number of mRNA/10,000 copies of rpL32 mRNA. Each RT-PCR data point is the average of three independent biological replicates of 3-20 tissue samples.

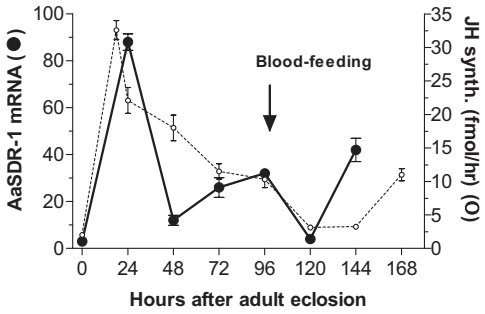

Fig. 5. Developmental expression of *Aa*SDR-1 mRNA. *Aa*SDR-1 mRNA in CA of sugar-fed and bloodfed females (solid lane) are expressed as copy number of *Aa*SDR-1 mRNA/10,000 copies of rpL32 mRNA. Each RT-PCR data point is the average of three independent biological replicates of 10-12 CA. JH synthesis values (dotted lane) are based on Li et al. (2), and are expressed as femtomoles/CA/hour. Females were blood fed 3 days after adult emergence (arrow).

tissues and the oxidation of farnesol to farnesal is not a JH biosynthesis-specific reaction. Long considered a "waste" product, farnesol has been found in many organisms to be a potential signaling molecule implicated in the regulation of a wide variety of cell functions, including cell proliferation and apoptosis (20, 21, 22). Posttranslational modifications by attachment of a farnesyl group to C-terminal cysteines of target proteins by farnesyl-transferases are essential for the function of several main components in signal transduction and vesicular transport (23, 24). An excess of farnesol can be quite toxic for cells (25, 26); therefore regulation of farnesol metabolism is critical. Farnesol metabolism includes oxidation to farnesal, glucuronidation, hydroxylation, and transformation into FPP by a FPP kinase (27). The dephosphorylation-rephosphorylation of farnesol may serve important physiological regulatory functions in the cell; hydrolysis of farnesyl pyrophosphate (FPP) to farnesol by allyl-pyrophosphatase has been described in several organisms (28).

Because of the multiple roles of farnesol it is not a surprise that in addition to the CA, we detected high expression of *Aa*SDR-1 mRNA in other mosquito tissues. Transcripts were abundant in midgut and the nervous system. Farnesol oxidation was confirmed in the midgut and Malpighian tubules using the in vitro colorimetric assay (Fig. S4). In summary, *Aa*SDR-1 is not CA specific because *Aa*SDRs might play important roles in farnesol homeostasis in other mosquito tissues, besides controlling the flux of precursors in JH synthesis.

***Aa*SDR-1 Is a Rate-Limiting Enzyme in JH Synthesis.** Transcriptional regulation of JH biosynthetic enzymes plays a key role in the control of JH synthesis (8, 19). Changes in *Aa*SDR-1 mRNA levels in the CA are consistent with the role of this gene in JH synthesis. *AaSDR*-1 mRNA and its closest ortholog in *D. melanogaster* (CG 1386) were undetectable in pupae of mosquitoes and fruit flies (29). It has been described that transcription of genes coding for JH synthetic enzymes is suppressed or significantly reduced during pupal development to guarantee a successful completion of metamorphosis (8, 19, 30). On the other hand, JH synthesis in adult mosquitoes is a very dynamic and nutrient-dependent process (31). JH titers (32) and spontaneous CA synthetic activity (2) are very low in newly eclosed females, and match in a timely manner with low *Aa*SDR-1 mRNA levels. JH synthesis, JH titer, and *Aa*SDR-1 mRNA levels increase during the first day after adult emergence; this initial rise in JH synthesis is essential for female's reproductive maturation; it signals that ecdysis of the adult has finished and reproductive processes should begin (1).

We propose that *Aa*SDR-1 is a rate-limiting enzyme in JH synthesis. There are remarkable differences in its patterns of mRNA expression and rates of stimulation by an exogenous substrate when we compared it to a nonrate-limiting enzyme, such as JHA methyl transferase (*Aa*JHAMT) (19). *Aa*SDR-1 transcripts are undetectable in the inactive CA of the newly emerged female and showed a 30-fold increase by 24 h after adult eclosion; in contrast, *Aa*JHAMT is highly expressed in the CA of the newly emerged female and mRNA levels only double by 24 h (19, Fig. S5). Even when transcription is stimulated in the CA at the peak of JH synthesis there are 1,000 copies of *Aa*JHAMT per each copy of *Aa*SDR-1 (Fig. S5).

The rates of stimulation of JH synthesis by farnesol and FA seem to confirm that farnesol-SDRs are less abundant than JHAMTs; addition of high concentrations of farnesol to CA cultures stimulated JH synthesis only 1.8-fold in the adult stink bug *Perillus bioculatus* (33), 4-fold in the adult stick insect *Carausius morosus* (34), and 3-fold in adult and embryos of *D. punctata* cockroaches (35, 36). On the other hand, addition of FA increased the rate of JH synthesis 100-fold by CA of the locust *Schistocerca gregaria* (37) and 40-fold in CA of *A. aegypti* females (19).

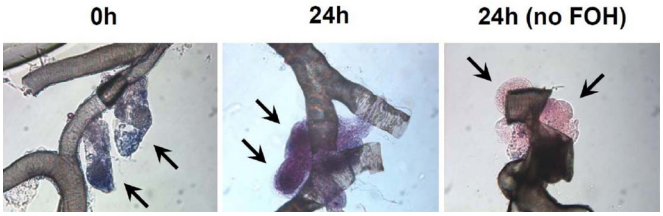

Fig. 6. Farnesol dehydrogenase activity in isolated corpora allata. Whole mount preparations of adult female CA stained for farnesol SDR activity using farnesol as substrate. At 0 h: CA dissected immediately after adult emergence: 24 h: CA dissected 24 h after adult emergence; 24 h (no FOH): CA dissected 24 h after adult emergence incubated without farnesol. Arrows indicate CA attached to tracheae. Purple color indicates enzyme activity. Magnification, ×200.

Conclusions

This is a characterization of an enzyme that catalyzes the oxidation of farnesol into farnesal in the CA of an insect. The *AaSDR-1* isolated from a mosquito CA EST library is structurally and functionally a cP2 SDR and presents typical features of these enzymes, including the presence of multiple paralogs with broad substrate specificity and tissue distribution. The combination of transcriptional studies and oxidase assays showed that this gene plays a key role in the regulation of JH synthesis in adult mosquitoes, and therefore could be an excellent target for strategies of control.

Materials and Methods

Insects. *Aedes aegypti* of the Rockefeller strain were reared as described in ref. 19.

Secondary Structure and Phylogenetic Analysis. The *Aa*SDR-1 predicted secondary structure was obtained using PSIPRED v 2.6 Protein Structure Prediction Server (38). Phylograms were generated using a Neighbor-Joining analysis with the program Mega 3.1 with a bootstrapping of 500 (39). Pairwise deletion method was selected for the gap/missing data.

Expression and Purification of the Recombinant *Aa*SDR-1. The coding region of the *Aa*SDR-1 cDNA was cloned into the expression vector pET28a (+) (Novagen). *E. coli* BL21 (DE3) strain cells were transformed with the construct and expressed as described in ref. 19. Recombinant His-tagged protein was purified from the supernatant by using a His-tagged cobalt column (Pierce), as described in ref. 19. A more detailed description of the expression system is provided in *SI Text*.

Protein Identification and Characterization. Quantification and SDS/PAGE analysis of the purified protein was performed as described in ref. 19. Purified recombinant enzyme was transferred onto a nitrocellulose membrane and detected using a mouse Anti-His Antibody (Amersham Biosciences) (diluted 1:3,000), followed by a Goat Anti-Mouse secondary antibody conjugated with

HRP (diluted 1:3,000) (Upstate). Native 10% PAGE and gel filtration chromatography using a Superdex 75 HR/10 column (Amersham Biosciences) were used to study the native state of the protein and the existence of dimerization.

Enzyme Assays. The characterization of the enzymatic properties of *Aa*SDR-1 was done using two different types of assays, a chromatographic method (HPLC) and a spectrophotometric assay. Purified recombinant *Aa*SDR-1 was used to test substrate specificity, optimal pH and cofactor requirement following a chromatographic protocol described by Mayoral et al. (19) with minor modifications. Briefly, (*E*,*E*)-FOH, (*Z*,*Z*)-FOH, geraniol, nerol, geranyl-geraniol, citronellol, and additional substrates were assayed in 500 μL of Stauffer buffer (40). The reaction mixture was incubated for 1 h at 30 °C, stopped by adding 500 μL of acetonitrile and vortexing. Samples were cleared by centrifugation and the supernatants were analyzed for oxidized products directly by RP-HPLC.

To estimate kinetic properties we used a SDR spectrophotometric assay based on the different optical properties of nicotinamide adenine dinucleotide phosphate ($NADP^+$) and reduced form of NADP (NADPH) at 340 nm, as described by Inoue et al. (17) with minor modifications. Briefly, assays were conducted with 1.5 μg of recombinant enzyme in a final volume of 500 μL of Stauffer buffer at pH 10.0. The substrate concentrations in the assay ranged from 5 to 10,000 μM, using a $NADP^+$ concentration of 2 mM. The reaction was incubated 1 h at 30 °C. Reactions were stopped and the organic phase extracted by adding 500 μL of hexane and vortexing for 2 min. After spinning the samples for 10 min (14,000 $\times$ *g* at 4 °C), the organic phase was removed and the OD of aqueous fractions was measured at OD_{340} nm in a spectrophotometer (SmartSpec™3000, Bio-Rad). Reactions were performed in duplicate and blanks (in which extraction was performed before the enzyme was added) were prepared for each assay. Two to four independent experiments were carried out for each substrate or treatment assayed. The K_m values were obtained by double reciprocal Lineweaver–Burk plots of the amount of total product formed at increasing concentrations of the substrate.

Mass Spectrometric Characterization of Oxidized Products. Unequivocal identification of enzymatic products by accurate mass measurements was obtained using Direct Analysis in Real Time (DART)-Time-of-Flight (TOF) mass spectrometry (41). A detailed description of the DART-TOF methodology is described in *SI Text*.

Quantitative Real-Time PCR (qPCR). RNA isolation and PCR were performed as described in ref. 19. The primer probes for the house keeping gene 60S ribosomal protein L32 (*rpL32*; AAEL003396 from VectorBase) and for *Aa*SDR-1 are described in *SI Text*.

Staining for Dehydrogenase Activity. Visualization of farnesol dehydrogenase activity in PAGE and whole mount CA preparations were performed as described by Madhavan et al. (42). CA were isolated as described in ref. 43 and farnesol oxidase activity was visualized using the staining system developed by Ursprung and Leone using farnesol and $NADP^+$ as substrates (44). Controls were incubated in the same solution without farnesol. Samples were rinsed in PBS, mounted, and examined using a Leica DMBR microscope. Farnesol dehydrogenase activity in PAGE gels was visualized using the same staining solution as described for CA (42). Staining of midguts and Malpighian tubules is described as *SI Text*.

Statistical Analysis. Statistical analysis of the data were performed by *t* test using GraphPad Prism version 3.00 for Windows, GraphPad Software. The results were expressed as mean $\pm$ SEM, and considered significantly different at $P < 0.05$.

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