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 (AaSDR-1) that oxidizes farnesol into farnesal, a precursor of JH, in the CA. AaSDR-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 AaSDR-1 into the “classical” NADP$^+$-dependent c2 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. AaSDR-1 acts as a homodimer and efficiently oxidizes C10 to C15 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 AaSDR-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.

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 (AaSDR-1) and 4 additional sequences with a high degree of similarity (72-83%) (AaSDR-2 to AaSDR-5) (Fig. 1). AaSDR-1 is clustered in the same contig with two of the closest paralogs (AaSDR-2 and AaSDR-4). Analysis of the five AaSDRs 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 AaSDRs have several conserved sequence motifs that place them in the “Classical” SDR family and the subfamily c2, for example the pattern of three glycine residues that are distinctive of the nucleotide-binding region (TGxGxG), 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 closely related 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
event. Members of these two clusters have between 67 and 72% similarities with AaSDR-1. There are six orthologs in Anopheles gambiae that have between 61–67% similarity with AaSDR-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 AaSDR-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 AaSDR cP2 (AaSDR-6, 14% similarity with AaSDR-1) expressed in the CA of A. aegypti adult female was included in the analysis as an outgroup.

Functional Characterization of AaSDR-1. Recombinant AaSDR-1 (rAaSDR-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. 3A). 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. 3B). Purified rAaSDR-1 oxidized farnesol (FOH) to farnesal in the presence of NADP⁺ 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 Km 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 AaSDR-1 mRNA. Real time PCR was used to analyze the transcript tissue specificity. In adult females the highest levels of AaSDR-1 mRNA were detected in the midgut and brain (Fig. 4); relatively high levels were
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 AaSDR-1 transcripts in pupae. When AaSDR-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).

**Discussion**

**AaSDR-1 Is a Classical cP2 SDR Expressed in the CA of Insects.** One EST was found in a CA library of *A. aegypti* encoding AaSDR-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 xenobiotoxic metabolism. Sequence similarities are low, and the most conserved feature is an αβ 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 AaSDR-1 and four additional paralogs with a high degree of sequence similarity (72-83%). Two of these genes (AaSDR-4 and -5) were not expressed in the CA of the adult mosquito; of the additional three (AaSDR-1, -2, and -3), only AaSDR-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 AaSDR-1, as well as the four additional paralogs (AaSDR-2 to AaSDR-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.

**AaSDR-1 Efficiently Oxidizes Farnesol to Farnesal.** Our enzymatic assays demonstrated that AaSDR-1 efficiently oxidizes C10 to C15 isoprenoid and aliphatic compounds, although it had the highest affinity for farnesol. Similar farnesol dehydrogenases that oxidize farnesol, as well as C10 to C15 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 AaSDR-1 was compared with those of two additional cP2 NADP+ dependent SDRs expressed in the CA of the adult mosquito. The closely related AaSDR-2 exhibited substrate specificity, pH optimum, and catalytic properties similar to those described for AaSDR-1. To the contrary, AaSDR-6 had no activity on farnesol; confirming that not all cP2 AaSDRs 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. Glind homogenates from adult female moths were found to have some substrate specificity for the 2E isomers of farnesal 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...
by a metal and/or flavin dependent alcohol oxidase that also metabolized geraniol and geranylgeraniol (12, 13). The discrepancies between \textit{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$^{+}$/H$^{+}$ dependent, the only ortholog that was found in the Lepidoptera \textit{B. mori} belongs to the NAD$^{+}$/H$^{+}$-dependent cD1e subfamily.

Farnesol Homeostasis Determines AaSDR-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 \textit{A. aegypti}, and the highest transcript levels of the two enzymes are found in the CA (19).

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

\begin{table}
\centering
\begin{tabular}{llll}
\textbf{Substrate} & \textbf{C} & \textbf{K_M} & \textbf{V_{max}} \\
(\textit{E},\textit{E}) Farnesol & 15 & 91 ± 27 & 29.1 ± 0.7 \\
(\textit{Z},\textit{Z}) Farnesol & 15 & 97 ± 3 & 37.7 ± 1.4 \\
2-Decanol & 10 & 184 ± 6 & 112.3 ± 3.5 \\
Geraniol (\textit{E}) & 10 & 208 ± 26 & 80.9 ± 0.4 \\
Nerol (\textit{Z}) & 10 & 109 ± 4 & 48.8 ± 2.9 \\
Citronellol & 10 & 133 ± 17 & 3.3 ± 0.2 \\
Octanol & 8 & 195 & 4.9 \\
\end{tabular}
\caption{Enzymatic activity of the recombinant AaSDR-1 for different substrates}
\end{table}
AaSDR-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 AaSDR-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 AaSDR-1 mRNA levels. JH synthesis, JH titer, and AaSDR-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 AaSDR-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 (AaJHAMT) (19). AaSDR-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, AaJHAMT 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 AaJHAMT per each copy of AaSDR-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).

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 AaSDR-1 predicted secondary structure was obtained using PSI-PRED v2.6 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 gapmissing data.

Expression and Purification of the Recombinant AaSDR-1. The coding region of the AaSDR-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 AaSDR-1 was done using two different types of assays, a chromatographic method (HPLC) and a spectrophotometric assay. Purified recombinant AaSDR-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 Stuffer buffer (40). The reaction mixtures were 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 Stuffer 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. 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 (13400 × g at 4 °C), the organic phase was removed and the OD of aqueous fractions was measured at OD[340] nm in a spectrophotometer (SmartSpecTM3000, Bio-Rad).

Reactions were performed in duplicate and blanks (in which extraction was performed but no reaction was performed) were prepared for each assay. Two to four independent experiments were carried out for each substrate or treatment assayed. The V_max 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 (pL32; AAE003936 from VectorBase) and for AaSDR1 are described in SI Text.

**Staining for Dehydrogenase Activity.** Visualization of farnesol dehydrogenase activity in PAGE and whole mount CA preparations was 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 Urspurg 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 DMRB 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 ± SEM, and considered significantly different at P < 0.05.

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