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Aldehyde dehydrogenase 3 converts farnesal into farnesoic acid in the *corpora allata* of mosquitoes

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ABSTRACT

The juvenile hormones (JHs) play a central role in insect reproduction, development and behavior. Interrupting JH biosynthesis has long been considered a promising strategy for the development of target-specific insecticides. Using a combination of RNAi, *in vivo* and *in vitro* studies we characterized the last unknown biosynthetic enzyme of the JH pathway, a fatty aldehyde dehydrogenase (*Aa*ALDH3) that oxidizes farnesal into farnesoic acid (FA) in the *corpora allata* (CA) of mosquitoes. The *Aa*ALDH3 is structurally and functionally a NAD^+-dependent class 3 ALDH showing tissue- and developmental-stage-specific splice variants. Members of the ALDH3 family play critical roles in the development of cancer and Sjögren–Larsson syndrome in humans, but have not been studies in groups other than mammals. Using a newly developed assay utilizing fluorescent tags, we demonstrated that *Aa*ALDH3 activity, as well as the concentrations of farnesol, farnesal and FA were different in CA of sugar and blood-fed females. In CA of blood-fed females the low catalytic activity of *Aa*ALDH3 limited the flux of precursors and caused a remarkable increase in the pool of farnesal with a decrease in FA and JH synthesis. The accumulation of the potentially toxic farnesal stimulated the activity of a reductase that converted farnesal back into farnesol, resulting in farnesol leaking out of the CA. Our studies indicated *Aa*ALDH3 plays a key role in the regulation of JH synthesis in blood-fed females and mosquitoes seem to have developed a "trade-off" system to balance the key role of farnesal as a JH precursor with its potential toxicity.

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1. Introduction

Juvenile hormone (JH) is a key regulator in insect morphogenesis and reproduction (Goodman and Cusson, 2012). For this reason, interfering with its biosynthesis has long been considered a promising strategy for the development of target-specific insecticides (Cusson et al., 2013). The biosynthetic pathway of JH III in the *corpora allata* (CA) of the Dengue mosquito *Aedes aegypti* involves 13 discrete enzymatic steps (Nouzova et al., 2011); the early steps follow the mevalonate pathway to form farnesyl pyrophosphate (FPP) (Bellés et al., 2005). During the late steps, FPP is transformed sequentially to farnesol, farnesal, farnesoic acid (FA), methyl farnesoate and JH III (Goodman and Cusson, 2012). The last 2 enzymes of the pathways have been identified and used to screen for inhibitors (Shinoda and Itoyama, 2003; Helvig et al., 2004); however, the oxidation of farnesal to FA remains as one of the less understood steps in JH synthesis, and it has been predicted to be catalyzed by an aldehyde dehydrogenase (Baker et al., 1983; Goodman and Cusson, 2012). The aldehyde dehydrogenase superfamily (ALDH, E.C. 1.2.1.X) comprises a cluster of evolutionary related sequences with a wide phylogenetic distribution (Dayhoff, 1976). It includes $NAD(P)^+$-dependent enzymes catalyzing the oxidation of a broad spectrum of aldehyde substrates to their corresponding acids (Pietruszko, 1983). The human ALDH3A2 is able to convert long-chain aldehydes, including farnesal, to their related acids (Lloyd et al., 2007); mutations of ALDH3A2 gene causes Sjögren–Larsson syndrome, a disorder associated with impaired fatty alcohol oxidation due to deficient activity of fatty alcohol oxidoreductase and its consequent oxidation to fatty aldehyde and fatty acid (Rizzo and Craft, 1991; De Laurenzi et al., 1996; Kelson et al., 1997). Farnesal oxidation in the CA was first revealed in *Drosophila melanogaster* using an *in vitro* colorimetric assay (Madhavan et al., 1973). Subsequently the conversion of *2E*- and *2Z*-farnesal to FA by CA homogenates of the adult female sphinx moth, *Manduca sexta* was reported (Baker et al., 1983).

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Further characterization of the CA ALDH has been hindered by the minute size of the endocrine gland. Recently, we described a new and sensitive assay for the JHs and their precursors using fluorescent tags (Rivera-Perez et al., 2012); this assay allowed the quantification of farnesal and FA pools in the CA, as well as ALDH enzymatic activities from CA extracts. Here, we report the molecular and biochemical characterization of an *A. aegypti* ALDH3 (*Aa*ALDH3) expressed in the CA. Our results suggest that in the female of the Dengue mosquito *A. aegypti*, this enzyme plays a critical role in the regulation of JH biosynthesis after blood feeding; in CA of blood-fed females the low catalytic activity of *Aa*ALDH3 limited the flux of precursors and caused a major increase in the pool of farnesal with a decrease in FA and JH synthesis. The accumulation of the potentially toxic farnesal stimulated the activity of a reductase that converted farnesal back into farnesol, resulting in farnesol leaking out of the CA. This aldehyde scavenging enzyme prevents aldehyde toxicity in CA cells and plays a key role in farnesal homeostasis.

2. Materials and methods

2.1. Insects

A. aegypti of the Rockefeller strain were reared as described by Nouzova et al. (2011).

2.2. Identification of two farnesal dehydrogenase genes in A. aegypti

We screened the *A. aegypti* genome (Assembly AaegL1) for homologue sequences to the Human ALDH3A2 (P51648) that has been reported to transform farnesal into FA (Lloyd et al., 2007). The search revealed an area in the supercontig 1.662 containing three predicted genes in close proximity (AAEL012161, AAEL012162 and AAEL012165), which showed more than 50% protein identity with the human ALDH3A2 gene. Closer examination of this contig disclosed that there were only two genes, with AAEL012161 and AAEL012165 being parts of the same gene. There was also one predicted gene in the supercontig 1.417 with a DNA sequence almost identical to AAEL012162 (with a few point mutations). Primers were designed to amplify the full coding sequences of the two genes from cDNA of *corpora allata* (Supplemental Table 1). They were named *Aa*ALDH3-1 and *Aa*ALDH3-2. Sequencing of the cDNAs revealed five different transcripts from the two different genes. The gene *Aa*ALDH3-1 produces four different transcripts (A–D) and the gene *Aa*ALDH3-2 produces only one transcript. The individual transcripts from gene *Aa*ALDH3-1 differ only in exon six (Supplemental Fig. 1).

2.3. Secondary structure and phylogenetic analysis

Secondary structures for ALDHs were predicted using PSIPRED version 2.5 (Jones, 1999) and transmembrane helices using TMHMM server version 2.0 (Nugent and Jones, 2009). A maximum-likelihood tree was built using MEGA software version 5.1 (Tamura et al., 2011), with a bootstrapping value of 1000. A pairwise deletion method was selected for the gap/missing data.

2.4. Expression of recombinant AaALDH3s

*Aa*ALDH3 cDNAs were expressed in *E. coli* cells as described by Mayoral et al. (2009b). Recombinant His-tagged proteins were identified by Western blot using a mouse anti-His antibody as described by Mayoral et al. (2009b).

2.5. Enzyme assays

Bacterial crude extract containing overexpressed proteins were used to test substrate specificity and cofactor requirements. The ALDH activity assay was optimized based on a protocol described by Lloyd et al. (2007). Reaction mixtures (750 μL) contained 50 mM glycine buffer (pH 9.5), 2 mM NAD^+ and 50 μM substrate (octanal, decanal or farnesal). Reactions were started by the addition of 5 μg of bacterial extracts. After 2 h incubation at 37 °C, reactions were stopped with 500 μL of hexane, vortexed 1 min and centrifuged for 10 min at 14,000*g* and 4 °C; supernatants were used for analysis of production of octanoic acid, decanoic acid and FA by reversed-phase high performance liquid chromatography (RP-HPLC). Negative controls included boiled enzyme, no enzyme or bacterial cells without constructs. ALDH and aldo-keto reductase (AKR) activities in mosquito CA were measured by HPLC coupled to a fluorescent detector (HPLC-FD), monitoring the production of FA and farnesol respectively. The AKR activity assay was optimized based on a protocol described by Endo et al. (2009). Glands were dissected in buffer solution (50 mM glycine buffer pH 9.5 for ALDH and 0.1 M phosphate buffer pH 7.4 for AKR). CA were homogenized for 1 min, sonicated 3 min and centrifuged at 4 °C and 10,000*g* for 10 min. Supernatant was recovered and used as crude extracts for ALDH activity assays as described before; for AKR assays, the reaction mixture consisted of 0.1 M phosphate buffer, 0.1 mM NADPH and 50 μM farnesal, both reactions mixtures were incubated 1 h at 37 °C. The reactions products were recovered as described before and labeled with AABD-SH (farnesoic acid) and DBD-COCl (farnesol) for HPLC-FD analysis (Rivera-Perez et al., 2012).

2.6. Precursor pools and JH III quantification

Farnesol, farnesal, FA and JH III quantification by HPLC-FD and mass spectrometry characterizations were done as described by Rivera-Perez et al. (2012).

2.7. RNAi experiment

Specific target sequences for dsRNA synthesis were designed for each splice variant of *Aa*ALDH3-1 by selecting regions in the splice variant-specific exons. We also designed dsRNA for *Aa*ALDH3-2 as well as dsRNA against a common region to all splice variants of *Aa*ALDH3-1 (*Aa*ALDH3-1-RA, *Aa*ALDH3-1-RB, *Aa*ALDH3-1-RC, *Aa*ALDH3-1-RD) and a Yellow Fluorescent Protein (YFP), that was used as control. Targeted regions were amplified using the primers included in Supplemental Table 1. dsRNA was synthesized using a MEGAscript RNAi kit (Ambion), and 1.6 μg of dsRNA were injected intrathoracically to 0 h female mosquitoes using a Drummond Nanoject II microinjector and a micromanipulator. The effect of dsRNA was evaluated 4 days after injection, a time selected based on the analysis of dsRNA depletion experiments.

2.8. Quantitative real-time PCR (qPCR)

RNA isolation and PCR were performed as described by Nouzova et al. (2011). The primers and probes for the housekeeping gene 60S ribosomal protein rpL32 and *Aa*ALDH3 transcripts are included in Supplemental Table 1.

2.9. Statistical analysis

Data were analyzed for statistical significance using GraphPad Prism (GraphPad Software, San Diego, CA). The results were expressed as means ± S.E.M. Significant differences ($P < 0.05$) were determined with a one tailed students *t*-test performed in a