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Biochemical properties, expression profiles, and tissue localization of orthologous acetylcholinesterase-2 in the mosquito, *Anopheles gambiae*

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ABSTRACT

Acetylcholinesterases (AChEs) catalyze the hydrolysis of acetylcholine, a neurotransmitter for cholinergic neurotransmission in animals. Most insects studied so far possess two AChE genes: *ace-1* paralogous and *ace-2* orthologous to *Drosophila melanogaster ace*. We characterized the catalytic domain of *Anopheles gambiae* AChE1 in a previous study (Jiang et al., 2009) and report here biochemical properties of *A. gambiae* AChE2 expressed in *Sf*9 cells. An unknown protease in the expression system cleaved the recombinant AChE2 next to Arg^{110}, yielding two non-covalently associated polypeptides. A mixture of the intact and cleaved AChE2 had a specific activity of 72.3 U/mg, much lower than that of *A. gambiae* AChE1 (523 U/mg). The order of V_{max}/K_M values for the model substrates was acetylthiocholine > propionylthiocholine ≈ acetyl-(β-methyl) thiocholine > butyrylthiocholine. The IC_{50}'s for eserine, carbaryl, BW284C51, paraoxon and malaoxon were 1.32, 13.6, 26.8, 192 and 294 nM, respectively. *A. gambiae* AChE2 bound eserine and carbaryl stronger than paraoxon and malaoxon, whereas eserine and malaoxon modified the active site Ser^{232} faster than carbaryl or paraoxon did. Consequently, the k_i's were 1.173, 0.245, 0.029 and 0.018 $\mu M^{-1}min^{-1}$ for eserine, carbaryl, paraoxon and malaoxon, respectively. Quantitative polymerase chain reactions showed a similar pattern of *ace-1* and *ace-2* expression. Their mRNAs were abundant in early embryos, greatly decreased in late embryos, larvae, pupae, and pharate adult, and became abundant again in adults. Both transcripts were higher in head and abdomen than thorax of adults and higher in male than female mosquitoes. Transcript levels of *ace-1* were 1.9- to 361.8-fold higher than those of *ace-2*, depending on developmental stages and body parts. Cross-reacting polyclonal antibodies detected AChEs in adult brains, thoracic ganglia, and genital/rectal area. Activity assays, immunoblotting, and tandem mass spectrometric analysis indicated that *A. gambiae* AChE1 is responsible for most of acetylthiocholine hydrolysis in the head extracts. Taken together, these data indicate that *A. gambiae* AChE2 may play a less significant role than AChE1 does in the mosquito nervous system.

1. Introduction

Acetylcholinesterases (AChEs) are serine hydrolases mainly associating with the basement membrane around cholinergic nerve terminals of vertebrates and invertebrates, which breakdown acetylcholine to terminate excitatory neurotransmission (Soreq and Seidman, 2001). Due to this critical physiological function and related practical applications, insect AChEs have been purified to various degrees from nerve tissue or expression systems for testing enzyme properties and inhibition by AChE targeting insecticides (Zhu and Zhang, 2005; Walsh et al., 2001; Zhao et al., 2010; Temeyer et al., 2010). Certain mutations in the AChE genes rendered their carriers resistant to insecticides (Oakeshott et al., 2005) and investigations of such resistance led to the discovery of AChE genes and mechanisms of target site insensitivity (Fournier et al., 1989; Weill et al., 2002; Menozzi et al., 2004). We now know most insects have two AChE genes (*ace-1* and *ace-2*) that arose from ancient gene duplication prior to the radiation of Arthropoda (Weill et al., 2002; Kozaki et al., 2008). While a close association of insecticide-resistant AChEs with mutations in *ace-1* suggests its physiological importance (Kono and Tomita, 2006), the role of *ace-2* remains elusive. However, in the lineage of Aristoceran flies, since

Abbreviations: AChE, acetylcholinesterase; DTT, dithiothreitol; ATC, acetylthiocholine iodide; AβMTC, acetyl-(β-methyl)thiocholine iodide; PTC, propionylthiocholine iodide; BTC, butyrylthiocholine iodide; DTNB, 5,5-dithio-bis(2-nitrobenzoic acid); BW284C51, 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide; NBT, nitro-blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indoryl phosphate; BSA, bovine serum albumin; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EST, expressed sequence tag.

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ace-1 was lost in the evolution, *ace-2* became indispensable in regulating cholinergic neurotransmission (Huchard et al., 2006).

Are *ace-1* and *ace-2* both transcribed and translated in nerve tissues of other insects? If so, do they play a similar role by complementing each other? Molecular biological tools have been used to address these questions. For instance, feeding the cotton bollworm *Helicoverpa armigera* with small interfering RNA identical to *ace-2* somehow caused *ace-1* silencing, mortality, growth inhibition, weight loss, and fecundity reduction (Kumar et al., 2009). In the German cockroach *Blattella germanica*, *ace-1* mRNA level was ~10-fold higher than *ace-2*'s in the central nervous system (Kim et al., 2006). Knockdown of *ace-1* expression by RNA interference reduced its protein level and significantly increased the sensitivity to chlorpyrifos, whereas *ace-2* silencing in the cockroach did not affect mortality to the organophosphate (Revuelta et al., 2009). This result agrees with the observation that *B. germanica* AChE1 represents ~70% of the total activity in nerve tissue (Kim et al., 2010). Since, unlike other insects, the cockroach AChE1 has lower catalytic efficiency (V_{max}/K_M: 58.8 $min^{-1}ml^{-1}$) than AChE2 does (234 $min^{-1}ml^{-1}$) (Kim et al., 2010), the AChE1 level has to be much higher in the cockroach tissues to account for the majority of acetylcholine hydrolysis. In another study, silencing *ace-1* in 20-day-old *Tribolium castaneum* increased larval susceptibility to AChE inhibitors and caused 100% mortality within two weeks after eclosion (Lu et al., 2012b). Silencing *T. castaneum ace-2* delayed development and reduced egg laying and hatching. One of the two AChEs detected in the mosquito *Culex pipiens* had much lower sensitivity to malaoxon than the other (Bourguet et al., 1997). So far, thorough comparisons of gene expression and enzyme properties are not available in other insects possessing both *ace-1* and *ace-2*.

The mosquito *Anopheles gambiae* is a major vector of malaria parasites, containing both AChE genes (Weill et al., 2002). We previously expressed and characterized the catalytic domain of AChE1, which has a specific activity much higher than AChEs from other orders of insects (Jiang et al., 2009). It tightly binds eserine, rapidly reacts with a carbamate, and exhibits product inhibition by choline. In this study, we characterized *A. gambiae* AChE2 (38% identical in amino acid sequence to *A. gambiae* AChE1) and compared its biochemical properties with those of the AChE1. We further examined their gene expression patterns by quantitative real-time polymerase chain reaction (qRT-PCR), detected the proteins by immunohistochemistry, and discussed their relative contributions to cholinergic neurotransmission.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide (ATC), acetyl(β-methyl)thiocholine iodide (AβMTC), propionylthiocholine iodide (PTC), *S*-butyrylthiocholine iodide (BTC), 5,5-dithio-bis(2-nitro- benzoic acid) (DTNB), carbaryl, eserine hemisulfate, and 1,5-bis(4-allyldimethyl-ammonium phenyl)-pentan-3-one dibromide (BW284C51) were purchased from Sigma–Aldrich. Paraoxon (O,O′-diethyl *p*-nitrophenyl phosphate) and malaoxon (O,O′-dimethyl S-(1,2-dicarbethoxy)ethyl phosphorothioate) (Chem Service), Clear-Rite 3 (Richard-Allan Scientific), paraformaldehyde, goat-anti-mouse/rabbit IgG-conjugated to alkaline phosphatase (Sigma–Aldrich), nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoryl phosphate (BCIP) (Bio-Rad) were purchased from the enlisted vendors.

2.2. Mosquito culture and tissue sectioning

The G3 strain of *A. gambiae* was obtained from Mark Benedict and Malaria Research and Reference Reagent Resource Center (MR4)/American Type Culture Collection (ATCC). The mosquitoes were reared as described by Benedict (1997) with minor modifications. Larvae were reared at 27 °C and fed a mixture of baker's yeast and ground fish food (Vitapro Plus Staple Power Flakes, Mike Reed Enterprises). Adults were maintained at 27 °C with 85% relative humidity and were fed 10% sucrose. Adult females were fed heparinized equine blood with a membrane feeder (Hemotek). Tissue sections were prepared from 40 adults (day 5). Wings and legs were removed and two gaps were made in the cuticle of each adult with a forceps, one from the thorax and the other from the abdomen, to allow the fixative (0.25% Triton-X100, 4% paraformaldehyde, and 0.1 M sodium phosphate, pH 7.2) to enter. After fixation for 3.5 h at room temperature, the specimens were treated with 70% ethanol overnight, stored at 4 °C, and embedded in melted paraffin. Embedding and sectioning were performed in Oklahoma Animal Diseases and Diagnosis Laboratory.

2.3. A gambiae AChE2 cDNA cloning and sequence analysis

Three cDNA clones (BM632651 from RSP strain; BX621591 and BX619729 from a mixture of PEST, 4arr, M2, Kisumu and RSP strains at different life stages) (Fig. 1) were kindly provided by Dr. Neil Lobo in the Department of Biological Sciences at University of Notre Dame and completely sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). One 5′ fragment (missing in the EST clones) was amplified by semi-nested PCR using *ace2*-specific primers (Table S1, PCR1 and PCR2) and a cDNA pool of adult *A. gambiae* G3, a generous gift from Dr. Susan Paskewitz in the Department of Entomology at University of Wisconsin–Madison. After electrophoresis, the PCR product at expected size was recovered from agarose gel, cloned into pGem-T (Promega), and sequenced. Exon-intron organization of *A. gambiae ace-2* was deduced by comparing the assembled cDNA with its corresponding genomic sequence (GenBank accession number BN000067). Signal peptide and glycosylation sites were predicted using SignalP, NetNGlyc, and NetOGlyc (http://www.cbs.dtu.dk/services/). The 25–30-residue region unique to some insect AChE2s (Weill et al., 2002), catalytic triad, disulfide connectivity, and conserved hydrophobic residues lining the active site gorge were identified through sequence comparison using BLASTP (http://blast.ncbi.nlm.nih.gov/).

2.4. Construction of AChE2/pMFH6 and recombinant baculovirus

Another 5′ AChE2 fragment, not found in the three cDNA clones, was amplified from the adult cDNA pool (Table S1, PCR3 and PCR4), cloned into pGem-T (Promega), confirmed by sequence analysis, and digested with *Eco*RI and *Kpn*I. The 3′ cDNA fragment was amplified using BX621591 as template (Table S1, PCR5) and inserted into pGem-T. Plasmid DNA was prepared from transformants, confirmed by DNA sequencing, and digested with *Kpn*I completely and *Xho*I partially. DNA fragments at correct sizes (*Eco*RI-*Kpn*I, 1256 bp; *Kpn*I-*Xho*I, 503 bp; $pMFH_6$ digested with *Eco*RI and *Xho*I, 4780 bp) were recovered from the gel and ligated. The cloning strategy allows in-frame fusion with the amino-terminal honeybee mellitin signal peptide and the carboxyl-terminal hexahistidine tag, both encoded by the vector (Lu and Jiang, 2008). After transformation, plasmid isolation, and restriction site verification, the recombinant plasmid AChE2/$pMFH_6$ was used to generate a high-titer baculovirus stock ($1–2 \times 10^8$ pfu/ml) as described previously (Zhao et al., 2010).

2.5. Expression and purification of A. gambiae AChE2

*Spodoptera frugiperda Sf*9 cells (3.0×10^6 cells/ml) in 820 ml of Sf-900™ II serum-free medium (Invitrogen Life Technologies) were