Cloning, sequencing and functional expression of an acetylcholinesterase gene from the yellow fever mosquito *Aedes aegypti*

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Abstract A degenerate PCR strategy was used to isolate a fragment of the acetylcholinesterase gene (*Ace*) homolog from *Aedes aegypti* and screen for a cDNA clone containing the complete open reading frame of the gene. The predicted amino acid sequence of the *Aedes* gene shares 64% identity with *Ace* from *Drosophila* and 87% identity with the acetylcholinesterase gene from another mosquito species *Anopheles stephensi*. High levels of expression of the *Aedes* gene were achieved by infection of *Sf*21 cells with a recombinant baculovirus containing the *Aedes Ace* cDNA. The catalytic properties and sensitivity of the recombinant enzyme to insecticide inhibition are described and discussed in relation to the role of insensitive AChE in conferring resistance to organophosphorus and carbamate insecticides.

Key words: Acetylcholinesterase; Yellow fever mosquito; *Aedes aegypti*; Baculovirus expression; Insecticide resistance

1. Introduction

Acetylcholinesterase (AChE) catalyzes the hydrolysis of the excitatory neurotransmitter acetylcholine and is a key component at cholinergic synapses in the insect nervous system [1]. In insects, AChE is the only cholinesterase [2] and possesses a substrate specificity that is intermediate between that of vertebrate acetylcholinesterases and butyrylcholinesterases [2,3]. In contrast to vertebrate cholinesterases which display a variety of molecular forms [4], the predominant form of AChE in insects is a globular amphiphilic dimer bound to membranes by a glycolipid anchor at the C-terminal of each catalytic subunit [2,5].

AChE is also the major molecular target for organophosphorus and carbamate insecticides which inhibit enzyme activity by phosphorylating or carbamoylating the serine residue within the active site gorge [6]. In many insect species, insensitive AChE constitutes an important target site based mechanism of resistance to organophosphorus and carbamate insecticides [7,8]. Interestingly, biochemical studies have shown that several insect species also possess more than one form of insensitive AChE [9–13].

The 3-D structure of AChE from *Torpedo californica* has recently been elucidated [14]. Structural analysis of this protein in combination with site-directed mutagenesis studies have revealed the precise location of the active site gorge within the molecule and the identity and relative position of the three residues constituting the catalytic triad: serine 200, histidine 440 and glutamic acid 327 [14,15]. Recently, several point mutations conferring resistance to organophosphorus and carbamate insecticides have been identified in the acetylcholinesterase gene (*Ace*) from *Drosophila melanogaster* [16]. In the light of existing knowledge of the 3-D structure of AChE and the identification of several point mutations conferring insecticide resistance, we were interested in developing a gene expression system to test the effects of resistance associated mutations on enzyme structure and function. Here we report the cloning and functional expression of the *Ace* gene from an important disease vector, the yellow fever mosquito *Aedes aegypti*. An analysis of the catalytic properties and sensitivity of the enzyme to inhibition by carbamate and organophosphorus insecticides is described as a prelude to site-directed mutagenesis studies.

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Abbreviations: AChE, acetylcholinesterase enzyme; *Ace*, acetylcholinesterase gene; PCR, polymerase chain reaction.

2. Materials and methods

2.1. Cloning and functional expression

A degenerate PCR strategy was employed to isolate a fragment of the *Ace* gene from *Aedes aegypti* (Fig. 1). This PCR fragment was cloned into the pCRII vector (Invitrogen), according to manufacturer's instructions, and used to screen an adult cDNA library constructed in the vector λgt10 [17]. The library was screened at high stringency (16–20 h hybridization at 65°C in 1 × Denhardt's solution, 6 × SSC and 0.5% SDS; three washes at 65°C in 2 × SSC, 0.5% SDS for 30 min each) using a ^{32}P radiolabeled PCR fragment isolated from the cloning vector by *Eco*R1 digestion and electrophoresis in low melting temperature agarose (NuSieve, FMC Bioproducts). The complete sequence of both strands of the *Aedes Ace* cDNA was determined from a series of nested deletions generated by the Erase-a-Base kit (Promega). DNA sequencing was carried out by the dideoxy chain termination method [18] using the Sequenase kit (United States Biochemicals).

In order to insert the *Aedes* gene into a recombinant baculovirus, the cDNA was engineered to remove most of the 5′ and 3′ flanking DNA. *Pst*1 sites were introduced immediately upstream of the *Sun*1 site (S) and downstream of the *Eco*N1 site (E) (Fig. 2). This *Pst*1 fragment was subcloned into the *Pst*1 site of the pEV/35K/polybsmer transfer vector downstream of the polyhedrin promoter (Fig. 2) and cotransfected into *Sf*21 cells with the vΔ35K/lacZ parent viral DNA as previously described [19]. For mock-infections, cells were infected either with the wild-type virus or a recombinant baculovirus containing the *Drosophila* gene *Resistance to dieldrin* (*Rdl*) that codes for a γ-aminobutyric acid (GABA) receptor [20]. For uninfected controls, tissue culture medium alone was added to cell cultures.

2.2. Biochemical assay of AChE activity

Cells were infected with recombinant or wild-type baculovirus at the late exponential growth phase. At 72–96 h post-infection, cells were washed 3 × in ice cold phosphate buffered saline and resuspended at a density of 1000 cells/μl in 0.1 M sodium phosphate buffer (pH 7.5). Cells were then sonicated for 15 s and the remaining debris removed by centrifugation at 2500 rpm. Biochemical assay of AChE activity [21] was carried out using 0.5 mM acetylthiocholine (ASCI) as substrate and 1 mM 2,2′-dinitro-5,5′-dithiodibenzoic acid in the assay buffer (0.1 M sodium phosphate buffer, 0.1% Triton X-100; pH 7.5). Kinetic parameters of the enzyme were estimated by measuring reaction rates (V) over a range of substrate concentrations and Eadie–Hofstee plots (V vs. V/S) were constructed to determine the K_m and V_{max} values of the expressed protein.

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Progressive inhibition of AChE activity by organophosphorus and carbamate insecticides (irreversible cholinesterase inhibitors) was examined within the context of the following reaction scheme [22]:

$$\text{AChE} + \text{AB} \underset{K_i}{\rightleftharpoons} \text{AChE}\cdot\text{AB} \xrightarrow{k+2} \underset{\text{(inactive)}}{\text{AChE-A}} + \text{B} \xrightarrow{k+3} \text{AChE} + \text{A}$$

Where AChE is the free enzyme, AB is an insecticide, K_i is the equilibrium constant of the reversible combination of the enzyme with a competitive inhibitor, K+2 is the inactivation rate constant and k+3 the rate of spontaneous reactivation of the inactive enzyme. Under this scheme, formation of the inactive enzyme–insecticide complex (AChE-A) proceeds through an intermediary reversible complex designated AChE*AB. Measurements of K_i for each insecticide were derived by plotting $1/V$ against insecticide concentration at several different substrate values [23].

3. Results and discussion

3.1. Cloning and sequence analysis of Aedes Ace gene

A 308-bp fragment of the *Aedes Ace* gene, encompassing most of the coding region between the histidine and glutamic acid residues of the catalytic triad, was amplified using degenerate primers based on knowledge of conserved regions of insect AChE sequence data [24–26]. This fragment was used to probe an adult cDNA library at high stringency. A positive clone containing an insert size of ~4 kb was subcloned into Bluescript KS+ (Stratagene). Sequence data confirmed that the cDNA contained the entire open reading frame of the *Aedes Ace* gene. The predicted amino acid sequence of the open reading frame of the *Aedes* gene is illustrated in Fig. 3. The sequence shows a high degree of amino acid identity with that previously reported for both *Anopheles stephensi* (87%) [25] and *Drosophila melanogaster* (64%) [24].

Several features common to all cholinesterases are evident in the predicted amino acid sequence of the *Aedes* gene. The three conserved residues equivalent to those constituting the catalytic triad in *Torpedo* AChE are found in positions corresponding to those likely to constitute the catalytic triad in other invertebrate AChE sequences [24–27]. These residues, relative to the equivalent residues in *Drosophila Ace* gene are: serine258(276), glutamate387(405) and histidine 501(518). Another conserved feature of the *Aedes* sequence is the six amino acid consensus sequence FGESAG surrounding the active site serine. This peptide motif is common to all vertebrate [28–35] and invertebrate [24–27] cholinesterases studied to date.

As noted by Hall and Malcolm [25], both the putative signal peptide domain and C-terminus of the insect AChE protein are highly divergent. Previous studies in *Drosophila* have shown that the start of the mature polypeptide is at V39, giving a signal peptide of 38 amino acids [5]. By analogy, the mature *Anopheles* protein is predicted to begin at I30 [25] or at the equivalent site I29 in *Aedes*. Studies in *Drosophila* have also shown that the non-homologous C-terminal domain of the protein is removed and replaced by a glycolipid anchor [5,36,37]. As the predominant form of AChE in insects is a globular amphiphilic dimer attached to the membrane via a glycolipid anchor [2], it is therefore likely that *Aedes* AChE gene undergoes the same post-translational modifications at its C-terminus as those described for the *Drosophila* AChE protein.

An additional region of sequence divergence between *Drosophila* and other insect acetylcholinesterases [25,26] is the hydrophilic region located between Arg-148 and Pro-180 in *Drosophila* AChE, where endoproteolytic cleavage of the 75KDa precursor protein into two non-covalently linked polypeptides takes place [37]. Although lacking sequence homology with the *Drosophila* hydrophilic peptide, the corresponding region in the two mosquito AChE peptide sequences (Fig. 3) is also hydrophilic and interestingly, exhibits complete amino acid identity. This hydrophilic insertion appears to be unique to insect acetylcholinesterases and is absent in both nematode *Ace-1* [27] and vertebrate cholinesterase sequences [28–35], lending support to the hypothesis that proteolytic cleavage of the AChE precursor protein could be a common mechanism in insects [25,26].

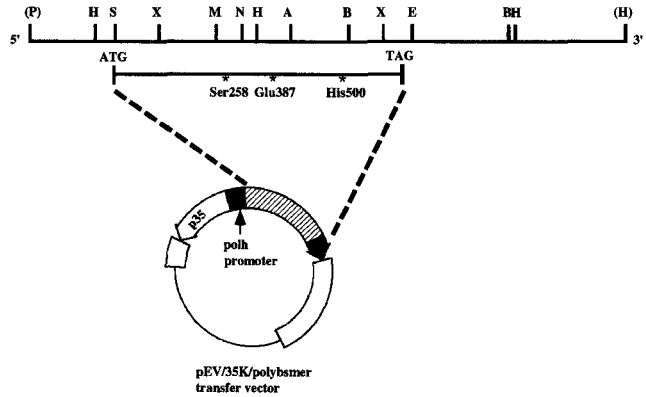

Fig. 2. Restriction map of the full-length *Aedes aegypti Ace* cDNA, including the *Sun*1 site (S) *Eco*N1 site (E), used to remove most of the 5′ and 3′ flanking DNA. The engineered cDNA was subcloned into the pEV/35K/polybsmer transfer vector and cotransfected into *Sf*21 cells with the vΔ35K/lacZ parent viral DNA.

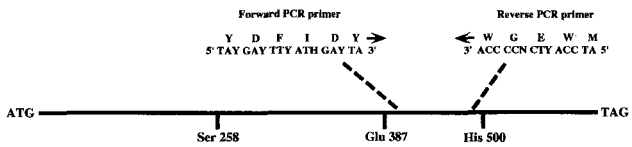

Fig. 1. Degenerate PCR strategy used to clone a fragment of the *Aedes aegypti Ace* gene. PCR primers were designed from sequences conserved between the *Drosophila* and *Anopheles stephensi* genes and amplify a 308-bp fragment between two putative members of the catalytic triad: glutamic acid 387 and histidine 501. (The location of the degenerate primers in relation to the predicted *Ace* amino acid sequence is indicated in Figure 3).

Table 1
Kinetic analysis of acetylcholinesterease activity in *Sf*21 cells infected with recombinant baculovirus containing the *Aedes Ace* cDNA

K_m (μM)	13.79 ± 1.78
V_{max} (mOD/min/10^4 cells)	36.79 ± 4.28
Carbofuran	
K_i (μM)	0.084 ± 0.003
Propoxur	
K_i (μM)	0.238 ± 0.078
Azinphosmethyl oxon	
K_i (μM)	0.266 ± 0.069
Paraoxon	
K_i (μM)	4.633 ± 2.070

Kinetic constants ± S.E.M. (kinetic constants derived from 3–5 separate experiments).

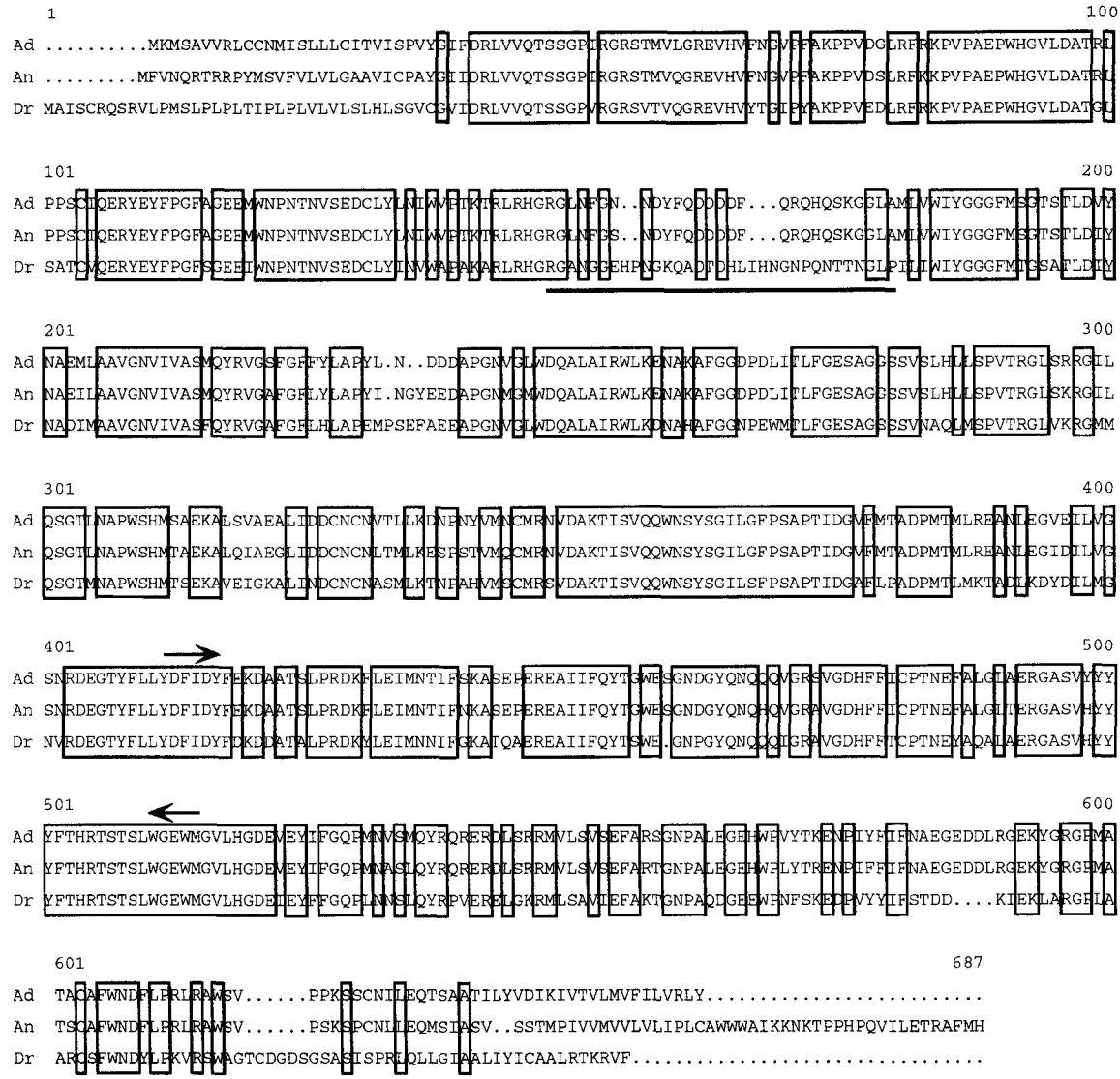

Fig. 3. Comparison of the predicted amino acid sequences of the *Ace* gene from *Aedes aegypti* (Ad), *Anopheles stephensi* (An) and *Drosophila melanogaster* (Dr). Boxed residues indicate sequence identity. The region corresponding to the hydrophilic peptide sequence (underlined), absent in vertebrate cholinesterase sequences, where endoproteolytic cleavage of the *Drosophila* precursor protein occurs [37], is shown. (Arrows show the location and direction of the degenerate primers used to amplify a fragment of the *Aedes Ace* gene.).

3.2. *In vitro expression of* Aedes Ace *cDNA*

Previous expression studies of the *Drosophila Ace* gene have demonstrated that the baculovirus insect cell system is capable of carrying out correct post-translational modification of AChE protein [38]. We therefore chose this system to express the *Aedes Ace* gene. No measurable levels of AChE activity were detected in uninfected cells or in cells infected with either the wild-type or recombinant *Rdl* baculovirus (Fig. 4). The catalytic properties and sensitivity of the expressed AChE protein to insecticide inhibition were examined. Eadie–Hofstee plots of the transformed data yielded a mean K_m value of 13.79 ± 1.78 μM and a mean V_{max} value of 36.79 ± 4.28 mOD/min/1.0 × 10^4 cells (Table 1, Fig. 5). Estimates of K_i of the enzyme in the presence of several organophosphorus and carbamate insecticides are presented in Table 1. Both organophosphorus and carbamate insecticides inhibit AChE activity competitively although the affinity of the enzyme for these inhibitors varies over more than one order of magnitude. The order of effectiveness of the insecticides examined in this study as enzyme inhibitors was as follows: carbofuran > azinphosmethyl oxon = propoxur > paraoxon. Analysis of the effects of different insecticides on the kinetic parameters of this enzyme will greatly facilitate future studies aimed at determining the changes in kinetic constants brought about by specific resistance associated mutations.

3.3. Aedes Ace *expression system as a tool for site-directed mutagenesis studies*

Since the early documentation of insensitive AChE as a mechanism of insecticide resistance in the two spotted spider mite *Tetranychus urticae* [39], subsequent studies have demonstrated the importance of altered AChE as a mechanism of

resistance to organophosphorus and carbamate insecticides in many insects [7], including several mosquito species [40]. More recently, a number of resistance associated mutations have been identified in the *Ace* gene from insecticide resistant strains of *Drosophila* [16,41]. From the latter study, the authors postulate that high levels of resistance to organophosphorus and carbamate insecticides are achieved through a combination of weak mutations within the same gene, some of which may thus remain unidentified [16]. We are therefore interested in examining the role that specific resistance associated mutations may play in altering enzyme function.

Previous biochemical studies have examined the effects of altered AChE on substrate hydrolysis and the kinetic parameters affecting insecticide inhibition in several insect species (cited in [8]). Site-directed mutagenesis of several *Ace* genes has also proved useful in examining the effects of introduced mutations on insecticide sensititivity and the role of different residues in substrate hydrolysis. Mutagenesis of the *Drosophila Ace* gene has demonstrated a correlation between the number of introduced mutations in the expressed protein and the bimolecular rate constant (k_i) ratio of the mutated vs. wild-type form of the enzyme [16]. Using the *Torpedo* acetylcholinesterase gene as a model, Radic and colleagues [42] conducted detailed kinetic studies examining the effects of two conservative substitutions of Glu199 on the kinetic properties of the enzyme. This residue is located deep within the active site gorge and close to the substrate binding site. Replacements of this residue with either aspartate or glutamine decreased affinity of the enzyme for cholinesterase inhibitors and reduced the rate of phosphorylation or carbamoylation of the enzyme, indicating that mutations at this residue in insect AChE could also confer resistance to organophosphorus and carbamate insecticides.

Site-directed mutagenesis has also identified several residues in the *Torpedo* AChE affecting substrate specificity [43,44]. Coincidentally, a mutation at one of these residues in *Drosophila* has been identified in more than one field collected insecticide resistant strain [16]. However, detailed studies on the functional consequences of putative resistance associated mutations on the kinetic properties, substrate specificity and affinity of an insect AChE to insecticide inhibitors are lacking. Our

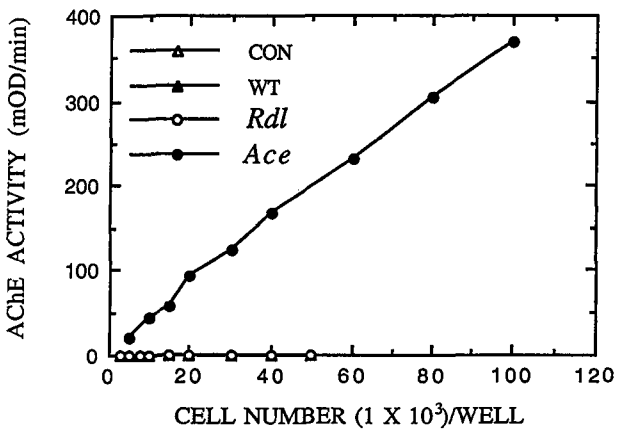

Fig. 4. The effect of increasing infected *Sf*21 cell density on AChE activity. Activity was monitored over a range of cell densities in uninfected cells (CON), cells infected with the wild-type virus (WT), cells infected with the GABA receptor gene *Rdl* 14.1 construct (*Rdl*) and cells infected with the recombinant acetylcholinesterase gene (*Ace*).

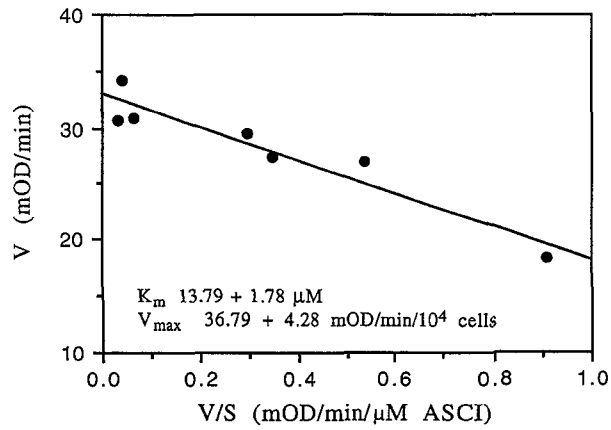

Fig. 5. Representative Eadie–Hofstee plot of AChE activity (V) over (V/S). Mean estimates of K_m and V_{max} are based on values derived from five separate experiments.

future work will therefore focus on investigating the functional consequences of introduced point mutations on substrate hydrolysis, and the kinetic parameters that influence the sensitivity of the enzyme to insecticide inhibitors. The gene expression system described in this study will thus be an invaluable tool in examining the role that key residues play in enzyme function and target site insecticide resistance.

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