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An amino acid substitution attributable to insecticide-insensitivity of acetylcholinesterase in a Japanese encephalitis vector mosquito, *Culex tritaeniorhynchus*☆

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Abstract

A cDNA sequence encoding a *Drosophila Ace*-paralogous acetylcholinesterase (AChE) precursor of 701 amino acid residues was identified as the second AChE gene (*Ace2*) transcript from *Culex tritaeniorhynchus*. The *Ace2* gene is tightly linked to organophosphorus insecticide (OP)-insensitivity of AChE on chromosome 2. The cDNA sequences were compared between an insecticide-susceptible strain and the resistant strain, TYM, that exhibits a 870-fold decrease in fenitroxon-sensitivity of AChE. Two amino acid substitutions were present in TYM mosquitoes. One is F455W whose homologous position in *Torped* AChE (Phe331) is located in the vicinity of the catalytic His in the acyl pocket of the active site gorge. The other substitution is located to a C-terminal Ile697 position that apparently seems to be excluded from the mature protein and is irrelevant to catalytic activity. The F455W replacement in the *Ace2* gene is solely responsible for the insecticide-insensitivity of AChE in TYM mosquitoes.

Keywords: Acetylcholinesterase; Insecticide resistance; Organophosphate; Mosquito; Linkage map

Acetylcholinesterase (AChE, EC 3.1.1.7) hydrolyzes the neurotransmitter acetylcholine (ACh) to terminate neuronal excitement at the postsynaptic membrane. AChE is a target of organophosphorus (OP) and carbamate compounds. These compounds remain widely used pesticides, even though novel compound groups and biopesticides are currently available. Insecticide-resistance due to insensitive AChE was first reported for the two-spotted spider mite in 1964 [1,2]. More than 33 insect and acari species have developed insecticide-resistance through decreased sensitivity of AChE [3].

Since the first cloning of an insect AChE gene (*Ace*) from *Drosophila melanogaster* in 1986 [4], a focus of interest in insect toxicology has been to elucidate mutations responsible for insecticide-insensitivity and 41 *Ace*-orthologous AChE (AO-AChE) sequences have been registered with databases to date (http://bioweb.ensam.inra.fr/ESTHER/general?what=index). Several resistance-associated protein polymorphisms were confirmed as conferring decreased sensitivity of the AO-AChEs in the three flies (Cyclorrhapha dipterans), *D. melanogaster* [5], *Musca domestica* [6,7], and *Lucilia cuprina* [8]. In *D. melanogaster*, no other cholinesterase family member than the single *Ace* gene has been identified [9]; *Ace*-defective homozygous mutants are embryonic lethals [10]; a major amphiphilic globular dimer isoform of AChE is localized in the central nervous system [11,12]. Based largely on available information

☆ Amino acid position numbers appearing dually in parentheses denote the homologous positions of mature acetylcholinesterase in *Torped californica*.

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from flies, it has been suggested that only a single AChE locus existed in all insect genomes [13,14]. However, several studies documented the presence of insensitive AChE mediated insecticide resistance, but were unable to identify any insensitivity-specific mutation in the ortholog to the *D. melanogaster Ace* gene including transcripts obtained from two mosquitoes (Orthorrhapha dipterans), *Culex pipiens* [15] and *Culex tritaeniorhynchus* [16], two aphids, *Myzus persicae* [17] and *Aphis gossipii* [14], the green rice leafhopper, *Nephotettix cincticeps* [13], and the rice leaf beetle, *Oulema oryzae* (Tomita, unpublished). Furthermore, the *Ace* gene and the insecticide-insensitivity of AChE were localized to different linkage groups in the two mosquito species [15,16]. The apparent contradictory results for the Cyclorrhapha dipterans and the other taxonomic groups indicated that the molecular mechanisms underlying insensitive AChEs in insects had not been elucidated completely.

A breakthrough in this toxicological riddle was achieved in 2002. An *Ace*-paralogous AChE (AP-AChE) cDNA was cloned from the greenbug *Schizaphis graminum* [18] and a putative homolog was identified following genome sequence determination in the mosquito *Anopheles gambiae* [19]. Since these determinations, four additional AP-AChE cDNA sequences have been reported for insects including the mosquitoes *Cx. pipiens* [20] and *Aedes aegypti* (GB: Accession No. AJ428049), and the aphids *A. gossipii* [21] and *M. persicae* [17]. In *Cx. pipiens*, the amino acid substitution G119S has recently been shown to be responsible for an elevated level of propoxur-insensitivity [20].

Culex tritaeniorhynchus is a primary vector of Japanese encephalitis virus, with a distribution throughout Southeast Asia and South Asia. Continuous application of pesticides to rice fields has resulted in countrywide insecticide-resistance throughout Japan [22]. The principal mechanism of OP- and carbamate-resistance in these regions is due to insensitive AChE [23]. Insensitive AChE in the resistant Toyama strain has been well characterized in previous studies including the demonstration of: a 870-fold decrease in fenitroxon-sensitivity as well as a comparable level of decreased sensitivity to several in vivo active forms of OPs and to a lesser extent to carbamate insecticides; loss of optimum pH and substrate concentration; and lowered affinity to artificial substrates such as acetylthiocholine (ATCh), yet retaining a high affinity to the natural substrate, acetylcholine (ACh) [24].

In this study we isolated an AP-AChE cDNA sequence from *Cx. tritaeniorhynchus*, demonstrated tight linkage between the AP-AChE locus and genetic insensitivity of AChE, and identified a unique insensitivity-associated single amino acid substitution compared with that reported for *Cx. pipiens*. We have designated the AO- and AP-AChE genes in *Cx. tritaeniorhynchus* as *Ace1* and *Ace2*, and their deduced protein sequences as CtAChE1 and CtAChE2, respectively.

Materials and methods

Mosquitoes. Two *Cx. tritaeniorhynchus* strains maintained in the National Institute of Infectious Diseases were used to prepare enzyme assays, cDNA analysis, and linkage analysis. An insecticide-susceptible strain, Kochi (KCH), was originally provided by Dr. Matsuzaki at the Kochi Women's College in Kochi Prefecture, Japan [16]. The Toyama (TYM) strain was established with mosquitoes collected from an insecticide-resistant field population in Toyama prefecture, Japan, in 1991 by Dr. Watanabe at the Toyama Institute of Health [25]. Mosquitoes were reared under constant conditions at 25 °C and 16L:8D without insecticide selection.

Enzyme assays. Whole bodies of adult mosquitoes were individually homogenized on ice in 600 μl of 50 mM Tris–HCl (pH 8.05) containing 0.1% Triton X-100 with a polytron. The homogenate was centrifuged at 900*g* for 10 min at 4 °C and then the supernatant was tested for AChE activity. The DTNB method of Ellman et al. [26] was adopted with minor modification in order to detect inhibition of AChE activity by microtiter assay. AChE inhibition was assayed in a 240 μl reaction containing 40 μl of the supernate in each well, at final concentration of 40 mM Tris–HCl (pH 8.05), 1.8 mM ATCh iodide, 1 mM of 5,5′-dithiobis 2-nitrobenzoic acid, 1.8 mM $NaHCO_3$, and either 1–1000 μM of fenitroxon or edrophonium, or a comparative volume of H_2O. Residual AChE activity was measured at 40 min following preparation of reaction mixtures. Procedures for reaction conditions, measurements of OD, and calculations of enzyme activity followed a previous study [24]. Fenitroxon and edrophonium were purchased from Wako Pure Chemical Industries (Tokyo, Japan).

cDNA analysis. RNA was extracted from a pool or individual homogenates of adults within 7 days after emergence with Isogen (Nippon Gene, Tokyo, Japan). Poly(A)$^+$ RNA was selected from the pooled RNA extract from the KCH strain with Oligotex-(dT) Super (Takara, Ohtsu, Japan). The oligonucleotides used for RT-PCR in primer walking are shown in Table 1. cDNA was synthesized with ReverTra Ace (Toyobo, Tokyo, Japan) and oligo(dT) primer (step #1 in Table 1). cDNA was amplified with Ex*Taq* DNA polymerase (Takara). Degenerate primers (step #2) were used for amplifying the first cDNA fragment. Then RACE methods were conducted to amplify the rest of the coding sequence (step #3.1–4.2). 5′ RACE following the cRACE method [27] was performed with the 5′-Full RACE Core Set (Takara). Total RNAs derived from individual KCH and TYM mosquitoes were reverse-transcribed and an inner cDNA segment (base #555–2868) was obtained for confirmatory sequencing by nested PCR (step #5–5.2). Amplified sequences were determined by direct sequencing. Sequencing reactions were performed with the BigDye Terminator v1.1 Cycle Sequencing Kit (ABI, Tokyo, Japan) and then analyzed on a Genetic Analyzer 310 system (ABI). The cloning strategy of the *Ace1* cDNA and its protein structural characteristics will be described elsewhere (DDBJ No. AB122151; Harada and Hidoh, unpublished).

Linkage analysis. Genetic linkage analysis was performed using BC_1 backcross progeny ($n = 166$) initiated from KCH females and TYM males. F_1 males were backcrossed to TYM females. Southern blots containing *Eco*RI-digests for the segregating population were prepared in a previous study that mapped the AO-AChE locus (utilizing an *Ae. aegypti* AChE cDNA probe) and insecticide-insensitivity of AChE [16]. The same nylon membranes were reused in this study using the endogenous *Ace1* and *Ace2* cDNAs as probes. High stringency hybridizations were performed at 65 °C and membranes were washed for 15 min each at room temperature and at 65 °C in 2× SSC–0.1% SDS followed by 15 min at 65 °C in 0.2× SSC–0.1% SDS as previously described [28].

Computer programs. Multipoint linkage analysis was performed using the MAPMAKER/QTL computer program [29]. The default minimum threshold LOD score of 3.0 was used to identify linkage between loci. Presence of a signal peptide was predicted using SignalP

Table 1
Primers used for RT-PCR

Step #	Reaction	Forward primer	Reverse primer	Synthesized inner base #[a]
Primary cDNA walking				
1	RT[b]	–	Q0–Q1′-$(dT)_{15}$	⩽3439
2	PCR	GTiACiATGTGGAAyCCiAA	GGrTTiCCiGTyTTiGCrAA	1382–2543
3.1	3′ RACE	TATCTGTACACGCACAGAAGCA	CCAGTGAGCAGAGTGACG (=Q0)	2401–3439
3.2	3′ RACE	ACGAGATCAACTACGTGTTTGG	GAGGACTCGTGCTCAAGC (=Q1′)	2465–3439
4	RT[c]	–	(5′-phosphorylated-) CTGGAACAGGTC	⩽1766
4.1	5′ RACE	GCGTAACACTGTTCGGCGAAAG	TGTTGTCGCGGACCCATCTC	1–1648, 1718–1766
4.2	5′ RACE	GTCTCGGTTTCGCTGCATCT	GCCAGATTCTGATCAAACAGTC	1–1623, 1748–1766
Confirmatory cDNA walking				
5	RT	–	Q0–Q1′-$(dT)_{15}$	⩽3439
5.1	PCR	CTTCTGCGTTACAACAGTGAG	CCGCTAGTGCATAAATCGCTC	449–3314
5.2	PCR	GAGTCATCGTCGTAGTTGGT	GTGTGTTGTAACGGAAGGGA	555–2868

"i" denotes deoxyinosine.
[a] Base #744 and 2849 correspond to the first position of start codon (ATG) and the third position of stop codon (TAA), respectively.
[b] Provided cDNA for step 2 and 3.1.
[c] Provided ligation-mediated circularized first strand cDNA for step 4.1.

v1.1 [30]. A molecular phylogenetic tree was constructed with ClustalX v1.8 [31] using the bootstrap N–J tree option (number of bootstrap trials = 1000). The 3D modeling of the AChE active center was performed by MOE 2000.2 (CCG, Montreal, Canada) based on the *Torpedo* AChE (PDB: 1EA5).

Results

AChE inhibition

Fenitroxon is an in vivo active metabolite of an OP insecticide, fenitrothion, that irreversibly acylates the serine hydroxyl group of AChE to halt hydrolysis. Fig. 1 shows in vitro AChE inhibition with fenitroxon using individual mosquitoes of the insecticide-susceptible KCH and -resistant TYM strains. The results indicate genetic homogeneity in the inhibitory response within each strain and a distinctive difference in sensitivity between the two strains. A previous study estimated IC_{50} values of fenitroxon at 1.48×10^{-7} and 1.29×10^{-4} M for a different susceptible reference strain and the TYM strain, respectively [24]. Inhibitory responses observed in the present study were similar to those from the previous study.

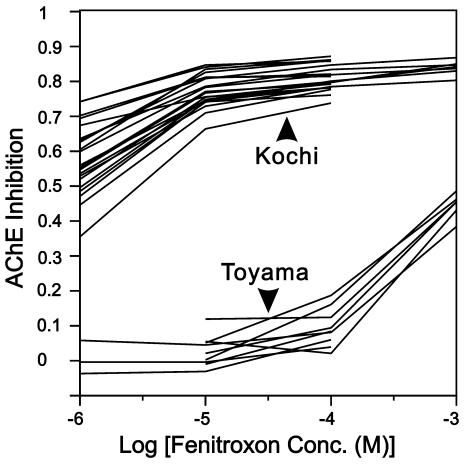

Fig. 1. In vitro AChE inhibition with fenitroxon based on individual mosquitoes. KCH ($n = 21$) and TYM ($n = 9$) are insecticide-susceptible and -resistant strains, respectively.

Edrophonium is a competitive AChE blocker that inhibits the enzyme by binding to the active site without reacting with the enzyme and is often used for isolating AChE as a ligand for affinity chromatography. The TYM strain mosquitoes showed a 16-fold decrease in sensitivity to edrophonium inhibition (Table 2).

cDNA and protein sequences of Ace2

As many as 3439 bases of the *Ace2* cDNA sequence were determined from a pool of KCH mosquitoes by primer walking, initially based on conserved peptide sequences of AP-AChEs from *S. graminum* [18] and *An. gambiae* [20], and registered with the DDBJ (Accession No. AB122152). The cDNA sequence contained a 2106 bp ORF (base #744–2849) encoding a 701 amino acid residue AP-AChE precursor, 743 bases of partial 5′ UTR containing multiple stop codes prior to the first Met, and 590 bases of 3′ UTR containing three copies of the standard polyadenylation signal, AATAAA.

The deduced protein precursor sequence of *Ace2* (that is CtAChE2 sequence) from the KCH strain is

Table 2
AChE inhibition with edrophonium

Strain	KCH	TYM
IC_{50} (M)	6.2×10^{-6}	1.0×10^{-4}
Ratio	–	16

shown in Fig. 2, with three other AP-AChEs from *Cx. pipiens*, *An. gambiae*, and *S. graminum*, and AChEs from *D. melanogaster* and *Torped californica*. The CtAChE2 precursor includes a putative 42 amino acid signal peptide and a vertebrate H-peptide like segment. Common features of AChE are conserved in CtAChE2: the Ser325, Glu451, and His565 residues of the catalytic triad; the 6 Cys residues for forming 3 intra-subunit disulfide bonds; 12 out of the 14 aromatic residues that are located in the active site gorge of *T. californica*.

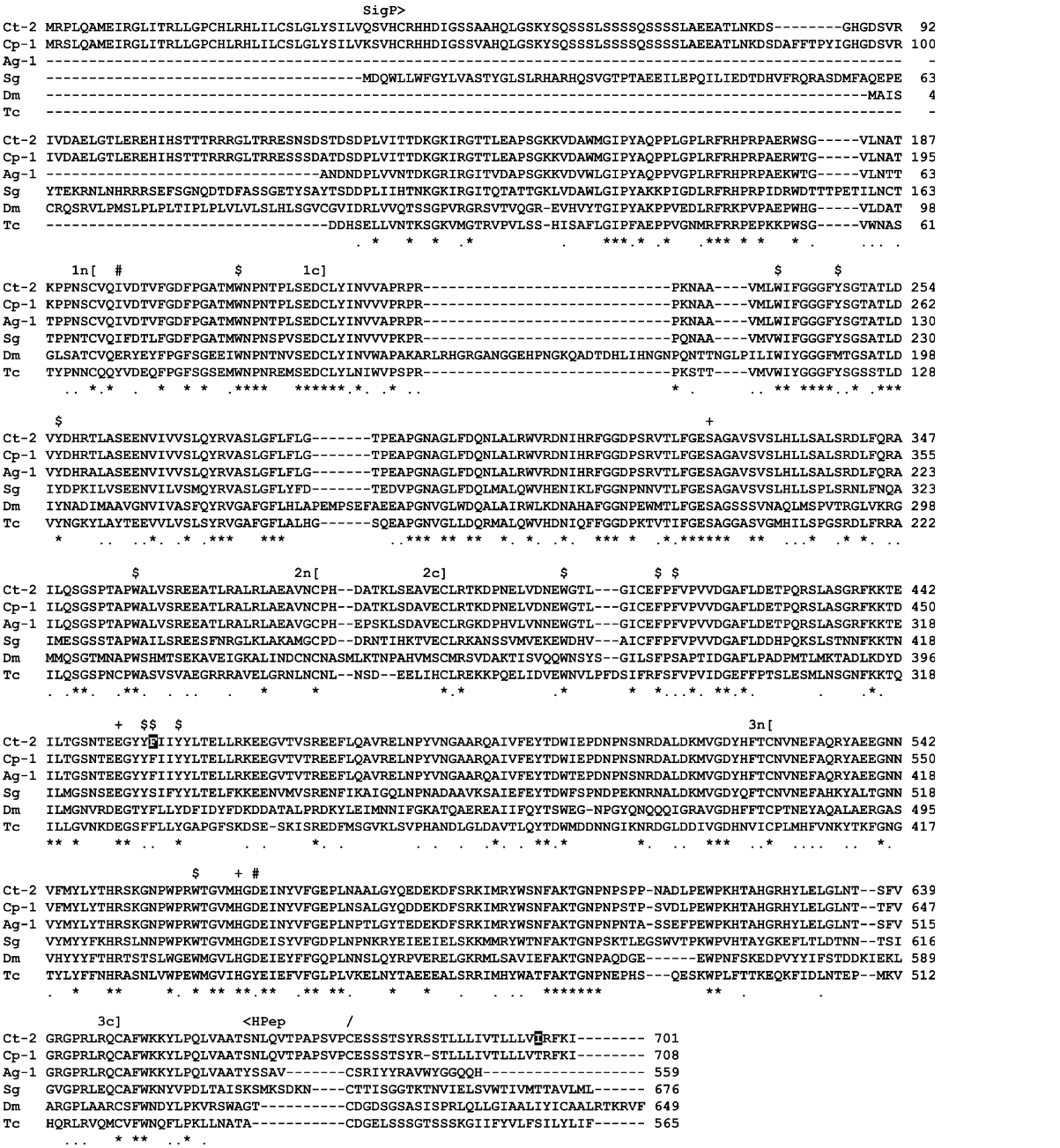

Fig. 2. Alignment of acetylcholinesterase sequences. Ct-2, Cp-1, Ag-1, and Sg show *Drosophila Ace*-paralogous AChEs from *Culex tritaeniorhynchus* and *Cx. pipiens*, *Anopheles gambiae*, and *Schizaphis graminum*. Dm and Tc show AChEs from *Drosophila melanogaster* and *Torped californica*. *T.c.* AChE sequence starts from N-terminal of the matured protein. See legend to Fig. 3 for their Accession numbers. Asterisks and dots denote the amino acid positions that are identical among all sequences and identical except for one sequence within the range from #1 to 535 in *T.c.* AChE sequence, respectively. Residues involved in catalytic triad of *T.c.* AChE are shown in crosses. Aromatic residues of the active site gorge in *T.c.* AChE that are conserved and not involved in insect AChEs are shown in dollar and sharp symbols, respectively. Square brackets and a slash denote the Cys residues forming intra- and inter-subunit disulfide bonds in *T.c.* AChE, respectively, and codes aside square brackets denote numbers of intra-subunit disulfide bond (#1–3) as well as N- or C-proximity of the Cys involved ("n" or "c"). Signs of G.T. and L.T. inequalities denote C-terminal of the predicted signal peptide in *C.t.* AChE and N-terminal of the H-peptide in *T.c.* AChE, respectively. The alignment corresponding to H-peptide sequences was manually edited.

A molecular phylogenetic tree involving CtAChE1 and CtAChE2 as well as currently available insect AO- and AP-AChEs whose registered sequences nearly cover the expected mature protein sequences was constructed and is shown in Fig. 3. CtAChE1 and CtAChE2 are located within mosquito subgroups of the respective insect AChE gene families. CtAChE2 precursor has the most similarity (97% identity) with AP-AChE from *Cx. pipiens* (Fig. 2).

An inner *Ace2* cDNA fragment (#555–2868) involving the complete coding sequence was analyzed for genotype by direct sequencing using four mosquitoes each from the KCH and TYM strains. The cDNA sequences examined appeared to be homozygous and no intra-strain genetic polymorphisms were observed. There were 18 base substitutions in TYM mosquitoes (not shown) and three of them were involved in nonsynonymous substitutions which were related to two amino acid replacements, F455W (TTT–TGG) and I697M (ATA–ATG). I697M, near the C-terminus (Ile701), occurs in the H-peptide-like segment that is characterized by the Cys forming inter-subunit disulfide bond at its C-distal half and a series of hydrophobic residues at its C-proximal half. The H-peptide is involved in all the insect AO- and AP-AChE precursors known to date, independent of alternative-splicing, in contrast to the vertebrate AChEs. It is known that the hydrophobic C-proximal half of the H-peptide becomes replaced with a GPI anchor and is embedded in the postsynaptic membrane in vertebrate AChEs [32] and *Drosophila* AChE [4]. Thus, the I697M replacement is probably ruled out as the cause of AChE insensitivity. The other replacement, F455W, is the sole nonsynonymous substitution within the predicted mature enzyme of CtAChE2 and likely confers insecticide insensitivity in TYM.

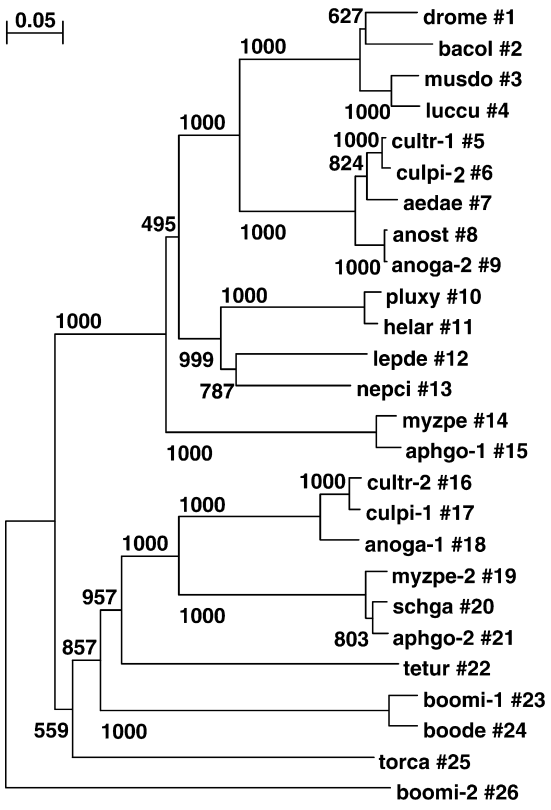

Fig. 3. Molecular phylogenetic tree of acetylcholinesterases from arthropoda. *Drosophila Ace*-orthologous insect sequences, #1–15, are derived from X05893 (*Drosophila melanogaster*), AF452052 (*Bactrocera oleae*), AF281161 (*Musca domestica*), U88631 (*Lucilia cuprina*), AB122151 (*Culex tritaeniorhynchus*), [15] (*Cx. pipiens*), G1245693 (*Aedes aegypti*), 1808210A (*Anopheles stephensi*), AAAB01008846 (*An. gambiae*), AY061975 (*Plutella xylostella*), AF369793 (*Helicoverpa armigera*), L41180 (*Leptinotarsa decemlineata*), AF145235 (*Nephotettix cincticeps*), AF287291 (*Myzus persicae*), and AF502081 (*Aphis gossypii*). *Drosophila Ace*-paralogous insect sequences, #16–21, are derived from AB122152 (*Cx. tritaeniorhynchus*), Q869C3 (*Cx. pipiens*), EAA01151 (*An. gambiae*), AF287291 (*M. persicae*), AF321574 (*Schizaphis graminum*), and AF502082 (*A. gossypii*). AChE sequences from arthropoda other than insecta, #22–24 and 26, are derived from AY188448 (*Tetranychus urticae*), AJ223965 (*Boophilus microplus*), AJ006337 (*B. decoloratus*), and AJ278342 (*B. microplus*). A fish AChE sequence of *Torped californica*, #25 from P04058 was set to be an outgroup to insect AChEs. The tree was constructed with the segments corresponding to #1 to 535 in *T.c.* AChE (see Fig. 2 for the range).

Genetic mapping of two AChE loci

In our previous study, the genetic insecticide-insensitivity of AChE ($AChE^R$) and an AO-AChE locus were mapped on different chromosomes, although, the latter was performed by probing with a heterologous AO-AChE cDNA from *Ae. aegypti* [33]. Two endogenous cDNA probes for *Ace1* and *Ace2* genes were used in the

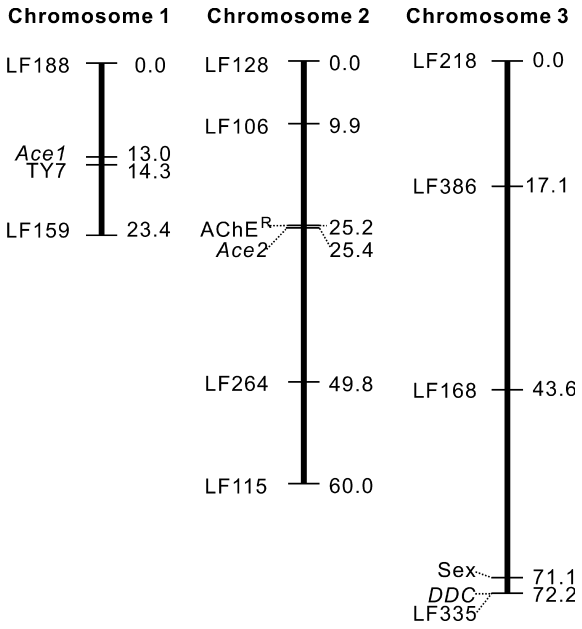

Fig. 4. Linkage map of *Cx. tritaeniorhynchus*. Structural loci for two AChE genes and a locus for insecticide-insensitivity of AChE are denoted by *Ace1*, *Ace2*, and $AChE^R$, respectively.

present study. *Eco*RI RFLP patterns obtained with the *Ace1* probe on Southern blots were identical to those previously obtained with the *Ae. aegypti* probe (not shown) [16], which confirms the position of the *Cx. tritaeniorhynchus Ace1* locus on chromosome 1 as previously reported (Fig. 4). The *Ace2* locus was mapped to a region within 0.2 cM of the $AChE^R$ phenotype on chromosome 2 (Fig. 4). Further, there were no amino acid replacements in CtAChE1 from TYM mosquitoes except for a segregating amino acid substitution polymorphism (Val/Ala7) in a predicted signal peptide (Hidoh, unpublished). Thus, only mutation(s) in the *Ace2* gene are associated with the insensitive AChE phenotype.

Discussion

The integrated results of sequence and linkage analyses indicate that CtAChE2 is likely the primary insecticidal target and the F455W replacement is likely responsible for conferring insecticide-insensitivity of AChE in TYM mosquitoes. The Phe455 position in CtAChE2 being homologous to the Phe331 of AChE in *T. californica* (abbr. TcAChE) is located in the acyl pocket neighboring the active center in the active site gorge [34] (Fig. 5). This position is not homologous to other reported amino acid substitutions responsible for conferring decreased sensitivity: Phe78 in the choline-binding site, Gly227, and Phe290 in the acyl pocket of AO-AChEs from three Cyclorrhapha fly species [5–8]; and Gly119 in the oxyanion hole of AP-AChE from *Cx. pipiens* [20] (all these positions are shown in TcAChE numbering). The novel substitution in CtAChE2, as well as G119S of AP-AChE in *Cx. pipiens*, is associated with an extreme level of insecticide-insensitivity while those identified in AO-AChEs confer moderate decreases in sensitivity (not more than 10^2-fold decreases in k_i value ratio with the compounds used). Therefore, the F455W substitution in CtAChE2 is of toxicological significance.

Two properties of the acyl pocket are responsible for ligand specificity. The first property is principally related to the formation of the enzyme–substrate intermediatary complex. The acyl pocket, being located at the bottom of the active site gorge and surrounded by sidechains of hydrophobic aromatic residues, attracts and orientates the acyl group of the substrate and inhibitors through its hydrophobicity during the catalytic reaction [34,35]. The F455W replacement in CtAChE2 makes the acyl pocket less hydrophobic and smaller and thus the replacement can potentially alter the accessibility of the substrates and inhibitors to this site. AChE from TYM mosquitoes exhibited a 30-fold decrease in edrophonium-sensitivity. Mutagenesis studies with human AChE also demonstrated that Phe338 (Phe331) to Ala replacement in the homologous position conferred a 2-fold decrease in edrophonium-sensitivity [36]. Decreased sensitivity of AChE to edrophonium as well as insecticide inhibitors may come from modified ligand transduction from the surface to the catalytic center because of antagonistic nature of edrophonium that has no esteratic site.

The second property of the acyl pocket derives from its electrostatic field. The pocket forming the sidechain of Phe331 attracts the catalytic His440 by cation–π interaction in TcAChE. X-ray crystallography and computer simulation demonstrated that the sidechain of His440 dynamically changes its orientation by

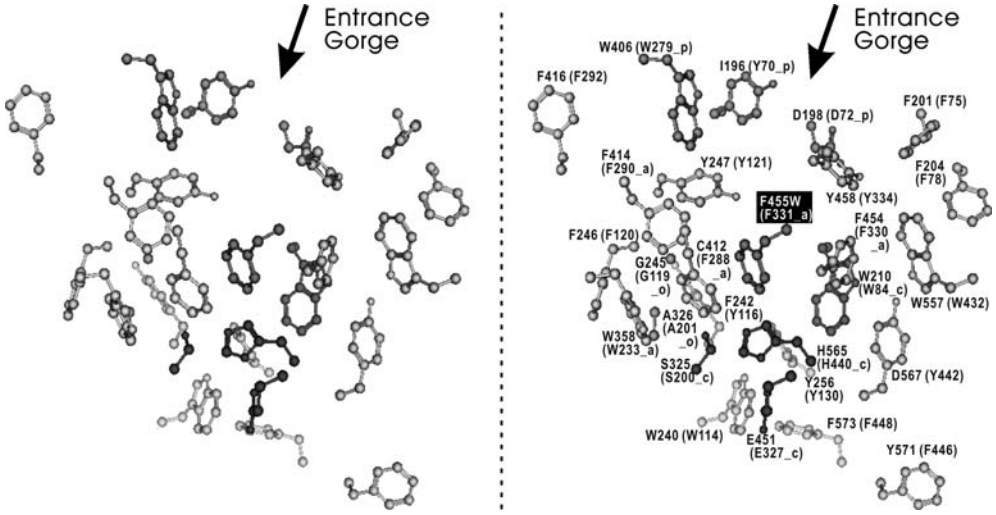

Fig. 5. Stereogram of the active site gorge in acetylcholinesterase. Deduced protein structure from *Ace2* in *Culex tritaeniorhynchus* was superimposed on that of *Torped californica* (PDB: 1EA5). Numbers in parentheses show those of *T.c.* AChE. Functional substructures are also denoted in parentheses: catalytic triad, "_c"; peripheral anionic site, "_p"; oxyanion hole, "_o", cholin-binding site, "_c"; and acyl pocket, "_a". Otherwise denoted are aromatic residues involved in active site gorge in *T.c.* AChE. F455W is the single substitution in the deduced mature enzyme from *Ace2*.

transporting a positive charge during the hydrolyzing process [37,38]. Mutagenesis studies followed by computer simulation demonstrated that the orientation of His447 in human AChE (His440) is changed by the substitution from the wild-type Phe338 (Phe331) to aliphatic residues [39,40]. Thus, Phe331 is considered to arrange the catalytic His so that a proper conformational change of the His can occur in hydrolyzing step. Cation–π interaction can occur between cationic and aromatic sidechains within a protein. However, it is not known how a F331W replacement effects cation–π interaction. F455W substitution in CtAChE2 is considered to have modified ligand specificity by the changes in either ligand affinity or interaction with catalytic His, or both.

Importance of substitutions at TcAChE Phe331-homologous positions for insecticide-insensitivity is also inferred from studies involving several mutagenized and naturally occurring substitutions in insect AChEs. In vitro mutagenesis studies with *Drosophila Ace* demonstrated that replacements from Phe409 (Phe331) to Ala, Gly, Ile, and Tyr resulted in 10^1- to 10^2-fold decreases in carbaryl (a carbamate)-sensitivity and the aromatic Tyr replacement among these tested substitutions resulted in 10^2-fold decreases in malaoxon- and paraoxon-(OP) sensitivity [41]. The Phe331 residue in TcAChE is conserved among most of the known sequences from vertebrate AChEs, mammalian butyrylcholinesterases and insect AO- and AP-AChEs. Interestingly, there are two cases in which resistance-associated amino acid replacements in AChE occur at the homologous position in arthropods. One is S331F (in TcAChE numbering) in pyrimicarb (a carbamate)-insensitive AP-AChE from an aphid *M. persicae* [17]. The aphid AP-AChE with the Phe331 residue was associated with 30- to 50-fold decreases in pyrimicarb-sensitivity [17], however, negative cross-resistance was also identified between pyrimicarb and other carbamate compounds (carbofuran and carbaryl) as well as several OPs that have been applied to control the aphid (Nabeshima, unpublished). The second is F331C (in TcAChE numbering) in OP-insensitive AChE from the two spotted spider mite *Tetranychus urticae* [42]. The AChE in a resistant strain having a single Cys331 substitution exhibited a 140-fold ratio of k_i values for an OP, dichlorvos. Further studies based on various replacements targeting the Phe331-homologous positions may bring us a clue to a deep understanding of the cross-resistance or negative cross-resistance among registered insecticides and exploitation of novel insecticides.

The physiological role of *Ace1* expression is still unknown along with AO-AChEs in non-Cyclorrhapha insects. Concordant with the results of our linkage analysis, no insensitivity specific nonsynonymous mutation has been found in the *Ace1* cDNA from highly OP-resistant field colonies (Tomita, unpublished). Furthermore, we have not identified any apparent dual inhibitory response of AChE from either the KCH and TYM mosquitoes (Fig. 1; Hidoh, unpublished data), though in *Cx. pipiens* such dual inhibitory kinetics are always obtained both in insecticide-susceptible and -resistant mosquitoes due to expressions of two AChE genes [43]. There may be a considerable difference in the expression level of AO-AChE genes between these two mosquito species. In the four AChE genes of *Caenorhabditis elegans*, only *ace-2* is expressed almost exclusively in neurons and *ace-4* exhibits a very low level of mRNA expression and undetectable enzyme activity [44]. To elucidate the functional differentiation among the AO- and AP-AChE gene families in non-Cyclorrhapha insects, tissue-specific expressions should be further studied.

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References

[1] H.R. Smissaert, Cholinesterase inhibition in spider mites susceptible and resistant to organophosphate, Science 143 (1964) 407–409.
[2] G. Voss, F. Matsumura, Resistance to organophosphorus compounds in the two-spotted spider mite: two different mechanisms of resistance, Nature 202 (1964) 319–320.
[3] D. Fournier, A. Mutero, Modification of acetylcholinesterase as a mechanism of resistance to insecticides, Comp. Biochem. Physiol. 108C (1994) 19–31.
[4] L.M. Hall, P. Spierer, The *Ace* locus of *Drosophila melanogaster*: structural gene for acetylcholinesterase with an unusual 5′ leader, EMBO J. 5 (1986) 2949–2954.
[5] A. Mutero, M. Pralavorio, J.M. Bride, D. Fournier, Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase, Proc. Natl. Acad. Sci. USA 91 (1994) 5922–5926.
[6] T. Kozaki, T. Shono, T. Tomita, Y. Kono, Fenitroxon insensitive acetylcholinesterases of the housefly, *Musca domestica* associated with point mutations, Insect Biochem. Mol. Biol. 31 (2001) 991–997.
[7] S.B. Walsh, T.A. Dolden, G.D. Moores, M. Kristensen, T. Lewis, A.L. Devonshire, M.S. Williamson, Identification and characterization of mutations in housefly (*Musca domestica*) acetylcholinesterase involved in insecticide resistance, Biochem. J. 359 (2001) 175–181.
[8] Z. Chen, R. Newcomb, E. Forbes, J. McKenzie, P. Batterham, The acetylcholinesterase gene and organophosphorus resistance in the Australian sheep blowfly, *Lucilia cuprina*, Insect Biochem. Mol. Biol. 31 (2001) 805–816.
[9] M. Weill, P. Fort, A. Berthomieu, M.P. Dubois, N. Pasteur, M. Raymond, A novel acetylcholinesterase gene in mosquitoes codes for the insecticide target and is non-homologous to the *ace* gene in *Drosophila*, Proc. R. Soc. Lond. B. Biol. Sci. 269 (2002) 2007–2016.

[10] F. Hoffmann, D. Fournier, P. Spierer, Minigene rescues acetylcholinesterase lethal mutations in *Drosophila melanogaster*, J. Mol. Biol. 223 (1992) 17–22.
[11] J.P. Toutant, M. Arpagaus, D. Fournier, Native molecular forms of head acetylcholinesterase from adult *Drosophila melanogaster*: quaternary structure and hydrophobic character, J. Neurochem. 50 (1988) 209–218.
[12] E. Zador, J. Gausz, P. Maroy, Tissue specific expression of the acetylcholinesterase gene in *Drosophila melanogaster*, Mol. Gen. Genet. 218 (1989) 487–490.
[13] T. Tomita, O. Hidoh, Y. Kono, Absence of protein polymorphism attributable to insecticide-insensitivity of acetylcholinesterase in the green rice leafhopper, *Nephotettix cincticeps*, Insect Biochem. Mol. Biol. 30 (2000) 325–333.
[14] F. Villatte, T.T. Bachmann, How many genes encode cholinesterase in arthropods?, Pestic. Biochem. Physiol. 73 (2002) 122–129.
[15] C.A. Malcolm, D. Bourguet, A. Ascolillo, S.J. Rooker, C.F. Garvey, L.M. Hall, N. Pasteur, M. Raymond, A sex-linked Ace gene, not linked to insensitive acetylcholinesterase-mediated insecticide resistance in *Culex pipiens*, Insect Mol. Biol. 7 (1998) 107–120.
[16] A. Mori, T. Tomita, O. Hidoh, Y. Kono, D.W. Severson, Comparative linkage map development and identification of an autosomal locus for insensitive acetylcholinesterase-mediated insecticide resistance in *Culex tritaeniorhynchus*, Insect Mol. Biol. 10 (2001) 197–203.
[17] T. Nabeshima, T. Kozaki, T. Tomita, Y. Kono, An amino acid substitution on the second acetylcholinesterase in the pirimicarb-resistant strains of the peach potato aphid, *Myzus persicae*, Biochem. Biophys. Res. Commun. 307 (2003) 15–22.
[18] J.-R. Gao, S. Kambhampati, K.Y. Zhu, Molecular cloning and characterization of a greenbug (*Schizaphis graminum*) cDNA encoding acetylcholinesterase possibly evolved from a duplicate gene lineage, Insect Biochem. Mol. Biol. 32 (2002) 765–775.
[19] R.A. Holt et al., The genome sequence of the malaria mosquito *Anopheles gambiae*, Science 298 (2002) 124–149.
[20] M. Weill, G. Lutfalla, K. Mogensen, F. Chandre, A. Berthomieu, C. Berticat, N. Pasteur, A. Philips, P. Fort, M. Raymond, Comparative genomics: insecticide resistance in mosquito vectors, Nature 423 (2003) 136–137.
[21] F. Li, Z.-J. Han, Two different genes encoding acetylcholinesterase existing in cotton aphid (*Aphis gossypii*), Genome 45 (2002) 1134–1141.
[22] K. Yasutomi, M. Takahashi, Insecticidal resistance of *Culex tritaeniorhynchus* (Diptera: Culicidae) in Japan: a country-wide survey of resistance to insecticides, J. Med. Entomol. 24 (1987) 604–608.
[23] M. Takahashi, K. Yasutomi, Insecticidal resistance of *Culex tritaeniorhynchus* (Diptera: Culicidae) in Japan: genetics and mechanisms of resistance to organophosphorus insecticides, J. Med. Entomol. 24 (1987) 595–603.
[24] A. Mamiya, Y. Ishikawa, Y. Kono, Acetylcholinesterase in insecticide resistant *Culex tritaeniorhynchus*: characteristics accompanying insensitivity to inhibitors, Appl. Entomol. Zool. 32 (1997) 37–44.
[25] M. Watanabe, S. Takebe, R. Arakawa, K. Kamimura, K. Kobashi, Susceptibility to nineteen insecticides and esterase activity in *Anopheles omorii*, Med. Entomol. Zool. 42 (1991) 33–42.
[26] G.L. Ellman, K.D. Courtny, U. Andres, K.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95.
[27] I.N. Maruyama, T.L. Rakow, H.I. Maruyama, cRACE: a simple method for identification of the 5′ end of mRNAs, Nucleic Acids Res. 23 (1995) 3796–3797.
[28] D.W. Severson, RFLP analysis of insect genomes, in: J.M. Crampton, C.B. Beard, C. Louis (Eds.), The Molecular Biology of Insect Disease Vectors: A Methods Manual, Chapman & Hall, London, 1997, pp. 309–320.
[29] E.S. Lander, P. Green, J. Abrahamson, A. Barlow, M. Daly, S. Lincoln, L. Newberg, MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations, Genomics 1 (1987) 174–181.
[30] H. Nielsen, J. Engelbrecht, S. Brunak, G. Heijne, Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites, Protein Eng. 10 (1997) 1–6.
[31] J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, Nucleic Acid Res. 24 (1997) 4876–4882.
[32] Y. Maulet, S. Camp, G. Gibney, T.L. Rachinsky, T.J. Ekstrom, P. Taylor, Single gene encodes glycophospholipid-anchored and asymmetric acetylcholinesterase forms: alternative coding exons contain inverted repeat sequences, Neuron 4 (1990) 289–301.
[33] A. Mori, D.W. Severson, B.M. Christensen, Comparative linkage maps for the mosquitoes (*Culex pipiens* and *Aedes aegypti*) based on common RFLP loci, J. Hered. 90 (1999).
[34] N.A. Hosea, H.A. Berman, P. Taylor, Specificity and orientation of trigonal carboxyl esters and tetrahedral alkylphosphonyl esters in cholinesterases, Biochemistry 34 (1995) 11528–11536.
[35] A. Ordentlich, D. Barak, C. Kronman, H.P. Benschop, L.P. De Jong, N. Ariel, R. Barak, Y. Segall, B. Velan, A. Shafferman, Exploring the active center of human acetylcholinesterase with stereomers of an organophosphorus inhibitor with two chiral centers, Biochemistry 38 (1999) 3055–3066.
[36] A. Shafferman, B. Velan, A. Ordentlich, C. Kronman, H. Grosfeld, M. Leitner, Y. Flashner, S. Cohen, D. Barak, N. Ariel, Substrate inhibition of acetylcholinesterase: residues affecting signal transduction from the surface to the catalytic center, EMBO J. 11 (1992) 3561–3568.
[37] M.A. Massiah, C. Viragh, P.M. Reddy, I.M. Kovach, J. Johnson, T.L. Rosenberry, A.S. Mildvan, Short, strong hydrogen bonds at the active site of human acetylcholinesterase: proton NMR studies, Biochemistry 40 (2001) 5682–5690.
[38] C.B. Millard, G. Koellner, A. Ordentlich, A. Shafferman, I. Silman, J.L. Sussman, Reaction products of acetylcholinesterase and VX reveal a mobile histidine in the catalytic triad, J. Am. Chem. Soc. 121 (1999) 9883–9884.
[39] P. Bar-On, C.B. Millard, M. Harel, H. Dvir, A. Enz, J.L. Sussman, I. Silman, Kinetic and structural studies on the interaction of cholinesterases with the anti-Alzheimer drug rivastigmine, Biochemistry 41 (2002) 3555–3564.
[40] A. Shafferman, A. Ordentlich, D. Barak, D. Stein, N. Ariel, B. Velan, Aging of phosphorylated human acetylcholinesterase: catalytic processes mediated by aromatic and polar residues of the active centre, Biochem. J. 318 (1996) 833–840.
[41] Y. Boublik, T.P. Saint-Ague, A. Lougarre, M. Arnaud, F. Villatte, S. Estrada-Mondaca, D. Fournier, Acetylcholinesterase engineering for detection of insecticide residues, Protein Eng. 15 (2002) 43–50.
[42] Y. Anazawa, T. Tomita, Y. Aiki, T. Kozaki, Y. Kono, Sequence of a cDNA encoding acetylcholinesterase from susceptible and resistant two-spotted spider mite, *Tetranychus urticae*, Insect Biochem. Mol. Biol. 33 (2003) 509–514.
[43] D. Bourguet, M. Raymond, D. Fournier, Existence of two acetylcholinesterases in the mosquito *Culex pipiens* (Diptera: Culicidae), J. Neurochem. 67 (1996) 2115–2123.
[44] D. Combes, Y. Fedon, J.P. Toutant, M. Arpagaus, Multiple ace genes encoding acetylcholinesterases of *Caenorhabditis elegans* have distinct tissue expression, Eur. J. Neurosci. 18 (2003) 497–512.