Identification and characterization of mutations in housefly (*Musca domestica*) acetylcholinesterase involved in insecticide resistance

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Acetylcholinesterase (AChE) insensitive to organophosphate and carbamate insecticides has been identified as a major resistance mechanism in numerous arthropod species. However, the associated genetic changes have been reported in the AChE genes from only three insect species; their role in conferring insecticide insensitivity has been confirmed, using functional expression, only for those in *Drosophila melanogaster*. The housefly, *Musca domestica*, was one of the first insects shown to have this mechanism; here we report the occurrence of five mutations (Val-180 → Leu, Gly-262 → Ala, Gly-262 → Val, Phe-327 → Tyr and Gly-365 → Ala) in the AChE gene of this species that, either singly or in combination, confer different spectra of insecticide resistance. The baculovirus expression of wild-type and mutated housefly AChE proteins has confirmed that the mutations each confer relatively modest levels of insecticide insensitivity except the novel Gly-262 → Val mutation, which results in much stronger resistance (up to 100-fold) to certain compounds. In all cases the effects of mutation combinations are additive. The mutations introduce amino acid substitutions that are larger than the corresponding wild-type residues and are located within the active site of the enzyme, close to the catalytic triad. The likely influence of these substitutions on the accessibility of the different types of inhibitor and the orientation of key catalytic residues are discussed in the light of the three-dimensional structures of the AChE protein from *Torpedo californica* and *D. melanogaster*.

Key words: carbamate, insect, insensitive AChE, organophosphate.

INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7) is a serine esterase in the α/β hydrolase fold enzyme family [1] that terminates nerve impulses at cholinergic synapses by breaking down the neurotransmitter acetylcholine. It is the target for the largest group of insecticides, organophosphate (OP) and carbamate compounds, which phosphorylate or carbamylate the active site serine to block the hydrolysis of acetylcholine and so lead to the death of the insect [2]. Intensive use of these insecticides over the past 50 years has led to the development of resistance in many target species that are important in agriculture or as vectors of human and animal diseases [3]. This frequently involves changes in the AChE, rendering it less sensitive to the inhibitors. These mutant forms of AChE have been characterized biochemically and can show widely differing spectra of insensitivity between species, as well as a marked range of insensitivity to different compounds within a species [4,5].

Until now, little has been known of the underlying genetic changes that cause this insensitivity in pest species, in contrast with *Drosophila melanogaster* in which five point mutations have been found in the AChE gene of different strains [6]. Modelling these changes on the X-ray structure of AChE from Pacific ray, *Torpedo californica* [7], indicated that they all modify amino acid residues within the active-site gorge of the enzyme, close to the catalytic triad [6]. Preliminary reports on the mutations found in houseflies have identified two mutations in common with those in *Drosophila*, as well as two novel ones whose influence on the catalytic properties of the enzyme have not been established [8,9]. The Colorado potato beetle, *Leptinotarsa decemlineata*, is the only phytophagous insect for which an AChE mutation (serine to glycine) associated with insensitivity has been identified [10]. Unlike those described in *Drosophila*, this mutation is remote from the catalytic region of the enzyme and any influence on insecticide sensitivity was suggested to arise from a change in the secondary structure of the protein; however, this is yet to be confirmed.

Although the AChE genes from several other pest species have been cloned, data comparing the sequences from strains with sensitive and insensitive forms of the enzyme are available only for cattle ticks, *Boophilus microplus* [11], and green rice leafhoppers, *Nephotettix cincticeps* [12], in which no differences were found between the genes encoding the sensitive and insensitive forms of the enzyme. This has led to the suggestion that insensitivity in these species is either due to an alteration in the post-translational modification of the enzyme or alternatively that an AChE gene other than the one that had been cloned is critical for survival. Although there is no direct evidence of altered post-translational processing in either of these species, biochemical and molecular studies have independently provided evidence of multiple AChEs in cattle ticks [13,14] and the mosquito species *Culex pipiens* [15,16]. In the cattle tick this has been confirmed by the cloning of a second AChE-like gene [13], whereas in *C. pipiens* the existence of a second gene has been inferred from linkage mapping studies showing that the gene already cloned was sex-linked, whereas the gene involved in resistance mapped to chromosome II [15].

Only the amino acid replacements in *Drosophila* have been analysed by site-directed mutagenesis and functional expression [6], which confirmed their role in conferring insensitivity. A subsequent study showed that the *Drosophila* mutations also conferred similar levels of insensitivity when introduced into the

Abbreviations used: AChE, acetylcholinesterase; OP, organophosphate.

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cloned *Aedes aegypti* mosquito AChE gene [17], although 'natural' resistance mutations have not yet been found in this species. Furthermore, mutagenesis of the *Drosophila* gene to generate a series of novel substitutions at two of these naturally variant amino acids [18] led to a general, albeit mostly low-level, decrease in inhibition by various OP and carbamate inhibitors.

Here we report the identification of five mutations, singly or in combination, in the AChE gene of insecticide-resistant housefly strains and their effect on the kinetic properties of the enzyme after mutagenesis and expression of the gene *in vitro* with the baculovirus system. The likely influence of these substitutions on the accessibility of the different types of inhibitor and the orientation of key catalytic residues are discussed in view of the three-dimensional structures of the AChE protein from *T. californica* [7] and *D. melanogaster* [19].

EXPERIMENTAL

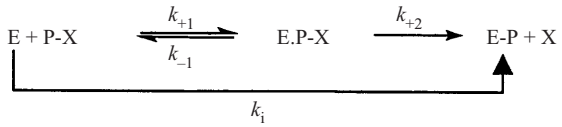
Housefly strains

With the exception of strains 77M and 690ab, the housefly strains used, and their biochemical characterization, have been described previously [4,20]. The 77M strain was established from flies collected on a farm in North Hertfordshire, U.K., in 1991 after selection with methomyl. This strain was not fully characterized but its AChE genotype and biochemical characteristics seemed similar to that of the Fm50 strain, with strong insensitivity to the carbamates methomyl and bendiocarb, but comparable to that of CH2 for the other three compounds used (dichlorvos, azamethiphos and malaoxon [9]). The 690ab strain was originally established from flies collected at a farm in Varde, Denmark, in 1984. Bioassays of F_2/F_3 flies with methomyl showed approx. 50-fold resistance at the concentration lethal to 95% of individuals compared with a control strain. Continued selection through subsequent generations was performed by feeding adults on methomyl-impregnated sugar. Adult houseflies were frozen in liquid nitrogen and stored at -80 °C for subsequent biochemical and molecular analyses.

Measurement of AChE activity and insensitivity to insecticides

AChE activity was assayed in microplates as described previously [21], with a colorimetric assay [22]. Reactions were performed at 25 °C in assay buffer (100 mM sodium phosphate, pH 7.5) containing 0.5 mM acetylthiocholine iodide and 0.5 mM 5,5′-dithiobis-(2-nitrobenzoic acid), in a total volume of 200 μl, with a Molecular Devices Thermomax microplate reader controlled by the SOFTmax program. Kinetic constants were determined from uninhibited AChE activities, measured at eight to ten acetylcholine iodide concentrations spanning the K_m, and calculated from the Michaelis–Menten equation with the computer program GraFit (version 3.0; Erithacus Software).

The reaction of AChE with OP and carbamate compounds is bimolecular but follows pseudo-first-order kinetics when the inhibitor concentration is at least 10-fold that of the enzyme. The progressive inhibition of AChE is described by the reaction scheme:



where E is the free enzyme, P-X is an OP (or carbamate) compound, E.P-X is the Michaelis complex, E-P is the phosphorylated (or carbamylated) enzyme, X is the 'leaving group' and k_i is the bimolecular rate constant. In houseflies the phosphorylated enzyme is stable and the carbamylated enzyme is hydrolysed only very slowly [20]. The k_i values were measured for each enzyme by incubation with the inhibitor in assay buffer and by assaying aliquots for remaining AChE activity at various time points. The inhibitors were added in acetone to give a final concentration of this solvent in the inhibitor–enzyme incubation of 2% (v/v). Pseudo-first-order rate constants were determined by non-linear regression with the computer program GraFit, and fitted by linear regression to the inhibitor concentrations in a double-reciprocal plot to obtain bimolecular rate constants. The first-order rate constants were measured with eight time-points and with five different inhibitor concentrations for each enzyme, in at least three independent experiments. S.E.M. values were always less than 10% of the mean. Insensitivity factors are the ratios of the bimolecular rate constants of the wild-type and mutant enzymes.

Reverse-transcriptase-mediated PCR analysis of AChE gene sequences

Total RNA was extracted from frozen adult houseflies of each strain for amplification of the AChE gene coding sequence by reverse-transcriptase-mediated PCR. Individual flies were ground in liquid nitrogen in 1.5 ml microtubes and homogenized in G-Mops buffer [8 M guanidinium-HCl/20 mM Mops/20 mM EDTA (pH 7.0)]. Homogenates were extracted twice with phenol/chloroform (1:1) and the RNA was precipitated with ethanol. First-strand cDNA was synthesized from the total RNA (5 μg) with reverse transcriptase (Superscript II, 200 units per reaction; Gibco BRL) with oligo(dT) (200 ng) as primer [23]. The AChE-coding region was amplified by PCR on two overlapping fragments (1.3 and 1.1 kb) with gene-specific primers directed against the housefly AChE cDNA sequence ([24], updated in EMBL accession no. AJ310134). Primer sequences for amplification of the 5′ (1.3 kb) fragment were 5′-CCTTAGTATGGCTCGGTCTG-3′ (sense) and 5′-ATCAGAATATCGTAACCGCT-3′ (anti-sense); for the 3′ (1.1 kb) fragment they were 5′-TCAAACGTGGCATGATGCAG-3′ (sense) and 5′-TGTGTATGTGTGGGTGAGTG-3′ (anti-sense). PCRs were performed in accordance with standard procedures with 2 μl of cDNA as the template, 200 ng of each primer and at an annealing temperature of 55 °C. The PCR fragments were fully sequenced in both directions with dye terminators on an Applied Biosystems 373A automated sequencer and the sequences were analysed with Staden [25] and GCG (Wisconsin Package Version 9; Genetics Computer Group, Madison, WI, U.S.A.) programs.

Site-directed mutagenesis, and expression in baculovirus

The 612-residue coding sequence of the housefly AChE gene (EMBL AJ310134) was cloned into the baculovirus transfer vector pK503.9 (a gift from Dr C. Oker-Blom, University of Jyvaskyla, Jyvaskyla, Finland) for the expression of recombinant housefly AChE in cultured insect (Sf9/Sf21) cells. The resulting pK503.9A5 construct contained N-terminal signal peptide and FLAG epitope sequences (from pK503.9) to enable secretion and purification (where desirable) of the expressed housefly AChE. Secretion was further facilitated by the removal of a 19-residue hydrophobic peptide sequence at the C-terminus of the protein (see [26]). The 65 kDa FLAG (DYKDDDK)-tagged recombinant AChE enzyme produced by the insect cells was found to be identical with the native housefly enzyme in its kinetic properties.

Putative resistance mutations were incorporated into the pK503.9A5 AChE construct with the QuikChange™ site-directed

mutagenesis kit (Stratagene Cloning Systems) in accordance with the supplier's recommended procedures. Mutations were introduced sequentially where combinations were required; all constructs were confirmed by resequencing across the region that had been mutagenized. Modified AChE proteins were produced by recombination of the mutated pK503.9A5 constructs into the baculovirus genome by using the Bac-to-Bac system (Gibco BRL), followed by transfection into Sf21 insect cells (Invitrogen). In brief, mutated plasmid DNA was transformed into the *Escherichia coli* strain DH10Bac for the selection of bacterial colonies carrying recombinant baculovirus DNA species. After purification the baculovirus DNA was transfected into Sf21 cells with Cellfectin reagent (Gibco BRL) in accordance with the supplier's recommended protocols. Initial, low-titre, viral stocks were collected as supernatants in the medium at 72 h and used to reinfect fresh cells at a cell density of 2×10^6 cells/ml with a multiplicity of infection (virus to cells) of 10. Culture supernatants were subsequently harvested at 48 h (protein) or 72 h (virus) after infection and either assayed for AChE activity (protein, assay as described above) or stored at -20 °C for future infections (virus). Sf21 cells were grown as monolayer cultures at 27 °C in L-glutamate SF900 II insect medium (Gibco BRL) containing 50 i.u./ml penicillin and 50 μg/ml streptomycin.

Analysis of AChE structure

The structure of *D. melanogaster* AChE was obtained from the Protein Data Bank (http://www.rcsb.org/pdb; ID ref 1Q09) and viewed with Sybyl (Tripos) and SwissPDBviewer (http://www.expasy.ch/spdbv) programs. Figure 2 is a cut-down model of the active site of the enzyme with acetylcholine substrate docked, showing some of the key residues and the locations of the resistance mutations. The figure is schematic, showing the probable side-group orientations of the mutated residues within the active site of the enzyme. Mutations were introduced using the 'mutate monomer' function within Sybyl.

RESULTS

Characterization of insensitivity of AChE to insecticide

Insensitivity factors to various insecticides, expressed as the ratio of the k_i values for the wild-type and mutated enzymes, were used to compare the AChEs (Table 1). The insecticide-insensitivity and kinetic properties of AChE from strains 49R and CH2 have been described previously [20,24]. The AChE of CH2 was characterized as moderately insensitive to dichlorvos but less so to malaoxon and bendiocarb. That of 49R was unique among the strains studied in showing slight (3-fold) insensitivity to azamethiphos in addition to the 17-fold insensitivity to malaoxon. The AChEs from all the other strains were slightly hypersensitive to azamethiphos. The original Fm50 [20] and 77M [9] strains both showed marked insensitivity to bendiocarb as well as enhanced resistance to dichlorvos. Although the Fm50 and 77M strains were lost from rearing culture before the molecular work (i.e. no live flies were available for testing), some frozen 77M individuals were recovered for sequence analysis (below) but their AChE was subsequently found not to show the enhanced bendiocarb insensitivity of the original strain. For this reason, another highly carbamate-resistant strain (690ab) was included in the present study and the sensitivity of its AChE to the same four insecticides was determined. It was found to have a greater than 500-fold insensitivity to dichlorvos, 8-fold insensitivity to malaoxon and 100-fold insensitivity to bendiocarb compared with the susceptible strain, Cooper (Table 1).

Table 1 Inhibition of AChE from housefly strains by insecticides

Bimolecular rate constants (k_i) were determined as described in the Experimental section. Insensitivity factors are the ratios of the k_i values of the wild-type (Cooper) enzyme to those of the other strains. Insensitivity factors are shown in parenthesis.

Housefly strain	k_i ($\mu M^{-1} \cdot min^{-1}$)			
	Dichlorvos	Azamethiphos	Malaoxon	Bendiocarb
Cooper*	2.2	41	0.87	0.91
49R*	0.42 (5)	16 (3)	0.05 (17)	0.39 (2)
CH2*	0.15 (15)	61 (0.7)	0.28 (3)	0.17 (5)
Fm50*; 77M†	0.04 (55)	63 (0.7)	0.14 (6)	0.0015 (610)
690ab‡	0.004 (550)	72 (0.6)	0.11 (8)	0.0089 (100)

* Data from [20].
† Strain 77M was similar to Fm50; see the text for details.
‡ Results from present study.

Table 2 AChE mutations in resistant housefly strains

Distribution of mutations between the four housefly strains analysed. Equivalent residue numbers in the AChEs of *Torpedo californica* [7] and *Drosophila melanogaster* [6] are shown for cross reference.

Housefly strain	Amino acid substitutions arising from mutations in the acetylcholinesterase gene				
49R					G365A
CH2		G262A		F327Y	
77M	V180L	G262A		F327Y	
690ab			G262V	F327Y	
Species	Residue no.				
Torpedo no.	150	227	227	290	328
Drosophila no.	220	303	303	368	406

Identification of mutations in the housefly AChE gene

The housefly AChE gene has been cloned as a full-length cDNA and contains an unusually long 80-residue signal peptide upstream of the 612-residue mature protein sequence (M. S. Williamson, unpublished work; EMBL accession no. AJ310134). Because only coding sequences corresponding to the mature protein were PCR-amplified and sequenced from the four resistant strains (49R, CH2, 77M and 690ab), the numbering used in this paper is based on this 612-residue mature protein sequence (previously shown in [9]). In comparison with the susceptible strain, five amino acid changes were identified (Table 2). The CH2 strain contained two mutations, Gly-262 → Ala (G262A) and Phe-327 → Tyr (F327Y). The 690ab strain also had mutations at the same two residues; however, whereas F327Y was conserved, the mutation at Gly-262 was to valine instead of alanine (G262V). The 77M flies that were tested had the same two mutations as CH2 (G262A, F327Y) together with the additional alteration of Val-180 → Leu (V180L). In contrast, the 49R strain had none of the above changes, with only a single mutation Gly-365 → Ala (G365A). For cross reference, the equivalent residues in the AChEs of *T. californica* and *D. melanogaster* are given in Table 2.

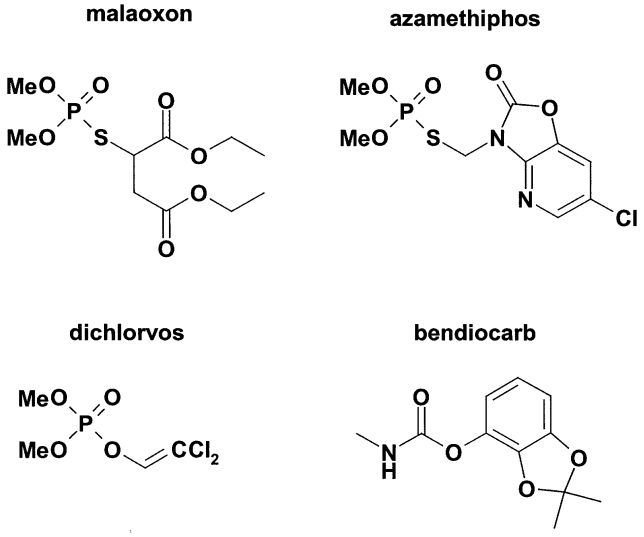


Figure 1 Structures of insecticide inhibitors

Expression in baculovirus and evaluation of mutations

To evaluate the individual contributions of these amino acid changes to insecticide insensitivity, the mutations were introduced individually and in combinations into the wild-type AChE gene by site-directed mutagenesis. These mutant proteins were expressed in the baculovirus system and all produced active AChE enzymes, which were assayed for their sensitivity to the same four insecticides: dichlorvos, azamethiphos, malaoxon (all dimethyl OPs) and bendiocarb (monomethylcarbamate) (Figure 1 and Table 3). Azamethiphos was by far the most potent of the four compounds tested; indeed it was better than all other anti-cholinesterase compounds evaluated previously. This is likely to reflect a high affinity of the molecule's leaving group for the quaternary binding site of the AChE, which seems to outweigh any effect of mutating the other residues near the catalytic triad and acyl-binding pocket of the enzyme (see the Discussion section). The potencies of dichlorvos and malaoxon were similar to each other for the sensitive enzyme and for some mutant enzymes, but differed markedly for others. The ratio of bimolecular rate constants illustrates the degrees of insensitivity (Table 3).

When introduced individually, the G262A and F327Y mutations of CH2 caused insensitivity to dichlorvos of only 4.3-fold and 1.8-fold respectively, but together gave 37-fold insensitivity. A much smaller effect was seen with malaoxon, for which the double mutant was only 2-fold insensitive compared with a barely detectable effect of the individual mutations. For bendiocarb, the carbamate inhibitor, both mutations were needed to achieve the relatively small (5-fold) insensitivity to this compound. In contrast, each mutation enhanced the sensitivity to azamethiphos (with a slight additive effect when combined). All of these effects are in line with the properties of the native CH2 enzyme (Table 1).

The alternative mutation of G262V instead of G262A in the 690ab strain had a much more marked effect on the properties of the AChE. A 240-fold insensitivity to dichlorvos was seen for the double mutant, mostly (58-fold) due to the G262V alone. Similarly, for bendiocarb the G262V mutation was the major contributor to the 100-fold insensitivity of the double mutant. The sensitivities to malaoxon and azamethiphos inhibition were similar to that of the CH2 flies.

The 49R strain had a single residue change, G365A, which was confirmed to be responsible for the azamethiphos insensitivity in the baculovirus-expressed mutant. This was the only mutation found that gave insensitivity to this compound, all others causing slight hypersensitivity. The G365A AChE showed the highest insensitivity to malaoxon, with little insensitivity to bendiocarb or dichlorvos.

The 77M strain had the same two mutations as the CH2 strain (G262A/F327Y) and an extra one at V180L. It is clear that the V180L mutation, alone or in combination with others, did not cause high resistance to bendiocarb as proposed previously [9] on the basis of its apparent similarity to the Fm50 strain. However, it did slightly enhance the insensitivity to dichlorvos and malaoxon when combined with the double mutant G262A/F327Y equivalent to the CH2 enzyme, or even when combined with just the F327Y mutation.

Substrate binding properties

The effect of the resistance-associated mutations on substrate binding was less marked than for the inhibitors (Table 3). The

Table 3 Effects of mutations on kinetic properties and insecticide sensitivity of recombinant housefly AChEs

Wild-type and mutated housefly enzymes were expressed with the baculovirus system. Bimolecular rate constant (k_i) and K_m values were determined as described in the Experimental section. Insensitivity factors (shown in parentheses) are the ratios of the bimolecular rate constants for the wild-type enzyme to that of the mutated enzyme for each of the inhibitors.

			Bimolecular rate constant ($\mu M^{-1} \cdot min^{-1}$)			
Recombinant AChE	Equivalent housefly strain	K_m (μM)	Dichlorvos	Azamethiphos	Malaoxon	Bendiocarb
Wild-type	Cooper	150 ± 19	1.34 (1)	37 (1)	1.6 (1)	0.44 (1)
G365A	49R	210 ± 17	0.55 (2.4)	10.3 (3.6)	0.12 (13)	0.35 (1.3)
G262A/F327Y	CH2	56 ± 7	0.036 (37)	84 (0.4)	0.75 (2.1)	0.085 (5.2)
V180L/G262A/F327Y	77M	129 ± 16	0.028 (48)	89 (0.4)	0.25 (6.4)	0.112 (3.9)
G262V/F327Y	690ab	267 ± 27	0.0057 (240)	112 (0.3)	0.24 (6.6)	0.0043 (100)
G262A	–	205 ± 12	0.31 (4.3)	50 (0.7)	1.07 (1.5)	0.14 (3.1)
G262V	–	1800 ± 97	0.023 (58)	51 (0.7)	0.35 (4.6)	0.0052 (85)
F327Y	–	44 ± 4	0.74 (1.8)	59 (0.6)	1.1 (1.5)	0.22 (2)
V180L	–	75 ± 8	1.04 (1.3)	25.8 (1.4)	0.74 (2.2)	0.48 (0.9)
V180L/G262A	–	153 ± 12	nd	40 (0.9)	nd	0.188 (2.3)
V180L/F327Y	–	84 ± 8	0.34 (3.9)	44 (0.8)	0.24 (6.7)	0.081 (5.4)

F327Y mutant was notable for enhancing the enzyme's affinity for acetylthiocholine (3-fold lower K_m), whereas the others generally decreased affinity with slightly increased K_m values (Table 3). The most marked effect of the G262V substitution was to increase the K_m 12-fold to almost 2 mM but this was modulated in the double mutant G262V/F327Y, in which it was less than twice that of the wild-type enzyme.

DISCUSSION

Distinct patterns of AChE insensitivity to different types of OP and carbamate insecticides are well documented in houseflies [20,24] and we have previously identified four point mutations (V180L, G262A, F327Y and G365A) in the housefly gene encoding AChE that are associated with these phenotypes [8,9]. Here we report the identification of a further mutation (G262V) in a strain displaying strong resistance to carbamates and clarify the relative roles of all five mutations in conferring OP/carbamate resistance by expression of the modified AChE proteins *in vitro* with the baculovirus system. The finding that two of these mutations (G262A and F327Y) are common to those reported previously for *Drosophila* AChE [6] not only reinforces the importance of these changes in causing resistance but also adds to the growing list of highly conserved mutations that are responsible for target site insensitivity to different classes of insecticide across a wide range of species [27]. All mutations described here were identified in strains isolated from housefly populations showing significant practical control problems in the field.

Mutations G262A and F327Y

As expected from the *Drosophila* study [6], the conserved G262A and F327Y mutations of the housefly CH2 strain each conferred a low level of insensitivity to three of the compounds. When combined, their effect was enhanced, especially for dichlorvos (Table 3). They also showed slight hypersensitivity to azamethiphos in line with the properties of the native enzyme. The X-ray crystal structures of AChE from *T. californica* [7] and *D. melanogaster* [19] have shown that the active site is buried deep within the enzyme at the bottom of a narrow cavity (or gorge) lined by aromatic residues. Initial modelling of these mutations within the *Torpedo* structure revealed that both are located close to the active-site triad at the base of the gorge, with G262A likely to affect the orientation of the catalytic serine (Ser-200 in *Torpedo*) and F327Y decreasing the available space within the acyl-binding pocket [6]. The changes are therefore predicted to cause resistance by restricting the access and/or binding of bulky insecticide inhibitors within the active site; this view is reinforced by the recent publication of the *Drosophila* AChE structure [19], which confirms the spatial conservation of key residues around the catalytic serine (Figure 2) despite extensive divergence (36% identity) in their primary sequences. This is further supported by a recent study in which the *Drosophila* Phe-368 residue (equivalent to housefly Phe-327 and to *Torpedo* Phe-290) was systematically mutated to a range of other side groups and the modified enzymes were examined for their inhibition by a series of OP and carbamate insecticides [18]. Replacement by tyrosine (as in the naturally occurring resistant strains) again showed only minor effects (less than 5-fold) on insensitivity, whereas the bulkier tryptophan consistently decreased sensitivity by at least 10-fold to all of the OPs tested. None of the other replacements had such a universal effect on OP sensitivity. However, it is worth noting that sensitivity to carbamates was often decreased by more than 10-fold after replacement of Phe-368 by leucine, isoleucine, valine, cysteine, serine and glycine, suggesting that the spatial constraints imposed by the amino acid at this position do not necessarily have a major role in limiting the access of carbamates to the acyl-binding pocket of these mutant enzymes.

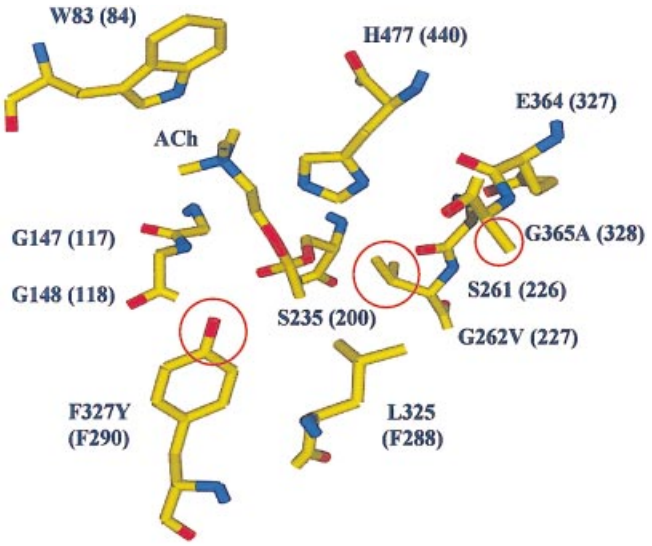


Figure 2 Locations of resistance mutations within the active site of AChE

Mutations are modelled on the X-ray structure of *Drosophila* AChE [19]. Residues are numbered in accordance with the housefly sequence; the corresponding numbering for *Torpedo* AChE (rather than *Drosophila*) is given in parentheses. Additional side groups from the mutated residues are circled.

Mutation G262V

Sequencing of the AChE gene from strain 690ab showed it to be similar to CH2 in that it carries mutations at Gly-262 and Phe-327; the difference is that valine rather than alanine replaces Gly-262. This was somewhat unexpected, given the greatly enhanced insensitivity of 690ab AChE to dichlorvos and bendiocarb (550-fold and 100-fold) compared with that of CH2 (15-fold and 5-fold) (Table 1). However, confirmation that this marked enhancement is caused mainly by the G262V mutation is clear from the analysis of the baculovirus-expressed single mutants (Table 3), with G262V alone conferring 58-fold and 85-fold insensitivity to these two inhibitors. As noted in the previous section, the side-group hydrogen of Gly-262 is oriented towards the active-site serine residue so that mutations at Gly-262 are predicted to move the serine and alter its configuration within the catalytic triad, thereby affecting its interactions with either the inhibitor or the substrate (Figure 2). Because valine carries a more bulky and hydrophobic side group than alanine, it is logical that G262V should have a more marked effect on inhibitor insensitivity than G262A. Also consistent with this is the greater than 10-fold decrease in substrate affinity of the G262V mutant compared with the wild-type enzyme, as shown by its increased K_m for acetylthiocholine (Table 3). It is interesting to note that this seems to be modulated by the F327Y mutation, which brings the K_m back to the same range as that of the wild-type enzyme in the G262V/F327Y double mutant. This might explain why the G262V mutation has not been found alone in housefly populations.

Gly-262 is also adjacent to another serine residue (Ser-261, equivalent to Ser-226 in *Torpedo* and Ser-302 in *Drosophila*),

which is completely conserved in 52 out of 53 esterases and lipases surveyed [28]. It is also well conserved in serine proteases, where it has been claimed to have a role in catalysis by constituting the fourth member of a catalytic 'tetrad' [29]. Its presence in the same three-dimensional orientation in the serine protease and α/β hydrolase fold enzyme families strongly points to its having a functional role. Recent homology modelling of this residue in the juvenile-hormone esterase structure has indicated that it serves to orient a water molecule known to be conserved in the AChE and lipase X-ray crystal structures [28]. Although this water most probably has a role in hydrolysis of the acylated enzyme, a role in the access of the ester when binding is also likely, which could be affected by the change of the glycine residue adjacent to the bulkier and more hydrophobic valine.

The most obvious effect of the F327Y mutation is to enhance the enzyme's affinity for substrate, which could be its primary contribution to the resistance. This would be achieved by counteracting the negative effects of the Gly-262 mutations on K_m and would enhance the affinity for substrate, protecting the enzyme from an inhibitor when the two molecules are competing for binding to the catalytic site. Both this residue and the neighbouring Leu-325 (equivalent to Phe-288 in *Torpedo* and to Phe-297 in mouse) have been shown to be important for substrate selectivity and binding in vertebrate AChEs. For example, in mouse the F297I substitution causes an increase in the K_m for acetylcholine from 39 to 357 μM [30], whereas F297Y decreases the K_m to 17 μM [31,32], which is similar to that of the housefly F327Y mutant.

Mutations G365A and V180L

The housefly G365A mutation is adjacent to the glutamate of the catalytic triad (Figure 2) and yielded insensitivity to all four insecticides, especially malaoxon. This was the only mutation to confer resistance to azamethiphos, in agreement with the results with the native enzyme from strain 49R, in which it was found (Table 1). This mutation also decreased the affinity of the enzyme for acetylthiocholine (Table 3) as would be expected if it were altering the configuration of the adjacent glutamate in the catalytic triad. Although the extra methyl group on G365A points away from the active site when modelled on the *Torpedo* and *Drosophila* structures, it is likely to alter the conformation in this part of the enzyme (Figure 2). The native 49R enzyme containing this mutation is also notable with respect to its interaction with carbamates. Although the mutation had no effect on inhibition by bendiocarb, previous work has shown that the monomethyl-carbamylated 49R enzyme is hydrolysed 4-fold faster than the wild-type enzyme or any of the other mutant enzymes [20]. This suggests that its effect on the orientation of the catalytic triad glutamate is to enhance the nucleophilic attack of water on the carbonyl group of the carbamylated serine. This is discussed further in the final section.

The V180L mutation identified previously in the 77M housefly strain was found to have little effect on the properties of the AChE. In particular, the 600-fold insensitivity to bendiocarb originally seen in the native Fm50 enzyme is difficult to reconcile with the 77M sequence data. Both strains had been lost from rearing culture before the present study, so it was not possible to confirm the original findings. The most likely explanation is that the original biochemical data for 77M were obtained from a heterogeneous population, with only some individuals carrying the strong bendiocarb insensitivity of the Fm50/690ab strains and the remainder showing only low to moderate resistance to this inhibitor, which would be more consistent with the G262A/F327Y genotype of the frozen individuals that were recovered for PCR and sequencing analysis.

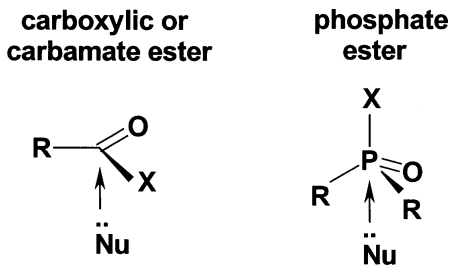


Figure 3 Reaction of the nucleophilic serine residue of AChE with OP and carboxylic esters

The contrasting conformations of carboxylic and phosphate ester groups and their orientations for nucleophilic attack by serine hydrolases (adapted from [35]). X, leaving group.

Conformation of enzyme–inhibitor complexes

A final question is why the mutations show such markedly different effects on the kinetics of the enzyme's interaction with different OP and carbamate inhibitors and with the substrate analogue acetylthiocholine. All the mutations cause changes to slightly larger residues, which implies a steric effect that still allows access for the smaller acetylcholine molecule but restricts the larger inhibitors. However, this is not always so, because all of the mutations except for G365A elicit hypersensitivity to azamethiphos, which was the largest and the most potent OP used. The differences between the three OPs were due entirely to the nature of their leaving groups because all were dimethyl-phosphates (Figure 1). The crystal structures of other α/β hydrolase fold enzymes inhibited by paraoxon [33,34] show that the ethyl groups are directed away from each other around the phosphorus atom so that only one of them needs to be accommodated in the acyl-binding pocket. This leads to another consideration relating to the conformation around the electron-deficient carbon or phosphorus atom to which the nucleophilic serine binds. Phosphate esters show sp^3 hybridization around the phosphorus (i.e. the bonds are tetrahedral), whereas the carbonyl carbon of the carbamates and substrate has sp^2 hybridization (i.e. the bonds are planar) [35,36]. This in turn radically changes the orientation in which the nucleophilic attack by the catalytic serine takes place (Figure 3). For phosphate esters, its approach must be directly opposite the leaving group, whereas with the carbonyl compounds it is perpendicular to the plane. This means that the usually bulky leaving group is oriented quite differently for OPs than for carbamates. Although this difference does not seem to affect most interactions studied here, it could contribute to the very marked bendiocarb insensitivity and high substrate K_m of the G262V/F327Y genotype in the 690ab strain, most of which is due to the valine substitution. The marked effect of this substitution on dichlorvos sensitivity could be due to the relatively small size of the leaving group in this compound. Similar considerations could explain the more marked effect on carbamates than on OPs of mutating the *Drosophila* Phe-368 (equivalent to housefly Phe-327) to acyclic lipophilic residues, and the converse effect of substituting a tryptophan at this position, which universally affects the OPs but not all carbamates [18].

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