Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase

ANNICK MUTERO*, MADELEINE PRALAVORIO, JEAN-MARC BRIDE, AND DIDIER FOURNIER†
Institut National de la Recherche Agronomique, Laboratoire de Biologie des Invertébrés, BP2078, 06606 Antibes, France

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ABSTRACT
Extensive utilization of pesticides against insects provides us with a good model for studying the adaptation of a eukaryotic genome to a strong selective pressure. One mechanism of resistance is the alteration of acetylcholinesterase (EC 3.1.1.7), the molecular target for organophosphates and carbamates. Here, we report the sequence analysis of the Ace gene in several resistant field strains of Drosophila melanogaster. This analysis resulted in the identification of five point mutations associated with reduced sensitivities to insecticides. In some cases, several of these mutations were found to be combined in the same protein, leading to different resistance patterns. Our results suggest that recombination between resistant alleles preexisting in natural populations is a mechanism by which insects rapidly adapt to new selective pressures.

Although insecticide resistance is an important agricultural problem, this phenomenon also provides a good model for studying adaptation of eukaryotes to a toxic environment. Resistance to insecticides results from three main mechanisms: reduction in the insecticide penetration; increased metabolism of the insecticide by esterases, mixed-function oxidases, or glutathione transferases; and modification of the insecticide target. So far, little is known of the genetic origins of resistance to insecticides at the molecular level. However, in some instances, resistance was shown to originate from gene amplification (1, 2), overtranscription (3, 4), or point mutations (5, 6).

Acetylcholinesterase (AChE; acetylcholine acethylhydro-lase, EC 3.1.1.7) terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. It is a key enzyme in the insect nervous system, which led to the development of inhibitors for this enzyme as insecticides. Two classes of compounds, organophosphorous and carbamate insecticides, are commonly used that quasi-irreversibly inhibit AChE. Their conformation is analogous to the natural substrate, acetylcholine, but they are hemisubstrates and they inactivate the enzyme by phosphorylating or carbamoylating the active-site serine (7). Following intoxication, the termination of impulse transmission at cholinergic synapses no longer takes place, causing the death of the insect. However, intensive insecticide use for >30 years has resulted in the appearance of several resistant insect species possessing altered AChEs that are less sensitive to the insecticide (8).

Materials and Methods

Materials. Three resistant D. melanogaster strains, Saltillo, Bygdeda, and Pierrefeu, were used. Their AChEs were characterized with respect to their cross resistance toward several insecticides. The patterns obtained permitted the suggestion that different types of proteins were present in these strains (9).

PCR Amplification. Exon 2 and groups of exons 3–5, 6 and 7, and 8 and 9 of Ace, the gene encoding AChE, were amplified from genomic DNAs extracted from the resistant flies, by using 20-mer oligonucleotides overlapping intron/exon boundaries (10). The products of PCRs were loaded onto agarose or polyacrylamide gels and bands of interest were cloned and sequenced. The sequence from the wild-type strain Canton-S was used as a reference (11).

Construction of Clones. We used the pX construction previously described (12). This plasmid contains the entire coding region of the Drosophila AChE gene, except for the sequence encoding the carboxyl-terminal 29 amino acids, which contains the signal for attachment of the glycosylphosphatidylinositol anchor. This construct encodes a soluble protein which is excrated into the medium by the Xenopus oocytes carrying the construct. The pX plasmid also contains the phage φ1 origin of replication, allowing the synthesis of single-stranded DNA. Oligonucleotide-directed mutagenesis was performed according to Nakamaye and Eckstein (13). Analysis of the mutant progenies was carried out by sequencing the mutated regions of the clones. Mutated clones were then expressed in Xenopus oocytes (14).

Microinjection into the Nuclei of Xenopus Oocytes. Injections into oocytes were performed according to Bertrand et al. (15). In brief, oocytes were isolated from dissected ovaries by 0.2% collagenase treatment and centrifuged at 400 × g for 10 min so that the nuclei became visible as a clear area at the animal pole. Fully grown oocytes were injected intranuclearly with 10 nl of plasmid preparation containing 1 ng of purified DNA suspended in 88 mM NaCl/1 mM KCl/15 mM Hepes, pH 7. Batches of 20 oocytes were kept at 20°C in 1 ml of OR2 medium (82.5 mM NaCl/2.5 mM KCl/1 mM Na2HPO4/15 mM Hepes, pH 7.5/0.05% polyvinylpyrrolidone/1 mM CaCl2/1 mM MgCl2 supplemented with penicillin (50 units/ml), streptomycin sulfate (50 μg/ml), and kanamycin sulfate (20 μg/ml). The medium was changed daily. Expression of active AChE was assayed for each oocyte by recording the activity in 5 μl of the oocyte incubation medium according to the procedure of Ellman et al. (16). Oocytes with the highest expression rates were conserved and their incubation medium was collected daily for analysis.

Abbreviation: AChE, acetylcholinesterase.

*Present address: Department of Pharmacology, School of Medicine, University of California at San Diego, 9500 Gilmor Drive, La Jolla, CA 92039-0636.
†To whom reprint requests should be addressed at the present address: Université Paul Sabatier, 118 Route de Narbonne, 31062 Toulouse, France.
**AChE Biochemistry.** Reaction of irreversible cholinesterase inhibitors, such as organophosphorous compounds, is pseudo-first order with respect to inhibitor concentration. The following reaction scheme holds for the progressive inhibition of cholinesterases by organophosphorous compounds:

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 \text{ChE} + \text{P-X} \rightleftharpoons \text{ChE-P-X} \rightarrow \text{ChE-P} + \text{X},
\]

where ChE is the free enzyme; P-X is an organophosphorous compound, ChE-P-X is the Michaelis complex, and ChE-P is the phosphorylated enzyme. The bimolecular velocity constant \((k_1)\) was estimated by the dilution method of Aldridge (7). In brief, AChE was incubated with the inhibitor for various times before tipping the inhibition mixture into a solution of acetylthiocholine (1 mM) to measure residual activity. A plot of the natural logarithm of residual activity \((V/V_0)\) versus time was linear for a given inhibitor concentration, and \(k_1\) values were obtained by dividing the slope of the curve by the concentration. This constant was estimated at five different concentrations for each inhibitor.

**RESULTS AND DISCUSSION**

We previously isolated several strains of *D. melanogaster* with AChEs highly resistant to organophosphorous and carbamate insecticides (9). To identify mutations associated with resistance, we determined the entire nucleotide sequence of the AChE coding regions after PCR amplification in three of these strains: Saltillo, Bygdéa, and Pierrefeu. Five mutations were identified: Phe-115(78) to Ser, Ileu-199(Val-129) to Val, Ileu-199(Val-129) to Thr, Gly-303(227) to Ala, and Phe-368(288) to Tyr (Fig. 1). (Amino acids are numbered from the N-terminal residue of the precursor and numbers in parentheses indicate the numbering of corresponding amino acid in the mature *Toledo AChE*.) The last mutation had already been found by sequencing the gene in another resistant strain, MH19 (3). We introduced these mutations into the wild-type gene by *in vitro* mutagenesis and expressed them in *Xenopus* oocytes. Proteins obtained were assayed for their sensitivity to four commonly used insecticides: two organophosphorous compounds (malaoxon and parathion) and two carbamates (carbaryl and propoxur). Inhibition of the enzyme by these compounds is irreversible. Inhibition rate constants \((k_i)\) were used to estimate the sensitivities of the mutated proteins. Ratios of the \(k_i\) value obtained for the mutant to the \(k_i\) value obtained for the wild-type protein allowed us to estimate the resistance of a mutated protein. Mutations occurring at different locations are responsible for AChE resistance to insecticides, with each of these mutations leading to a different pattern of resistance. However, the resistance levels observed for individual mutations are low (Fig. 2).

In the three-dimensional structure of *Toledo AChE* (17), the catalytic triad was found to be located near the bottom of a 20-A-deep cavity. As most of the residues facing this active-site gorge are conserved in the *Drosophila* sequence, we may speculate on the influence of the mutations observed in the resistant variants. Ile-199(Val-129) is close to Trp-121(84), which participates in the so-called anionic site and binds to the choline of the substrate or the leaving group of the insecticide (Fig. 3). Gly-303(227) seems to affect the orientation of Ser-276(200), which is phosphorylated or carbamoylated by insecticides. Phe-368(288) is near the acyl moiety of the bound substrate (acetylcholine or insecticide) and is implicated in the substrate specificity of cholinesterases (18, 19). Mutation to Tyr most likely reduces the size of the acyl pocket and restricts its accessibility to some bulky insecticides. Thus, the four sites of mutation affect the sensitivity of the enzyme by acting on different facets of the insecticide or substrate binding.

Besides the five mutations found in resistant strains, mutations of other amino acids located in the active-site gorge may result in insecticide resistance. For example, by studying the importance of Tyr-199(Asp-72) for catalytic properties of *Drosophila* AChE, we found that the mutation of this Tyr modifies the sensitivity of the enzyme to organophosphorous and carbamate compounds (20). Similarly, in studying the importance of Glu-199 in the *Toledo enzyme*, Radic et al. (21) found that replacement by Gln and, to a lesser extent by Asp, reduced the rates of phosphorylation and carbamoylation. This indicates that other potential mutations may occur in insect natural populations.

The resistant strains Bygdéa, Pierrefeu, and Saltillo were found to display several associated mutations, and some mutations were found in several strains (Fig. 1). These data suggest that novel resistances can originate from the combination of several mutations in the same protein. To test this hypothesis, we expressed proteins with various combinations of mutations. High resistances were observed for some of the combinations (Fig. 2). As the most resistant strains

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**Fig. 1.** Mutations responsible for resistance in the AChE gene (*Ace*) of *Drosophila*. The top line shows the genomic organization of the regions encoding the mature protein (6). Exons are numbered I–X; arabic numbers give positions in kilobases relative to the origin of the DNA walk. The lower part schematizes the protein sequence with the positions and nature of the five mutations analyzed. S, signal peptide; h, hydrophobic peptide exchanged in the mature protein with a glycolipid anchor.
showed combinations of several point mutations, we hypothesize that high-level resistances are more likely to originate from combinations of point mutations responsible for weak resistances than from the appearance of a point mutation giving a strong resistance.

Two lines of evidence suggest that recombination between preexisting isolated mutations has contributed to the combination of several mutations in the same protein. First, for two pairs of mutations, Gly-303 to Ala/Phe-368 to Tyr and Ile-199 to Val/Phe-368 to Tyr, the four gamete types are present (Fig. 1). This pattern is more easily explained by recombination than by successive mutations. Second, all the mutations observed correspond to only one nucleotide change, while a two-nucleotide change would sometimes result in a more resistant allele. For example in the case of Ile-199 (ATC) to Val (GTC) or Thr (ACC), a change to Gly (GGC) or Ala (GCC) would give a more resistant enzyme (unpublished data) but would correspond to a two-nucleotide change. Although the appearance of a new mutation in a gene already bearing a mutation may occur, these lines of evidence suggest that recombination events also take place to produce more-resistant enzymes. This possibility of acquiring resistance by intracistronic recombination is interesting since it would permit us to explain some features observed in insect resistance.

Examples of insects that are resistant by more than one mechanism, due to different genes, have been reported. It is commonly admitted that resistance can result from recombination events between two or more genes. Extension of this recombination mechanism to mutations within a single gene can help to explain the frequent occurrence of resistant insects. Moreover, intracistronic recombination is more frequent than point mutation: King and Jukes (22) estimated the substitution rate at about $10^{-8}$ to $10^{-9}$ per codon per generation, whereas the crossing-over frequency between two mutations in *Ace of Drosophila* has been shown to reach $10^{-3}$ (23). Further, the highly spliced structure of insect AChE genes (10, 24) greatly favors recombination. Recombination also requires that isolated point mutations preexist in field populations. Evidence for preexisting mutations at significant frequency in natural populations came first with the work of Oppenoorth et al. (25), who found different AChE sensitivities to paraaxon in various susceptible *Musca domestica* strains. Similarly, we found a high heterogeneity in the AChE sensitivity of several susceptible populations of *D. melanogaster* harvested in different parts of the world (9). These differences in sensitivity reflect the presence of different alleles associated with slight resistances.

Resistance to insecticides due to an altered AChE is not a feature common to all insect species. Indeed, among resistant insects, only some of them present a modification of the AChE, whereas others have developed other defense mechanisms—for example, an overproduction of a metabolizing enzyme. This species specificity was difficult to explain by stating that resistance arises from a point mutation, as this event is assumed to occur at random and in a nonspecific manner in regard to the species. To the contrary, intracistronic recombination would help to explain the species specificity. Indeed, several parameters affect recombination, as does, for example, the chromosomal pattern of the genome, the location of the gene on the chromosome, and the length and the structure of the gene. Outbreeding would also favor intracistronic recombination between different alleles, whereas parthenogenetic reproduction would prevent it. All these factors would contribute to an explanation of why resistance due to an altered AChE seems to be restricted to some species.

Intragenic recombination is thought to have important consequences for population and evolutionary genetics, mainly by increasing the number and frequency of different alleles (26). Although there is no evidence for a strict relationship between resistance and fitness cost, the insecticides
are substrate analogs and thus interact with the same site as the natural substrate. A single mutation is likely to be of little fitness cost, whereas an association of several mutations giving strong resistance is more likely to be of some fitness cost and thus to be counterselected in insecticide-free conditions. In natural conditions, populations are subdivided in treated and nontreated areas and are submitted to an alternance of periods with and without treatments. Recombinations which associate several mutations would be favored by an insecticide treatment while the reciprocal recombinations that isolate point mutations would be favored in the absence of treatment. It would be interesting to test whether this alternation of selected and nonselected generations and the possibility of recombination provides a way to maintain neutral point mutations at an unexpectedly high frequency.

Finally, the existence of several point mutations and the occurrence of intracistronic recombinations between the mutations have practical consequences in resistance-management programs. The previously hypothesized unique mutation suggested the use of oligonucleotide probes to survey the dynamics of resistance. On one hand, the presence of several point mutations makes the survey more complicated. But on the other hand, the prediction of resistance becomes easier. Indeed, finding characterized mutations in a population can preclude the utilization of insecticides which would select insects that have undergone recombination. Furthermore, identification of recombination as a mechanism of resistance points out the need to prevent mixing heterologous populations by migration even when they appear to be individually susceptible to insecticides.

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Fig. 3. Stereoview ribbon diagrams of the AChE active-site structure with paraoxon docked by energy minimization techniques. The corresponding Drosophila side chains of amino acids subject to mutations are shown alongside the catalytic-site Ser-276(200) and the anionic-site Trp-121(84). (Upper) Wild type. (Lower) Mutant.

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