BMC Evolutionary Biology

BioMed Central

Research article

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Mutations of acetylcholinesterase which confer insecticide resistance in *Drosophila melanogaster* populations

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Published: 05 February 2004

BMC Evolutionary Biology 2004, **4**:4

Received: 12 December 2003
Accepted: 05 February 2004

This article is available from: http://www.biomedcentral.com/1471-2148/4/4

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Abstract

Background: Organophosphate and carbamate insecticides irreversibly inhibit acetylcholinesterase causing death of insects. Resistance-modified acetylcholinesterases(AChEs) have been described in many insect species and sequencing of their genes allowed several point mutations to be described. However, their relative frequency and their cartography had not yet been addressed.

Results: To analyze the most frequent mutations providing insecticide resistance in *Drosophila melanogaster* acetylcholinesterase, the *Ace* gene was cloned and sequenced in several strains harvested from different parts of the world. Sequence comparison revealed four widespread mutations, I161V, G265A, F330Y and G368A. We confirm here that mutations are found either isolated or in combination in the same protein and we show that most natural populations are heterogeneous, composed of a mixture of different alleles. *In vitro* expression of mutated proteins showed that combining mutations in the same protein has two consequences: it increases resistance level and provides a wide spectrum of resistance.

Conclusion: The presence of several alleles in natural populations, offering various resistance to carbamate and organophosphate compounds will complicate the establishment of resistance management programs.

Background

Acetylcholinesterase (AChE; EC 3.1.1.7) is a key enzyme of the cholinergic system because it regulates the level of acetylcholine and terminates nerve impulses by catalyzing the hydrolysis of acetylcholine. Its inhibition causes death, so irreversible inhibitors have been developed as insecticides: organophosphates and carbamates. They have similar properties to acetylcholine but are hemisubstrates because they phosphorylate or carbamoylate the active-site serine leading to irreversible inhibition of the enzyme. This inhibition leads to an accumulation of acetylcholine in the synapses which in turn leaves the acetylcholine receptors permanently open, resulting in the death of the insect [1].

Table 1: Point mutations in acetylcholinesterase involved in insecticide resistance.

Position in mature AChE of *Torpedo californica*	Mutation	Species	Reference
78	F77(115)S	*Drosophila melanogaster*	[8]
119	G(247)S	*Culex pipiens*	[18]
	S(228)G	*Tetranychus urticae*	[19]
128	D(237)E	*Tetranychus urticae*	[19]
129	I161(199)V	*Drosophila melanogaster*	[8]
	I(214)V	*Bractocera oleae*	[20]
150	V180L(260)	*Musca domestica*	[21,22]
227	G265(303)A	*Drosophila melanogaster*	[8]
	G262(342)A	Musca domestica	[21,22]
	G262(342)V	Musca domestica	[21,22]
238	S(291)G	*Leptinotarsa decemlineata*	[23]
290	F330(368)Y	*Drosophila melanogaster*	[11]
	F327(407)Y	Musca domestica	[21,22]
328	G365(445)A	*Musca domestica*	[20]
	G368(406)A	*Drosophila melanogaster*	This study
331	S(431)F	*Myzus persicae* (MpAChE2)	[24]
	F(445)W	*Culex tritaeniorhynchus*	[25]
	F(439)C	*Tetranychus urticae*	[19]
396	G(488)S	*Bractocera oleae*	[20]

Numbers in brackets refer to the precursor numbering. Corresponding position in *Torpedo californica* sequence has been added since this sequence is used as reference for the cholinesterase family.

In 1961, Smissaert described the first case of AChE with a reduced sensitivity to pesticides [2]. Since then, resistance-modified AChEs have been described in many insect species [3-7]. Sequencing of the gene encoding AChE in resistant strains showed that the modifications arose from point mutations (Table 1). Combinations of several point mutations in the same protein were found in several alleles where they induced higher levels of resistance [8,9]. Most mutations were identical in several species, suggesting that a low number of mutations can actually provide resistance. These findings presented a striking contrast to experiments of *in vitro* expression of mutagenesized AChE, which revealed that insecticide resistance should have genetic diversity [10]. Besides qualitative modification of the enzyme, overproduction of AChE results in insecticide resistance as shown first experimentally by transforming *Drosophila* to increase the dose of enzyme and second by finding a positive correlation between the amount of AChE and resistance in natural populations [11,12].

The evolution of insecticide resistance in insects tends to be rapid because selection is strong, populations are large, and generation times are short. With the threat of insecticide resistance looming larger, it is absolutely necessary to investigate the molecular basis of AChE mutation distribution in natural populations from different parts of the world. In this paper, we report the most frequent point mutations of AChE genes associated with insecticide resistance and the different patterns of mutation combinations in natural populations of *D. melanogaster*.

Results

Point mutations detected in D. melanogaster *populations*

To identify mutations involved in insecticide resistance, the *Ace* gene encoding AChE in 30 strains of *D. melanogaster* harvested throughout the world was sequenced. Sequences were compared to a reference, the sequence from a strain harvested before the utilisation of insecticide (Canton-S strain). Three clones were sequenced per strain, when variability was detected, three new clones were sequenced. We found 18 mutations resulting in a change of the amino-acid sequence of the protein. These mutations were verified by PASA to eliminate the possibility of a PCR artefact. Among these mutations some were found only once and their positions were far from the active site, so should not be responsible for insecticide resistance (V19M, F187L, E193V, N228D, E281G, N300S, M305T, S325P, E453V, G489D, K507D).

Two mutations (E72G and E81K) were detected in the strains STIC from Italy and NY from the U.S.A. respectively. They are located near the entrance of the active site gorge in a position favourable to affect insecticide sensitivity. Similarly, another mutation located at the rim of the gorge (F77S) has already been reported from the strain Saltillo, which affects the sensitivity to insecticide [8], but this mutation was not found again in the present screening. As these three mutations (E72G, E81K and

Table 2: Alleles found in natural populations of *D. melanogaster*.

Strain	Origin	Sequence at position				Alleles
		161	265	330	368	
WC2	USA	ATC/**G**TC	GGC/G**C**C	TTT/T**A**T	GGC	IGFG, **V**GFG, I**A**FG, **VA**FG, **VAY**G
WC6	USA	ATC	GGC/G**C**C	TTT	GGC	IGFG, I**A**FG
WC97.1	USA	ATC/**G**TC	G**C**C	T**A**T	GGC	I**AY**G, **VAY**G
NY	USA	**G**TC/ **GC**C	GGC/ G**C**C	TTT/T**A**T	GGC	**A**GFG, **VAY**G
BHA	USA	ATC	GGC	TTT	GGC	IGFG
TORREON	Mexico	ATC/**G**TC	GGC/G**C**C	TTT/T**A**T	GGC	IGFG, **V**GFG, I**AY**G, **VAY**G
GUA	Guadeloupe	**G**TC	G**C**C	T**A**T	GGC	**VAY**G
RIC	Costa Rica	ATC/**G**TC	GGC/G**C**C	TTT/T**A**T	GGC	IGFG, I**A**FG, **V**G**Y**G, **VA**FG, **VAY**G
CART	Colombia	ATC	GGC	TTT	G**CT**	IGF**A**
MONT	Canada	ATC	GGC/G**C**C	TTT	G**CT**	IGFG, IGF**A**, I**A**FG
TBK72	Australia	**G**TC	GGC	TTT	GGC	**V**GFG
A12	Australia	ATC	GGC	TTT	GGC	IGFG
NOTT	U.K.	ATC	GGC	TTT/ T**A**T	GGC	IGFG, IG**Y**G
PIERREFEU	France	**G**TC	GGC	TTT	G**C**C	**V**GF**A**
CT	France	ATC	GGC	TTT	GGC	IGFG
NAU	France	ATC	GGC	TTT	GGC	IGFG
SANC	France	ATC	G**C**C	TTT	GGC	I**A**FG
STEG	France	ATC/**G**TC	GGC/G**C**C	TTT/ T**A**T	GGC	IGFG, **VAY**G
GRAM	France	ATC	GGC	TTT	G**C**C	IGF**A**
BEAU	France	**G**TC	GGC	TTT	G**C**C	**V**GF**A**
BIZIAT	France	ATC	GGC/G**C**C	TTT	GGC	IGFG, I**A**FG
FLO	Italy	ATC	G**C**C	TTT	GGC	I**A**FG
STIC	Italy	**G**TC	G**C**C	T**A**T	GGC/G**C**C	**VAY**G,**VAYA**
NORV	Norway	ATC	GGC/G**C**C	TTT	GGC	IGFG, I**A**FG
TUBINGEN	Germany	ATC	G**C**C	TTT	GGC	I**A**FG
BUD	Hungary	**G**TC	GGC	TTT	G**C**C	**V**GF**A**
MK	Marocco	ATC/**G**TC	GGC/G**C**C	TTT/T**A**T	GGC	I**A**FG, IG**Y**G, **VAY**G
NIMB	Ivory Coast	ATC	GGC	TTT	GGC	IGFG
NDL	India	ATC	GGC/G**C**C	TTT/T**A**T	GGC	IGFG, I**A**FG, I**AY**G, **VAY**G

The alleles correspond to the amino-acids found at the four positions 161, 265, 330, 368. Mutations are highlighted by the use of bold font.

F77S) were present only in one population, their effects were not studied in detail in this paper.

Four mutations, I161V, G265A, F330Y and G368A, which are located near the active site, were found in several natural populations (Table 2). These mutations have already been described in Drosophila and/or in other insects (Table 1). The reference sequence I161, G265, F330 and G368 (allele IGFG) most probably represents the wild type sequence since then same amino-acids were present in the other available AChE sequences from Brachycera (*Musca domestica*, *Lucilia cuprina* and *Bractrocera oleae*) and each mutation arose from a single mutation. At position 161, the mutation ATC to GTC changes the isoleucine to valine. In one strain (NY), we found another mutation (GTC to GCC) changing the valine to alanine (allele AGFG, Table 2). Compared to valine, this mutation provided higher resistance to some insecticides: for example, the alanine mutant exhibited 10- and 7-fold less sensitivity than the valine mutant to diazinon-oxon and monocrotophos respectively (data not shown). However, as this mutation has only been found once and is not widespread, we did not study it thoroughly. At position 368, two different codons (GCC or GCT) encode alanine suggesting either that the mutation of glycine to alanine originated from two independent events or that the C/T transition occurred in the GCC allele. The fact that we did not find any GGT alleles in natural populations favours the second hypothesis.

Other mutations may be present in natural populations. Only 30 populations were analyzed, and some parts of the world were missing from the study or weakly represented. Furthermore the rearing of some populations in laboratory conditions for a long time without insecticide treatments may have caused the loss of certain mutations. However, I161V, G265A, F330Y and G368A seem to be the most frequent mutations providing insecticide resistance. This is consistent with the observation that these mutations were also found in other species (Table 1).

Table 3: Number of each allele found in natural populations of *D. melanogaster*.

no mutation		single mutation		two mutations		three mutations		four mutations	
IGFG	16	**V**GFG	3	**VA**FG	2	**VAY**G	10	**VAYA**	1
		I**A**FG	11	**V**G**Y**G	1	**V**G**YA**	0		
		IG**Y**G	2	**V**GF**A**	3	**VA**F**A**	0		
		IGF**A**	3	I**AY**G	3	I**AYA**	0		
				I**A**F**A**	0				
				IG**YA**	0				

Mutations are highlighted by the use of a bold font.

The four mutations can be amassed in one allele as shown in Table 2 (the allele IGFG represents the wild type allele and VAYA the allele with the four mutations). Various combinations were found in natural populations, including the allele with all four mutations. Although screening was not performed to estimate the proportion of each allele, we did, however, note that allele IAFG with a single mutation at position 265 and allele VAYG with a triple mutation (at positions 161, 265 and 368) were the most frequent (Table 3).

Geographic distribution of point mutations I161V, G265A, F330Y and G368A

To tackle the geographic repartition of the four mutations, we checked their presence by PASA in several strains from several parts of the word (Table 4). At least ten flies were analyzed per strain. Some mutations were not detected by sequencing (Table 2) showing that the not all alleles in each population were detected. It appears that the four mutations are distributed all around the world. The worldwide distribution of these mutations might result from the ability of Drosophila to disperse either as larvae in fruits or as adults.

Effect of mutations I161V, G265A, F330Y and G368A on insecticide resistance

Site-directed mutagenesis was used to study the effect of the four mutations on insecticide sensitivity. Mutated proteins were expressed in the baculovirus system and proteins were purified to homogeneity. The four single mutations, I161V, G265A, F330Y and G368A, affect insecticide sensitivity (Table 5) but show a strong disparity with regard to the 17 insecticides tested. For example resistance to coumaphos-oxon was obtained by mutation G265A (allele IAFG) and resistance to diazinon-oxon by mutations F330Y (allele IGYG).

The combinations of mutations have two different types of effect. First, they provide higher resistance, as previously reported [8]. For each insecticide, the allele which provides higher resistance corresponds to a combination of three or four point mutations (Table 5). However, this effect is not systematic, for example, the double mutant (allele VGYG) does not change the sensitivity to insecticides except for paraoxon, when compared with sensitivity of single mutants (alleles VGFG and IGYG). Second, combinations decrease the specificity of resistance: a single mutation provides resistance to 76% of insecticides (and sensitivity in 24%), double mutations provide resistance to 78% of insecticides, triple mutations provide resistance to 97% of insecticides and the co-occurrence of all four mutations provides resistance to all the insecticides tested.

Discussion

Heterogeneity of populations and insecticide resistance

As shown by cDNA sequencing, several alleles coexist in most natural populations. For example, there were five alleles found in strain WC2 from USA and RIC from Costa Rica and four alleles in TORREON strain from Mexico and NDL strain from India. Because each allele manifested a specific resistance to certain insecticides, this allelic diversity in one natural population might enable the population to survive against a large variety of insecticide treatments. Correspondingly, subjecting field populations to a multiplicity of treatments might induce the emergence of multiple coexisting resistance alleles.

Practical consequences of the combination of mutations involved in insecticide resistance

In several cases, single mutations sensitised the enzyme to the insecticide (ki ratio was lower than 1.0, see Table 5). For instance, the mutation G368A rendered sensitivity to coumaphos oxon 4.0 times greater than that of the reference enzyme. The I161V/F330Y mutation caused 7.7 fold more sensitivity to monocrotophos than the reference enzyme.

One method to decrease the resistance of a field population is to apply an anti-resistance insecticide, which is an insecticide more active against the mutated protein than against the wild type protein. This strategy seems feasible when there is only one mutation in the population; for example, carbaryl can be used to decrease the frequency of

Table 4: Geographic distribution of point mutations at positions 161, 265, 330 and 368 determined by PASA.

Strain	Country	161	265	330	368
Europe					
BDX	France	I	G	F	G
BEAU	France	**V**	G	F	**A**
BIZIAT	France	I	G/**A**	F	G
BOB SS	France	I	G	F	G/**A**
BZH	France	I	G	F	G
GRAM	France	I	G	F	**A**
NAU	France	I/**V**	G	F	G
CO	France	I	G	F	G
CT	France	I	G	F	G
PIERREFEU	France	I/**V**	G/**A**	F	G/**A**
SANC	France	I	**A**	F	G
STEG	France	I/**V**	G/**A**	F/**Y**	G/**A**
ECO	U.K.	I	G	F	G
NOTT	U.K.	I	G/**A**	F/**Y**	G
TUBINGEN	Germany	I/**V**	G/**A**	F/**Y**	G
KU	Germany	I/**V**	G	F	**A**
BUD	Hungary	I/**V**	G/**A**	F/**Y**	G/**A**
NORV	Norway	I/**V**	G/**A**	F	G
POZ	Poland	I/**V**	G	F	G
STIC	Italy	I/**V**	G/**A**	F/**Y**	G/**A**
FLO	Italy	I/**V**	**A**	F	G
VAG(3875)	Greece	I	G	F	G
ES	Azores	I	G	F	G
BB 31	Australia	I	G/**A**	F	G
North America					
BHA	U.S.A.	I	G	F	G/**A**
BO	U.S.A	I	G/**A**	F	G/**A**
NY	U.S.A	**V**	**A**	**Y**	G
WC 97-8	U.S.A	I/**V**	G/**A**	F/**Y**	G
WC 2	U.S.A	I/**V**	G/**A**	F/**Y**	G
WC 6	U.S.A	I	G/**A**	F	G
WC 96-5	U.S.A	I/**V**	G/**A**	F/**Y**	G
WC 97-1	U.S.A	I/**V**	G/**A**	F/**Y**	G
WC 97-15	U.S.A	I/**V**	G/**A**	F/**Y**	G
MONT	Canada	I/**V**	G/**A**	F	G/**A**
RALEIGH	France	I/**V**	G	F	G
Central and South America, West Indies					
TORREON	Mexico	I/**V**	G/**A**	F/**Y**	G
CART	Columbia	I/**V**	G/**A**	F/**Y**	G/**A**
BOL	Bolivia	I	G	F	G/**A**
GUY	Guyana	I	G/**A**	F	G
RIC	Costa Rica	I/**V**	**A**	F/**Y**	G
GUA	Guadeloupe	I/**V**	G/**A**	F/**Y**	G
CUBA	Cuba	I/**V**	G/**A**	F/**Y**	G/**A**
MAR	Martinique	I	G/**A**	F	G
Africa and Indian Ocean					
MK	Morocco	I/**V**	G/**A**	F/**Y**	G/**A**
BISS	Guinea	I	G	F	G
DK	Senegal	I/**V**	G/**A**	F/**Y**	G/**A**
NIMB	Ivory Coast	I/**V**	G	F	G
GHE	Ethiopia	I	G/**A**	F	G
KEN	Kenya	I	G/**A**	F	G
MAD	Madagascar	I	G	F	G/**A**
Australia and Pacific islands					
A 12	Australia	I	G	F	G/**A**
A 80	Australia	I	G/**A**	F	G/**A**
INN 7	Australia	I	G/**A**	F	G
NB 18	Australia	I/**V**	G	F	G/**A**
RHS 2	Australia	I	G/**A**	F	G
TBK 72	Australia	**V**	G/**A**	F	G
TAH	Tahiti	I/**V**	G	F	G
TA	Vanuatu	I	G	F	G/**A**
Asia and Middle East					
HIKONE	Japan	I	G	F	G
2375	Japan	I	G	F	G/**A**
NDL	India	I/**V**	G/**A**	F/**Y**	G/**A**
NW	Arabia	I	G	F	G

In some cases the two amino-acids were found in the same population. For example at position 265 in the Strain Biziat, a glycine was found in some individuals and an alanine in others.

the G368A mutant (allele IGFA). However, with the combination of multiple mutations, this strategy is less and less feasible, since the protein becomes resistant to all the insecticides. Furthermore, field populations are composed of a mixture of different alleles with different sensitivities to each insecticide, so treatment with one pesticide would eliminate one allele, but would select another one. For instance, treatment with carbaryl will decrease the frequency of the allele IGFA but will increase the frequency of the allele VAYG. So, the anti-resistance insecticide strategy does not seem to be efficient in field conditions.

Material and Methods

Fly Strains

Strains of *D. melanogaster* were collected from several parts of the world (Table 2, 3, 4). They were maintained for several generations in laboratory conditions, without insecticide treatment. Canton-S strain, as a reference, has been cultured in the laboratory since its collection at the beginning of the 20th century in the USA.

RT-PCR, cloning, and sequencing

Total RNA was prepared using the RNeasy mini kit from Qiagen and mRNA was purified using oligodT-cellulose.

Table 5: Effects of different patterns of mutation combinations on insecticide resistance represented as *ki* ratio (*ki* reference/*ki* mutant enzyme).

	1 mutation			2 mutations							3 mutations				4 mutations
Alleles	IGYG	VGFG	IAFG	IGFA	VGYG	IAYG	IGYA	VAFG	VGFA	IAFA	VAYG	VGYA	IAYA	VAFA	VAYA
methyl-azinphos oxon	2.2	0.92	3.2	0.87	0.49	5.3	2.0	5.0	1.8	40	4.8	5.1	16	**77**	17
carbaryl	1.9	0.93	1.9	0.46	1.9	0.88	2.1	2.7	0.58	3.9	**12**	1.6	7.3	7.2	5.4
carbofuran	2.3	0.52	1.8	1.8	1.0	1.0	2.4	1.7	0.62	1.4	4.3	1.4	**7.2**	3.9	3.3
chlopyriphos oxon	1.7	0.80	1.5	10	0.92	5.5	5.7	1.9	4.5	10	11	2.9	23	**30**	8.6
coumaphos oxon	3.0	3.9	38	0.25	0.40	190	7.5	51	4.7	64	**1100**	210	230	95	250
diazinon oxon	91	1.9	2.2	1.3	2.2	45	4.7	12	7.0	32	8.3	**640**	13	3.7	12
dichlorvos	4.4	1.4	1.9	1.0	0.23	0.40	9.1	2.2	1.2	14	9.0	7.6	4.8	16	**30**
omethoate	0.35	0.95	1.0	8.4	0.66	1.2	2.8	0.71	21	**39**	12	11	21	14	29
malaoxon	3.9	6.4	1.7	10	1.0	5.7	37	16	6.5	40	32	48	83	1900	**5900**
methamidophos	2.53	0.29	4.2	11	2.53	2.65	16	1.8	5.9	76	0.37	150	84	150	**290**
methiocarbe	0.77	0.50	3.6	2.1	0.67	7.0	0.92	2.4	0.34	4.5	4.0	0.45	**16**	7.8	5.0
monocrotophos	0.58	0.78	5.6	2.1	0.13	0.69	2.3	8.7	0.44	30	5.9	4.9	6.7	**46**	21
paraoxon	2.2	2.3	8.9	8.7	6.0	10	4.3	41	4.7	67	**330**	9.1	47	97	93
methyl-paraoxon	2.3	3.4	6.5	11	0.28	3.7	2.4	2.1	1.3	**86**	14	5.4	26	31	58
pirimicarbe	2.6	1.3	5.6	5.3	0.83	6.9	2.4	32	3.7	40	28	3.2	42	42	**56**
propoxur	4.1	1.2	1.5	0.91	0.76	**8.6**	2.1	1.1	0.37	3.5	5.5	1.4	8.0	2.7	3.7
triazophos oxon	5.9	1.7	0.90	1.8	0.55	0.89	2.8	1.6	0.65	13	16	5.7	6.8	4.4	**17**

The most resistant alleles for each insecticide are in bold. All differences exceeding a factor of 2, (below 0.5 or above 2) are significant.

First strand cDNA was obtained with random hexanucleotides as primers using the first-strand cDNA synthesis kit from Pharmacia Biotech. *Ace* cDNA were PCR amplified using primers hybridizing on the sequence encoding the signal peptide and on the sequence encoding the C-terminal hydrophobic peptide. As these two peptides were taken off during the maturation of the protein, the whole sequence of the mature protein was available. RT-PCR products were cloned into the P3T vector digested by Xcm1 and Sma1 [13].

Genomic DNA extraction and point mutations confirmed by PASA

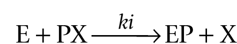
Genomic DNA was isolated from each population of *D. melanogaster* by phenol/chloroform/isoamyl alcohol extraction. PCR amplification of specific alleles (PASA) was used to detect point mutations in natural populations of *D. melanogaster*. PASA is a modification of PCR that depends on a PCR oligonucleotide primer that precisely matches one of the alleles but mismatches with the other. When the mismatch occurs at the 3' end of the PCR primer, amplification is inefficient. Therefore, only amplification of the perfectly matched allele is obtained. It was performed using three oligonucleotides to exclude the possibility of PCR artefacts [14].

Site-directed mutagenesis and protein preparation

We generated point mutations using a standard PCR based strategy and verified the identities of individual clones through double stranded plasmid sequencing. DNA encoding soluble AChEs, reference type or mutated, of *D. melanogaster* were expressed with the baculovirus system [15]. Secreted AChEs were purified to homogeneity and stabilized with 1 mg/ml BSA as previously reported [16]. Amino-acid numbering follows that of the mature protein as in the structure published by Harel *et al.* [17] (http://www.rcsb.org/pdb; PDB code: 1QO9) to facilitate the visualisation of the mutation on the structure. It corresponds to the numeration of the precursor used in a previous paper [8] minus the 38 amino-acids of the signal peptide.

Determination of enzyme sensitivity

The inhibition mechanism of AChE by organophosphate and carbamate compounds has been described by Aldridge [1].



$$E + PX \xrightarrow{ki} EP + X$$

with E = enzyme, PX = organophosphate or carbamate, X = leaving group. The insecticide phosphorylates or carbamoylates the active site serine of one enzyme molecule and the inhibition can be considered as irreversible during the first 30 min. Disappearance of free enzyme ([E]) follows second-order kinetics.

$$\frac{[E]}{[Eo]} = \frac{([PXo]-[Eo]).e^{-ki.t.([PXo]-[Eo])}}{[PXo]-[Eo].e^{-ki.t.([PXo]-.[Eo])}} \quad \text{(equation 1)}$$

where [PXo] is the initial concentration of inhibitor, [E] is the free enzyme remaining at time t, [Eo] is the initial con-

centration of enzyme, t represents the time of incubation, and ki the biomolecular rate constant. [PXo] and t are known, [E]/ [Eo] is estimated by incubating the enzyme with the insecticide at 25°C in 25 mM phosphate buffer pH7. The variation of the remaining free enzyme [E]/ [Eo] with time was estimated by sampling aliquots at various times and recording the remaining activity ([A]/ [Ao]) with 1 mM acetylthiocholine since [A]/ [Ao] = [E]/ [Eo]. Kinetics studies were performed with at least three concentrations of insecticide. The values of ki were estimated by multiple non-linear regression with [PXo] and t as variables. Data were collected until standard deviation came below 10% of the ki value. The resistance level of the mutated enzyme was expressed as the ratio of ki (ki reference/ ki mutant enzyme)

Authors' contributions

PM sequenced alleles and verified the occurrence of mutations by PASA. MAS purified the recombinant proteins and estimated their sensitivity. AL performed *in vitro* mutagenesis and production of mutants. ZHT participated in coordination and DF conceived the study. All authors read and approved the final manuscript.

Acknowledgments

We thank Tom Wilson (Colorado State Univ.), Eric Bonnivard (Univ. Paris 6), Philip Daborn (Melbourne Univ.) for providing *Drosophila* strains. This research was supported by grants from the European Community (ACHEB, QLK3-CT-2000-00650 and SAFEGUARD, QLK3-CT-2000-000481) and from the Chinese-French Advanced Research Program (PRA BT01-01).

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