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Research article

Acetylcholinesterase alterations reveal the fitness cost of mutations conferring insecticide resistance

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Abstract

Background: Insecticide resistance is now common in insects due to the frequent use of chemicals to control them, which provides a useful tool to study the adaptation of eukaryotic genome to new environments. Although numerous potential mutations may provide high level of resistance, only few alleles are found in insect natural populations. Then, we hypothesized that only alleles linked to the highest fitness in the absence of insecticide are selected.

Results: To obtain information on the origin of the fitness of resistant alleles, we studied *Drosophila melanogaster* acetylcholinesterase, the target of organophosphate and carbamate insecticides. We produced *in vitro* 15 possible proteins resulting from the combination of the four most frequent mutations and we tested their catalytic activity and enzymatic stability. Mutations affected deacetylation of the enzyme, decreasing or increasing its catalytic efficiency and all mutations diminished the stability of the enzyme. Combination of mutations result to an additive alteration.

Conclusion: Our findings suggest that the alteration of activity and stability of acetylcholinesterase are at the origin of the fitness cost associated with mutations providing resistance. Magnitude of the alterations was related to the allelic frequency in *Drosophila* populations suggesting that the fitness cost is the main driving force for the maintenance of resistant alleles in insecticide free conditions.

Background

Since the 1940's, the entire planet has been spread with insecticides and a lot of insects have developed resistance. As treatment is not continuous, insects have to adapt to be competitive in alternating periods with and without treatments. Thus, insecticide resistance offers the opportunity to study the adaptation of eukaryotes to variable environments. Resistance can be defined as the adaptation of a population from an environment free of insecticide to a new environment contaminated with new toxic molecules. Three main mechanisms of resistance to insecticides occur: reduction of insecticide penetration, increased degradation and modification of the insecticide target. One target is well documented: acetylcholinesterase (AChE, EC 3. 1. 1. 7). It is a key enzyme in the cholinergic synapses where it rapidly terminates nerve impulses by catalyzing

the hydrolysis of the neurotransmitter acetylcholine. Organophosphates are substrates of AChE and their hydrolysis results in the phosphorylation of the active serine followed by dephosphorylation [1]. This dephosphorylation is very long and takes several days, synaptic transmission remains blocked, whereas deacetylation of the acetylated enzyme by its natural substrate acetylcholine is a rapid process, 1000 s^{-1} in insects.

Consequently, blockage of AChE by organophosphate insecticides leads to the death of the insect. In 1964, Smissaert described a modified acetylcholinesterase less sensitive to inhibition by insecticide in the two-spotted spider mite [2], clearly demonstrating that organophosphate insecticides are poisonous to insects by inhibiting AChE. The work of Smissaert also showed for the first time that a pest could acquire resistance to insecticide through modification of AChE, the enzyme from the resistant strain being less inhibited than that from the susceptible strain. Such modifications have been reported for a large number of species [3]. Following the cloning of the gene encoding AChE, several mutations have been identified in *Drosophila* populations [4]. These mutations are near the active site of the enzyme and may affect the entrance of the insecticide into the active site [5]. A recent screening allowed the identification of four widespread mutations, I161V, G265A, F330Y and G368A. These four mutations were found either isolated or in combination in the same protein and most populations were heterogeneous, composed of a mixture of different alleles. Single mutations provide low level and specific insecticide resistance and combination of three or four mutations in the same protein provides high level and wide spectrum of resistance [6].

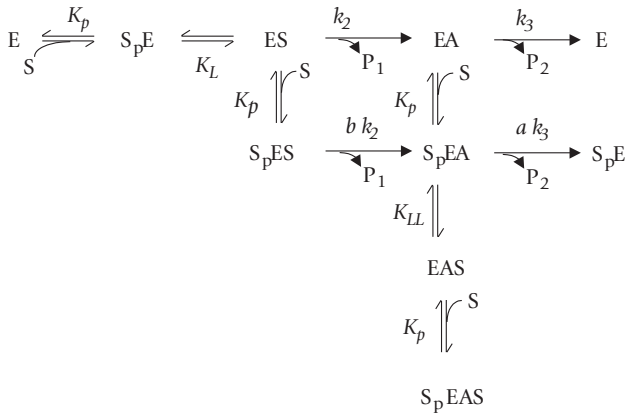
Resistance to insecticides usually appears to be unstable, associated with a genetic cost in the absence of selection [7-9]. In natural populations of *Drosophila*, we suspected that some alleles were associated with fitness cost following the observation that there were great discrepancies between the frequencies of the resistance alleles and the insecticide resistance levels they provided. To analyze the biochemical origin of this cost, we produced *in vitro* all the resistant proteins and tested their catalytic activity and enzymatic stability.

Results

Effect of single mutations on substrate hydrolysis

Do mutations have a cost? To answer this question, we first looked for the effect of mutations on the hydrolysis of acetylthiocholine by *in vitro* produced AChEs. When acetylcholine is released into the synapse cleft, the local concentration of acetylcholine is high, above 1 mM. At this concentration, AChE is inhibited by the substrate and hydrolysis of acetylcholine becomes low. When acetylcholine is diluted in the synaptic cleft to 1 mM, the activity of AChE is maximal and the acetylcholine concentration quickly decreases below 1 μM because the enzyme is able to hydrolyze its substrate with a wide range of concentrations [10].

Hydrolysis of acetylthiocholine by each molecule of the enzyme (v/Et) was recorded at substrate concentrations from 2 μM to 300 mM and analyzed according to the following scheme.



The substrate molecule (S) binds to the rim of the active site to form the complex SpE and then slides down to the bottom of the active site gorge (ES) [11-13]. It is cut to generate the acetylenzyme EA and choline (P_1) which leaves the active site. A water molecule near the active serine is used to deacetylate the enzyme to regenerate the free enzyme (E) and release acetic group (P_2) [14]. When there is a substrate molecule at the bottom of the active site (ES), a new molecule of substrate can bind at the rim to form the ternary complexes, SpES or SpEA. This binding has two effects: it inhibits acylation by hindering choline release ($b < 1$) [15] and accelerates deacetylation ($a > 1$) [16]. When choline is released in the complex SpEA, the substrate molecule at the rim of the gorge can slide down to the bottom to form the complex EAS. This molecule of substrate completely inhibits deacetylation and thus decreases the enzyme activity [17]. The rate equation derived from this scheme is:

Table 1: Effect of mutations on kinetic parameters of substrate hydrolysis by single mutations. Data were fitted on equation derived from scheme 1. Only parameters significantly modified in mutated proteins are shown.

	Wild type	I161V	G265A	F330Y	G368A
k_2 (s^{-1})	19000	15000	67000	30000	28000
k_3 (s^{-1})	400	154	860	120	180
K_p (μM)	180	-	-	-	300
K_L	1	-	-	-	16
K_{LL}	127	-	400	1150	-
a	4.2	-	-	-	-
b	0.16	-	-	-	-
k_{dec} (10^{-6} s^{-1})	108	57	122	61	59

$$v = \frac{\dfrac{k_2 k_3}{\dfrac{k_2\left(1+\dfrac{[S]}{K_p}+\dfrac{[S]}{K_p K_{LL}}+\dfrac{[S]^2}{K_p^2 K_{LL}}\right)}{1+a\dfrac{[S]}{K_p}}+\dfrac{k_3\left(1+K_L+\dfrac{[S]}{K_p}\right)}{1+b\dfrac{[S]}{K_p}}}[E]_0[S]}{[S]+\dfrac{\dfrac{k_3 K_p K_L}{1+b\dfrac{[S]}{K_p}}}{\dfrac{k_2\left(1+\dfrac{[S]}{K_p}+\dfrac{[S]}{K_p K_{LL}}+\dfrac{[S]^2}{K_p^2 K_{LL}}\right)}{1+a\dfrac{[S]}{K_p}}+\dfrac{k_3\left(1+K_L+\dfrac{[S]}{K_p}\right)}{1+b\dfrac{[S]}{K_p}}}}$$

The kinetic parameters were fitted with the equation and are presented in table 1. Kinetic constants for the wild type enzyme have been previously estimated using the effects of a substrate analogue on substrate hydrolysis and on decarbamoylation [18]. The same constants were used for the mutants and we searched the nearest set of parameters providing an acceptable fit, *i.e.* when estimated values fall inside the fiducial limits of experimental values. It appears that the four single mutations mainly affect acetylcholine hydrolysis via acylation rate constant (k_2) and deacylation rate constant (k_3), while initial binding at the rim of the gorge (K_p), sliding into the free site (K_L), or into the acetylated site (K_{LL}), acceleration of deacetylation (a) and inhibition of choline exit (b) remained slightly affected.

To verify the effect of mutations on deacetylation, we measured the rate of decarbamoylation of the enzymes. The reactivation could be described by a simple first-order rate equation. The decarbamoylation rate constant (k_{dec}), *i.e.* the number of μmoles of product formed per second per μmole of enzyme, was calculated by non-linear regression analysis using equation:

$$[E]_t = [Ec]_o(1 - e^{-k_{dec}\cdot t}) + [E]_o$$

where $[E]_t$ represents the free enzyme concentration at time t, $[E]_0$ the initial concentration of free enzyme and $[Ec]_0$ the initial concentration of mono-methylcarbamoylated enzyme. Results confirmed the effect of the single mutations on deacetylation of the enzyme, a decrease for I161V, F330Y and G368A and an increase for G265A (Table 1).

Effect of combined mutations on substrate hydrolysis

In populations, the four mutations are present with different types of combinations in the same allele. Some combinations, such as the triple mutant I161V / F330Y / G368A which combines three mutations which decrease the catalytic efficiency of the enzyme, have a drastic effect on enzymatic activity (Fig. 1). By contrast, other combinations result in a protein with the same activity as the susceptible wild type protein. For example, the double mutant G265A / F330Y, the triple mutant I161V / G265A / F330Y and the quadruple mutant have a similar activity to the wild type. In these examples, mutation G265A compensates the decrease of deacetylation produced by F330Y, I161V and G368A.

Effect of mutation on protein stability

The stability of the mutated protein was estimated by studying the irreversible thermal inactivation at several temperatures (from 37.5 to 57.5°C) and plotted the first-order denaturation rate constant (kd) against the reciprocal of the absolute temperature ($°K^{-1}$). It appeared that all four single mutations decreased the stability of the protein (Fig. 2). This effect strengthened with the number of mutations combined in the same protein: higher the number of mutations, the lower the stability of the mutant.

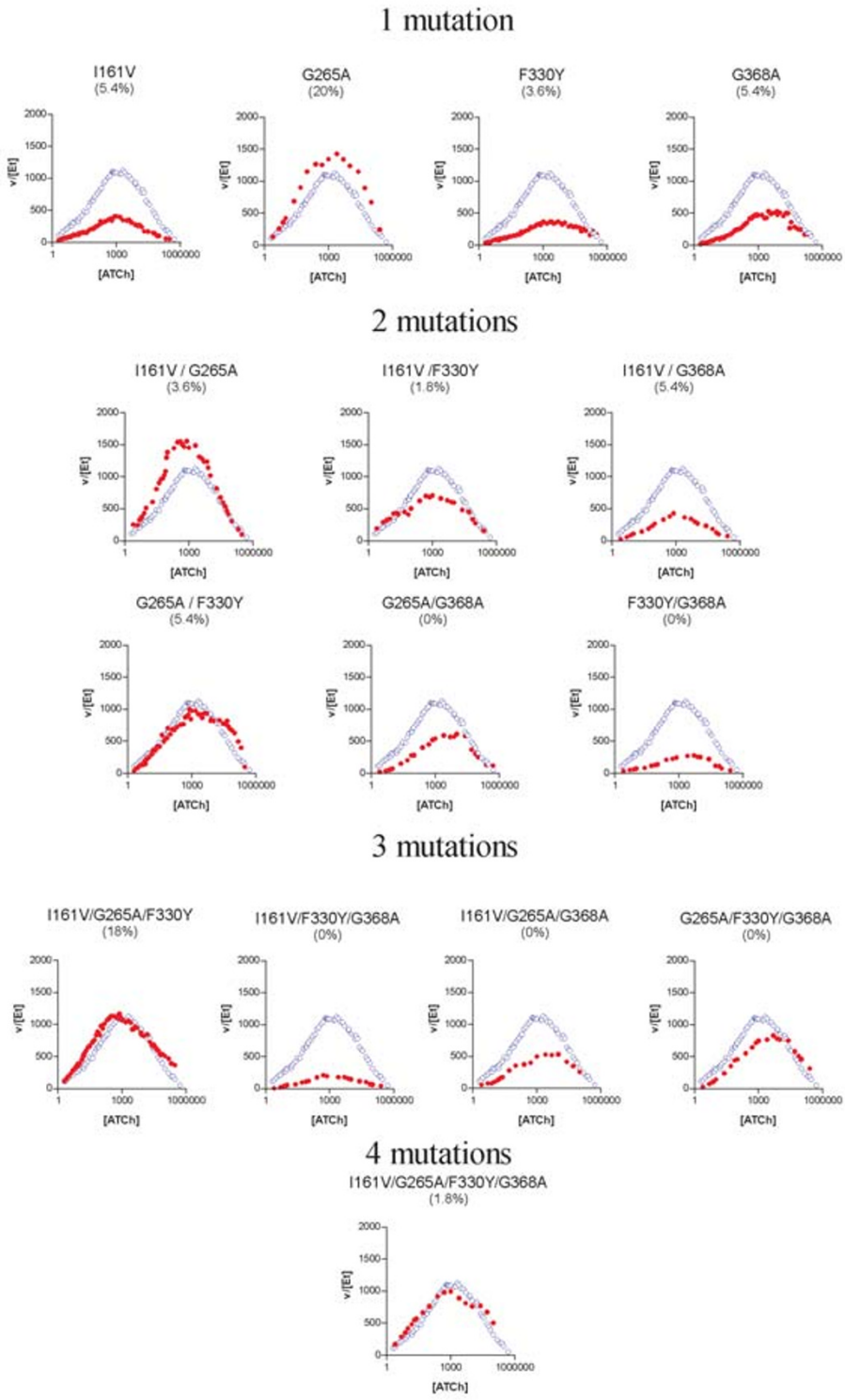


Figure 1
Effect of mutations and their combinations on acetylthiocholine hydrolysis versus substrate concentration (log scale). The frequency of each allele found by sequencing *ace* of 29 *Drosophila* populations from many parts of the world is provided for each mutant. (blue circle): wild type; (red circle): mutant. [ATCh]: Acetylthiocholine concentration in micromole per liter ; v/ [Et] specific activity in s^{-1}.

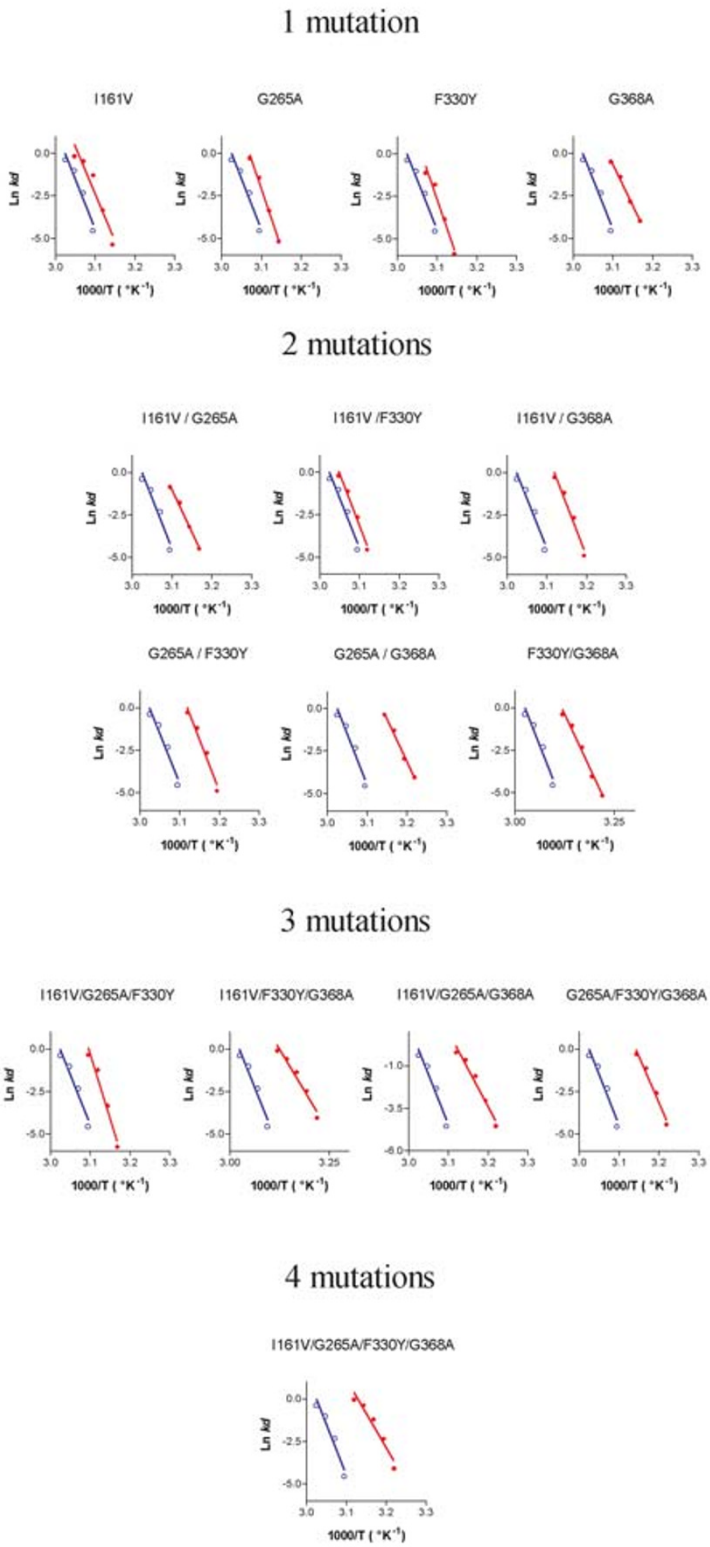


Figure 2
Arrhenius plots of thermal inactivation rate constants of mutated AChE compared to wild type. (blue circle): wild type; (red circle): mutant.

Discussion

Effect of mutations on protein activity and stability

The four single mutations affect the activity of the enzyme. Isoleucine 161 is located at the bottom of the active site behind tryptophan 83 which is the main component of the choline binding site. Mutation to valine increases the freedom of tryptophan 83, destabilizing substrate or insecticide binding in the Michaelian complex resulting in decreases of acetylation and deacetylation. As phosphorylation by insecticide corresponds to the acylation step, the mutated enzyme is less inhibited and decreased phosphorylation renders insects with this mutation resistant. But decreased deacetylation diminishes the ability to hydrolyze the neurotransmitter. Decreased stability was also observed for this mutant, since valine is smaller than isoleucine, this mutation creates a cavity which is energetically unfavorable for protein stability due to loss of van der Waals contacts [19].

Glycine 265 is positioned behind the active serine 238. This glycine is highly conserved in the cholinesterase family and its mutation to valine increases the activity of the enzyme mainly by modifying the orientation of serine 238. This changed orientation increases acetylation by the substrate as well as deacetylation, resulting in an improved efficiency of synapse cleaning. On the other hand, this mutation decreases the protein's stability most probably by steric hindrance. In consequence, this mutation should be near to neutrality for the fitness of the fly, due to the decreased stability compensated by the increased activity.

Phenylalanine 330 lines the active site gorge and is a component of the acyl pocket, which accommodates the acetyl moiety of acetylcholine. Mutation to tyrosine reduces the size of the acyl pocket and decreases the activity of the protein. To understand how this works, we mutated the residue to different amino acids differing in size and in hydrophobicity (G, A, V, L, I, W). With all these mutations, we observed decreased specific activities suggesting that phenylalanine is the most suitable amino acid to position the acetyl moiety of the acetyl-enzyme for its attack by a water molecule. Mutation to tyrosine also decreased the protein stability. This instability might be caused by a change of the hydrogen bond network due to the presence of the hydroxyl group on tyrosine.

Glycine 368 lies in the second layer of the active site wall and its mutation to alanine appears to change the architecture of the site, interfering with substrate translocation from the rim to the bottom of the gorge (K_L). Mutation of glycine to alanine destabilized the protein. This was unexpected since glycine 368 is situated at the C-terminal end of an α-helix. The glycine residue in helical regions has one of the lowest intrinsic α-helical propensities of all the amino acids (second only to proline) and in thermodynamic studies, alanine stabilizes a peptide or protein helix by up to 2 kcal/mol relative to glycine [20]. Most probably, unfavorable steric interactions have more destabilizing effects than α-helix stabilization.

A decrease in protein stability of the four single mutants should decrease the amount of protein in the synaptic cleft. Lower hydrolysis rates of some mutants prolongs the residence time of acetylcholine inside the synapse cleft. The association of decreased stability and decreased catalysis should have a severe effect on the efficiency of the cholinergic impulse, reducing the fitness of the flies.

Combination of mutations in the same protein results in combination of alterations provided by single mutation. As G265A increased the catalytic efficiency of the protein, its association with other mutations compensated their loss of efficiency. By contrast, as no mutation increased the stability of the enzyme, all combinations resulted in proteins still less stable.

Allelic frequencies and protein alterations

The same mutations are consistently found in the three major targets of conventional insecticides despite the large number of potential mutations which provide resistance and the wide range of insects studied [21,22]. When resistance originates from a combination of point mutations in the same allele, some combinations highly presented themselves while others were never found, suggesting that they are quite rare (Fig. 1). One hypothesis would be that some mutations or combinations of mutations too drastically affect the activity or the stability of the protein. To test this hypothesis, the frequency of each allele in field-caught populations has been compared to the protein characteristics. It appears that there is an intrinsic correlation between the allelic frequency and the alteration of the protein. The most frequent mutation in *Drosophila* populations is G265A. This mutation should be neutral if we consider that it increases activity and decreases stability: the decrease in the amount of enzyme in the synapse should be compensated by its increased activity. But single mutations provide only a low level of resistance. Higher level and wider spectrum of resistance is achieved by combining three or four mutations in the same protein. Among these alleles, the triple mutant I161V/G265A/F330Y is the most frequent in analyzed populations [6]. Compared to other mutants, it appears that this combination results in the least drastic effect on protein activity and stability, allowing its maintenance in the absence of insecticide selection. Thus, this result highly suggests that the low alteration of the protein by point mutations providing resistance is the main driving force responsible for the maintenance of resistant alleles in natural populations.

Evolution of fitness cost and characteristics of the proteins

The cost of the resistance often appear high at the beginning of the insecticide selection and resistance is unstable. With time elapsing and insecticide treatments, the cost disappears and resistance stabilizes. One explanation is the selection of modifier genes which diminish the effect of resistant mutation. Examination of alteration in the different alleles provides us with another explanation: at the beginning of the selection, mutations are isolated and for most of them associated with a high fitness cost. With time, mutations combine in the same protein, and this combination restores the initial catalytic properties of the protein and then restores the fitness of the fly. Thus in that case, the modifier gene is another mutation on the same protein.

Another modifier is the mutation controlling the amount of AChE in *Drosophila* populations. It varied by a factor from 0.9- to two-fold and is directly correlated with insecticide resistance [23,24]. It would be expected that this increased concentration of enzyme originates from higher stability, which could offset lower activity resulting in better mutation maintenance in the absence of insecticide. However, our data showed that the stability of all the mutated enzymes decreased relative to the wild type and there was not an inverse correlation between the catalytic activity and stability. Thus, the observed increased amounts of enzyme do not stem from an increase of protein stability but originate from other mechanisms such as over-transcription or increased stability of RNA. This would compensate for the decreased stability and activity provided by point mutations.

Methods

Site-directed mutagenesis and protein preparation

Site-directed mutagenesis was generated by PCR and the identities of individual clones were verified by sequencing. Truncated cDNA encoding wild type and 15 mutated *Drosophila* AChE were expressed with the baculovirus system [25]. In order to avoid the presence of detergent in kinetics analysis, we expressed soluble dimeric forms deleted from a hydrophobic peptide at the C-terminal end, which is exchanged for a glycolipid to obtain soluble proteins. Secreted AChEs were purified to homogeneity using the following steps: ammonium sulfate precipitation, ultrafiltration with a 30 kDa cut off membrane, affinity chromatography with procainamide as ligand, and gel filtration [26]. Residue numbering followed that of the mature protein [5].

Protein stability

Denaturation experiments were performed with 10 picomoles enzyme in one ml 25 mM sodium phosphate buffer pH 7, containing 1 mg/ml bovine serum albumin (BSA). AChE was incubated at different denaturing temperatures. At intervals, an aliquot was cooled quickly by 20-fold dilution in cold sodium phosphate buffer to stop the denaturation reaction and the remaining activity was recorded. The first-order denaturing rate constant (*kd*) was assessed by non-linear regression.

Kinetics of substrate hydrolysis

The kinetics of substrate hydrolysis was followed at 25°C in 25 mM sodium phosphate buffer pH 7, containing 1 mg/ml BSA. Hydrolysis of acetylthiocholine (ATCh), an analogue of the neurotransmitter allowing easy detection of the reaction product, was studied spectrophotometrically at 412 nm using the method of Ellman *et al.* [27], at substrate concentrations ranging from 2 μM to 300 mM, in 1 cm path length cuvettes. Activity was measured for 1 minute after addition of the enzyme to the reaction mixture. The concentration of the enzymes was determined by active site titration using 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide, a phosphorylating agent with high affinity [28].

Determination of the decarbamoylation rate constant

Enzyme was incubated at 25°C with carbaryl in 25 mM sodium phosphate buffer pH 7, 1 mg/ml BSA until more than 95% of the enzyme was inhibited. The mixture was loaded on a gel filtration column (PD10, Pharmacia) and eluted with 25 mM sodium phosphate buffer pH 7, 1 mg/ml BSA. Fractions with enzyme were collected. The decarbamoylation rate was followed with time for nine hours by sampling aliquots of the reaction mixture and estimating free enzyme concentration spectrophotometrically through its activity with 10 mM ATCh.

Abbreviations used

AChE: acetylcholinesterase; BSA: bovine serum albumin; ATCh: acetylthiocholine.

Authors' contributions

MAS performed biochemical analysis, AL performed *in vitro* mutagenesis, CA produced proteins, IF participated in protein production and biochemical analysis, ZHT participated in the design of the study, JS carried out fits of pS curves to the kinetic model and DF conceived and coordinated the study. All authors read and approved the final manuscript.

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