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Improvement of Drosophila acetylcholinesterase stability by elimination of a free cysteine

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Abstract

Background: Acetylcholinesterase is irreversibly inhibited by organophosphate and carbamate insecticides allowing its use for residue detection with biosensors. *Drosophila* acetylcholinesterase is the most sensitive enzyme known and has been improved by *in vitro* mutagenesis. However, it is not sufficiently stable for extensive utilization. It is a homodimer in which both subunits contain 8 cysteine residues. Six are involved in conserved intramolecular disulfide bridges and one is involved in an interchain disulfide bridge. The 8th cysteine is not conserved and is present at position 290 as a free thiol pointing toward the center of the protein.

Results: The free cysteine has been mutated to valine and the resulting protein has been assayed for stability using various denaturing agents: temperature, urea, acetonitrile, freezing, proteases and spontaneous-denaturation at room temperature. It was found that the C290V mutation rendered the protein 1.1 to 2.7 fold more stable depending on the denaturing agent.

Conclusion: It seems that stabilization resulting from the cysteine to valine mutation originates from a decrease of thiol-disulfide interchanges and from an increase in the hydrophobicity of the buried side chain.

Background

Acetylcholinesterase (AChE, EC 3.1.1.7) is a serine hydrolase, which catalyzes the hydrolysis of acetylcholine. This enzyme is the target of organophosphate and carbamate insecticides which phosphorylate or carbamoylate the serine of the active site blocking the hydrolysis of the neurotransmitter acetylcholine. The post-synaptic membrane then remains depolarised and synaptic transmission cannot take place so the insect dies. These compounds are used to control various agricultural pests: insects, acari and nematodes. One of the consequences is that pesticide residues remain in the environment and are potentially toxic for all animals including humans since cholinergic transmission is well conserved.

Insecticide residues can be detected with biosensors using AChE as biological element. Such a method has been described to detect low levels of contaminants in crops, soil, water or food samples using various kinds of biosensors [1,2]. Drosophila AChE was found to be the most sensi-

tive enzyme when compared to enzymes of non-insect origin and *in-vitro*-mutagenesis was used to select enzymes up to 300-fold more sensitive [3,4]. But like most enzymes from mesophilic organisms, Drosophila AChE is not stable, and this instability precludes its utilization in biosensors. It can be stabilized by additives: protein such as bovine serum albumin, reversible inhibitor or polyethylene glycol. Alternatively, stabilization can also be achieved by encapsulation in liposomes [5–8]. Another way to stabilize the enzyme is to use *in vitro* mutagenesis to modify the primary structure of the protein. This method has the advantage of stabilizing the enzyme during its synthesis leading to higher production and higher purification yields. Several kinds of mutation may stabilize a protein, among them there is elimination of free sulfhydryl groups.

Wang *et al.*[9] suggested that removal of nonessential free cysteines by serine could be a general method for preparing muteins with higher stability, specific activity and homogeneity. Subsequent assays have shown that mutation of free cysteines has been found to increase the half-life of proteins [10–14]. However, some exemples have been reported where elimination of a free cysteine did not improve the stability of protein and even decreased it [6,15,16].

There are eight cysteines in the wild type cholinesterase [17], six are involved in intrachain disulfide bonds, one is involved in an interchain disulfide bond and one, at position 290 (328 with the precursor numbering) remains free [18]. This free cysteine is half buried in the protein, located in a loop near the disulfide bond formed by cysteines 292 and 307 (Fig. 1 and 2) [19]. When comparing the primary sequence of cholinesterases, it appears that this cysteine is not conserved and thus is not essential for the function of the enzyme. In the present study, we used mutagenesis to change this free cysteine for a hydrophobic residue with a similar size, a valine to enhance the stability of the enzyme.

Results and discussion

Thermodynamic and thermal (also called long-term) stabilities are usually closely related since thermal denaturation is considered to be a two-step process involving a reversibly unfolded intermediate [20]. But the two stabilities do not necessarily correlate due to independent unfolding of microdomains [21,22]. Therefore, several denaturation methods were applied. In all situations analyzed, denaturation of AChE was irreversible and followed apparent first order kinetics. Stability was then characterized by the half-life (*t50*), the time at which 50% of an initial enzymatic activity is preserved. The effect of freezing was measured from the recovery after one freeze-thaw cycle (Table 1).

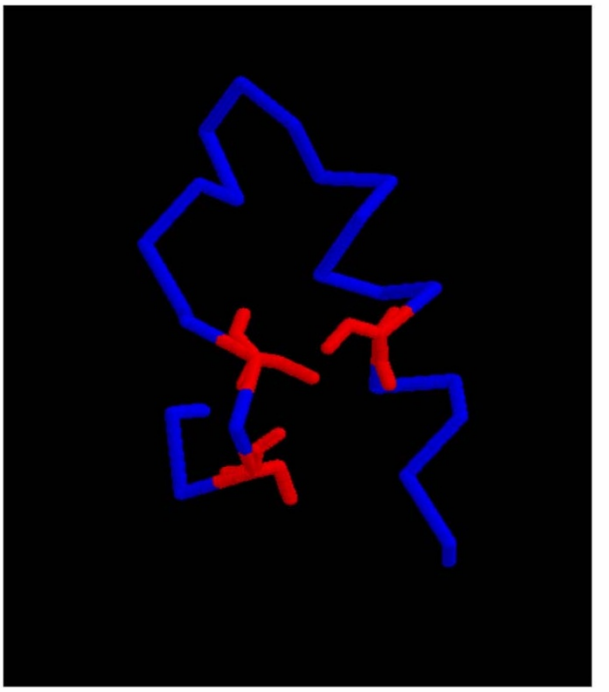

Figure 1
Position of cystein 290 near the disulfide bond formed by cysteines 292 and 307.

We first analyzed denaturation with the most common methods used to study protein denaturation: incubation at high temperature or in the presence of urea. Incubation of the wild type enzyme at 45°C resulted in the progressive irreversible denaturation of the enzyme with a 17 min half-life. Mutation of cysteine 290 to valine increased this half-life to 24 min. It also provided resistance to urea denaturation: the mutated enzyme was 2.7-fold more stable than the wild type if we consider the ratio of half-life time in 4 M urea (Table 1).

Lyophilisation is the most efficient method to store Drosophila AChE, however freezing denatures the enzyme at dilutions below the concentration of 1 mmole per liter [7]. We thus checked if the C290V mutation protects the enzyme at the concentration of 10 nM. Following one freezing to -20°C, 75% of the wild type enzyme was denatured while only 50% of the mutated enzyme activity was lost.

The utilization of AChE in a biosensor can last several days in some continuous flow devices. In fact, as the inhibition is irreversible, the longer AChE is incubated with the sample, the lower the detection limit of the biosensor. However, the enzyme activity has to decrease from inhibition and not from spontaneous denaturation of the protein. We thus incubated the wild type and the mutated

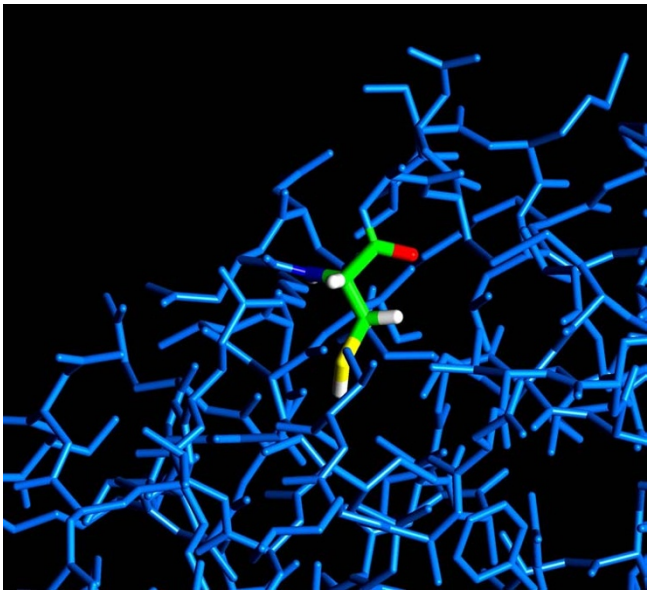

Figure 2
Orientation of cysteine 290 towards the center of the protein.

enzyme at 20°C. Half the enzyme was denatured in 1.5 days; the mutation doubled this half life.

Detection of insecticides in food requires their extraction with organic solvent. Although the solvent should be eliminated before the assay, low amounts may remain in solution and inactivate the enzyme. We used acetonitrile as model because it is soluble in water. This organic solvent strongly denatured the protein which had a half-life in 20% acetonitrile of 1.3 minutes. This half-life was slightly increased to 1.4 minutes by the mutation.

As some proteases may be present in the sample to be assayed for pesticide residues, we tested the effect of the mutation on the proteolytic sensitivity of the enzyme. Actually, it has been reported that a protein's resistance to proteolysis increases with its conformational stability [23,24] due to the fact that the susceptibily to proteolysis reflects the rate of local unfolding [25,26]. Our data support this relation between stability and proteolytic susceptibility since the mutated enzyme was 1.3 more resistant to proteolytic attack than the wild type enzyme in the experimental conditions used.

Several hypotheses can be put forward to explain this increased stability. A first hypothesis involves the deletion of the highly reactive sulfhydryl group which could interact with the neighboring disulfide bond. Thiol-disulfide interchanges have been reported in proteins [27–29] including acetylcholinesterase [30] and changes involving a free sulfhydryl group and a disulfide bond have been found to result in the irreversible denaturation of proteins [31–33]. As the disulfide exchange reaction originates from nucleophilic attack on a sulfur atom of the disulfide, the proximity of the disulfide bond formed by cysteines 292 and 307 enforces this hypothesis. The inter Cβ distance between Cys 290 and Cys 292 or Cys 307 are 5 Å or 7.7 Å respectively (Fig. 1). Actually, mutation of cysteine 292 to valine resulted in an inactive protein suggesting that disruption of the bond between cysteines 292 and 307 results in misfolding since the loop is not involved in the structure of the active site.

A free sulfhydryl group may also mediate the formation of dimer and oligomer. Although oligomerization usually has a stabilizing effect [34], it can sometimes lead to loss of biological activity [35]. As the side chain of cysteine 290 points towards the interior of the protein and is inaccessible to the solvent, it is unlikely that this cysteine is responsible for the formation of tetramers.

Air oxidation of thiols is an important source of thermal inactivation of lysozyme [36]. To test the relevance of this phenomenon for stability, we used the "foot printing system" of Tullius and Dombrowski [37] using the EDTA complex of iron(II) to generate hydroxyl radical form hydrogen peroxide as previously performed with AChE [38]. The C290V mutation did not increase the half life of the protein. Thus, stabilization does not seem to originate from the elimination of an oxidation site.

The contribution of individual amino acids to the stability of a specific protein has often been found to be difficult to assess since the elimination of one type of interaction often affects other types of interactions. This can happen in our mutation: by replacing a cysteine by a valine, we not only eliminated a reactive thiol, we also increased the hydrophobicity of the side chain which is oriented towards the heart of the protein (Fig. 2). As increasing the hydrophobicity of buried residues usually increases the stabilityof proteins [39–42], we may hypothesize that this rule applied here.

Thus, removal of the free cysteine 290 most probably stabilized Drosophila AChE by two mechanisms: inhibition of disulfide exchange and increase of the hydrophobicity of the side chain.

Methods

Protein production and purification

cDNA encoding *Drosophila melanogaster* AChE and mutant were expressed with the baculovirus system [43]. We expressed a soluble dimeric form deleted from a hydrophobic peptide at the C-terminal end with a 3 × histidine tag replacing the loop from amino-acids 103 to 136. This external loop is at the other end of the molecule with respect

Table 1: Stability of wild type and mutated AChE (mean ± SEM) and an index of the increase in stability i.e. the ratio between the measurement made for the mutant and the wild type enzymes.

Denaturation	Wild type	mutant	ratio
t_{50} at 45°C in min.	17.4 ± 2.1 (n = 10)	24.1 ± 4.3 (n = 5)	1.4
t_{50} in 4 M urea in min.	12.5 ± 1.2 (n = 7)	34.2 ± 2.0 (n = 5)	2.7
Recovery after freezing	0.24 ± 0.04 (n = 8)	0.49 ± 0.04 (n = 7)	2
t_{50} at room temperature in days	1.5 ± 0.2 (n = 5)	3.3 ± 0.5 (n = 4)	2.2
t_{50} in 20% acetonitrile in min.	0.94 ± 0.1 (n = 12)	1.4 ± 0.4 (n = 5)	1.5
t_{50} in presence of 0.1 mg/ml pronase	7.9 ± 0.2 (n = 9)	10.0 ± 0.6 (n = 9)	1.3
t_{50} in H_2O_2 in min.	4.6 ± 0.5 (n = 6)	4.0 ± 0.4 (n = 6)	0.9

to the active and its deletion does not affect either the activity or the stability of the enzyme. Secreted AChE was purified to homogeneity using the following steps, ammonium sulfate precipitation, ultrafiltration with a 50 kDa cut off membrane, affinity chromatography with procainamide as ligand, NTA-nickel chromatography and anion exchange chromatography [7]. Activity was recorded at 25°C in 25 mM phosphate buffer pH 7, with 1 mM acetylthiocholine iodide as substrate using the method of Ellman *et al.* [44]. Residue numbering followed that of the mature protein.

Denaturation

All denaturation experiments were performed with 10 picomoles enzyme in 1 ml 25 mM phosphate buffer pH7 at 25°C. AChE was incubated in denaturing conditions and the remaining activity was measured by taking aliquots at regular times since residual enzymatic activity is dependent on the time of incubation. To analyze thermosensitivity, enzymes were incubated at 45°C instead of 25°C and 1 mg/ml Bovine Serum Albumin was added to the buffer. Aliquots were mixed with cold buffer chilled on ice and the solution was incubated at 25°C for ten minutes before recording the remaining activity. For urea denaturation, unfolding of AChE was induced by adding 4 M urea to the incubation buffer. Aliquots were diluted 10-fold in 25 mM phosphate buffer pH7 before measuring the remaining activity. The effect of freezing was estimated by measuring the remaining activity after one day at -20°C. Stability at room temperature was measured by recording the activity after incubation at 20°C. The effect of organic solvent was followed by incubation of the enzyme in 20% acetonitrile. The effect of protease sensitivity was determined by incubation of AChE with 0.1 mg/ml pronase. To follow the effect of oxygen radical on denaturation 10 mM H_2O_2, 1 mM FeCl3, 2 mM EDTA and 40 mM ascorbic acid were added to the incubation medium following the procedure of Weiner et al. [38].

Authors' contributions

A.B-L performed the in vitro mutagenesis experiment, I.F. and M.A. produced and purified the proteins. S.M. and C.L. carried out the stability measurements, D.F. designed the study. All authors read and approved the final manuscript.

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