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Mutation of exposed hydrophobic amino acids to arginine to increase protein stability

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Abstract

Background: One strategy to increase the stability of proteins is to reduce the area of water-accessible hydrophobic surface.

Results: In order to test it, we replaced 14 solvent-exposed hydrophobic residues of acetylcholinesterase by arginine. The stabilities of the resulting proteins were tested using denaturation by high temperature, organic solvents, urea and by proteolytic digestion.

Conclusion: Although the mutational effects were rather small, this strategy proved to be successful since half of the mutants showed an increased stability. This stability may originate from the suppression of unfavorable interactions of nonpolar residues with water or from addition of new hydrogen bonds with the solvent. Other mechanisms may also contribute to the increased stability observed with some mutants. For example, introduction of a charge at the surface of the protein may provide a new coulombic interaction on the protein surface.

Background

Acetylcholinesterase (AChE, EC 3.1.1.7) is a serine hydrolyase, which catalyzes the hydrolysis of the neurotransmitter acetylcholine. This enzyme is irreversibly inhibited by organophosphate and carbamate pesticides leading to its use in biosensors to detect traces of these compounds in environment. *Drosophila* AChE was found to be the most sensitive enzyme when compared to enzymes of non-insect origin and *in-vitro*-mutagenesis was used to select enzymes up to 300-fold more sensitive [1,2]. 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 adding some molecules in the solution such as reversible inhibitors, polyethylene glycol or protein, provoking protein-protein

interactions. Alternatively, stabilization may also be achieved by encapsulation in liposomes [3,7]. Another way to stabilize the enzyme is to use *in vitro* mutagenesis to modify the primary structure of the protein [8]. This method could have the additional advantage of stabilizing the enzyme during its synthesis leading to higher production and higher purification yields.

Irreversible denaturation of AChE at room temperature can be minimized by increasing the protein concentration in the sample, either by increasing the enzyme concentration or by addition of another protein such as BSA [6]. This suggested that denaturation occurred by interaction of the hydrophobic region at the surface of AChE with tube walls or air-solvent interface. Addition of protein in

the solution would compete with hydrophobic surfaces and protect the enzyme. We thus hypothesized that decreasing the hydrophobicity at the surface of the protein may have some stabilizing effects.

Several examples showed that the change of hydrophobicity to hydrophilicity of amino-acid residues exposed to the solvent at the surface of proteins is an efficient strategy to stabilize proteins: i) Analysis of protein sequences showed a strong bias for hydrophilic residues and against large hydrophobic residues at most surface positions [9], ii) Mutagenesis showed that hydrophilic amino acids at surface positions is stabilizing Mutagenesis showed that hydrophilic amino acids at surface positions have stabilizing effect, while placing a hydrophobic residue in a solvent-exposed position causes destabilization [10-12], iii) The proportion of hydrophobic residues at the surface of proteins from hyperthermophilic species was found to be reduced compared to the proportion in their mesophilic counterparts [13-16], however, this observation is under debate [17].

Here we tested this strategy by mutating several hydrophobic residues scattered at the surface of *Drosophila* AChE to arginine. Hydrophobic residues were chosen by visual examination of the structure and arginine was chosen because the guanidinium group is the most polar of all the common amino-acid residues found in proteins.

Results and discussion

Effect of mutations on protein production

Production of the wild type was 60 nmoles per liter (5 mg/L). Several mutations affected the production of the protein (Fig. 1), most probably reflecting their influence on folding efficiency. One mutation drastically decreased production: the mutation M194R decreased production to 0.5% i.e. a level which was barely detectable. This amino-acid is partly buried behind proline 443 and we can assume that its hydrophobicity is important for the folding of the protein. The mutations V56R and L57R decreased production to 10%. It seems that these two residues are engaged in a hydrophobic bond with Val 14 helping to position the loop from Ala 50 to Thr 60 in connection with the N-terminus peptide of the protein (Fig. 2). Mutations to hydrophilic residue may disrupt the bond and thus decrease the probability of the protein reaching its active conformation. On the other hand, three mutations significantly increased the production: the mutations L62R, F225R and L471R. This increase of folding efficiency can be attributed to the reverse hydrophobic effect: if a hydrophobic residue is less exposed to the solvent in the denatured form than in the native form, it will oppose folding [18].

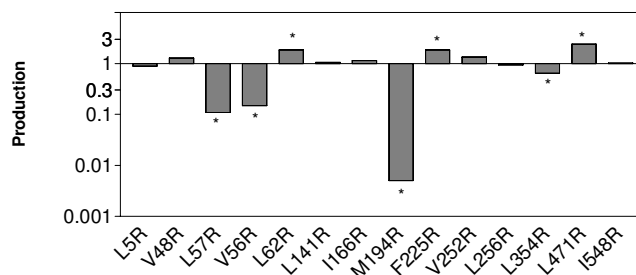


Figure 1
Effect of replacement of hydrophobic residues at the surface by arginine on relative production. Ratio of production represents the relative production of each mutant.

Effect of mutations on protein stability

Stability was assayed with four denaturing agents. In all cases, denaturation was irreversible and followed apparent first order kinetics. Stability was then characterized by the half-life (t_{50}), the time at which 50% of an initial enzymatic activity is preserved. The half life of the wild type protein is shown in Table 1. Stability was analyzed for all mutants except M194R for which the production was too low.

The effect of mutations on stability was homogeneous, a mutation either destabilizes or stabilizes the protein since we never found a mutation which significantly stabilizes the protein for one agent and significantly destabilizes it for another.

Most of the mutations significantly affect the stability of the protein (Fig. 3). However, differences were rather small. This is in accordance with literature, amino acid substitution usually does not significantly affect the stability [10,19], although important improvements of stability by mutagenesis of a single solvent-exposed residue have been reported [20,21].

Several mutations stabilize the protein. A possible explanation could be that the interactions of nonpolar residues with water present a thermodynamic disadvantage caused by the side chain being more exposed to solvent in the native than in the denatured state [18]. In addition, polar residues at the surface may provide additional hydrogen bonds with the solvent and then increase protein stability [16]. This explanation seems satisfactory for some mutations: when the hydrophobic residue is located inside a hydrophobic area made up of several residues. Thus, Valine 48 forms a small hydrophobic area at the surface of the protein with Valine 42, Phenylalanine 225 forms a hydrophobic region with Alanine 100, Alanine 224, Valine 6, Valine 7 and Isoleucine 174 (Fig. 4). This

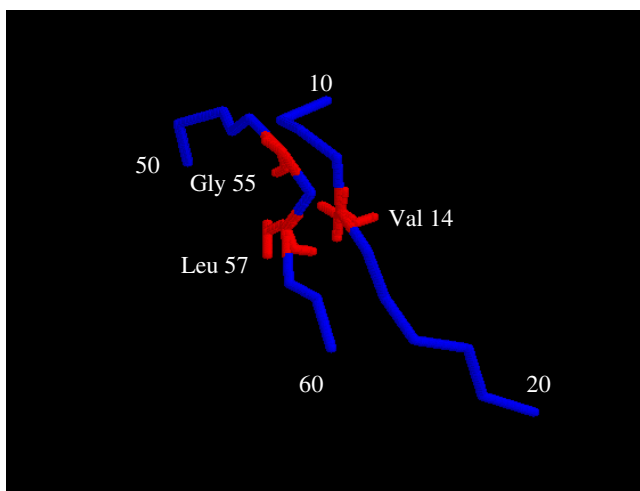


Figure 2
Position of Gly 55 and Leu 57, near Val 14.

Table 1: half-life (t_{50}) of the wild type protein.

Denaturing agent	t_{50} (min)
50°C	8 +/- 1.7
4 M Urea	11 +/- 1.6
20% acetonitrile	1.45 +/- 0.18
0.1 mg/ml pronase	14.4 +/- 2.2

hydrophobic area is broken when the hydrophobic Phe 225 is replaced with a hydrophilic arginine. Similarly, Val 252 forms a hydrophobic area with Gly 338, Ala 339 and Ala 212.

Some mutations destabilized the protein. The presence of hydrophobic residues at the surface may have stabilization properties by providing a shield from penetrating water molecules [22]. Or mutation to Arg may disrupt a hydrophobic bond. V56 and L57 are in hydrophobic contact with Val 14, mutation to Arg may disrupt this bond. Likewise, Ile 5 faces Gly 16 and this interaction may maintain the loop conformation.

But, other mechanisms may contribute to the increased or decreased stability observed with some mutants. Introduction of a charge at the surface of the protein may provide a new coulombic interaction on the protein surface. This strategy seems efficient since it is used by proteins from thermophiles [23-25] and several groups showed significant stabilization of proteins with substitutions of

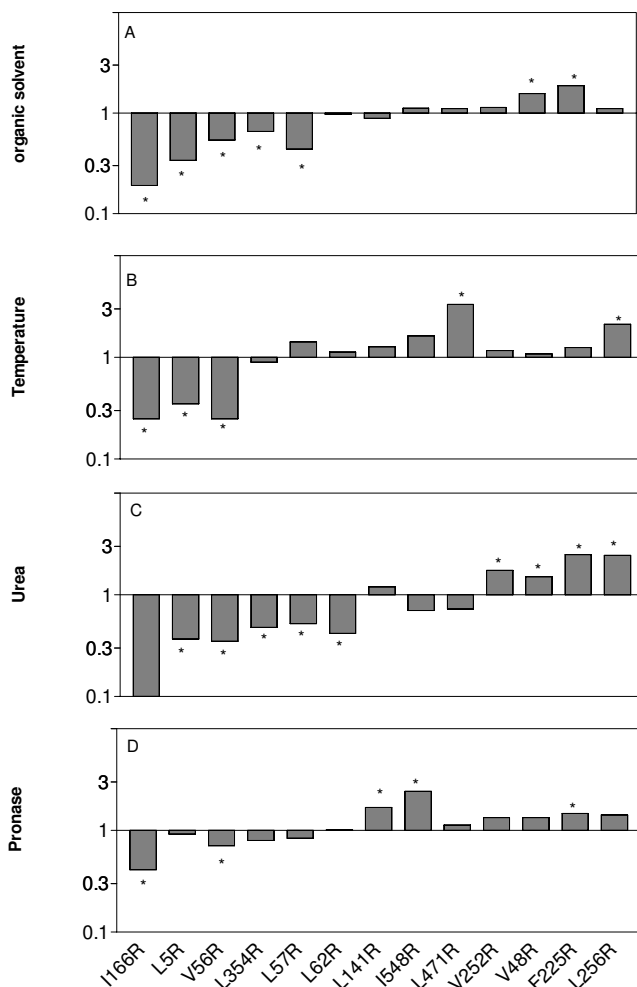


Figure 3
Effect of replacement of hydrophobic residues at the surface by arginine on relative stability. For each mutation, ratio of t_{50} (t_{50} mutant/ t_{50} wild type) was calculated for each denaturation agent. * indicates a significant difference with the wild type protein with $P < 0.05$. Mutations are arranged from the less to the more stable, considering a mean stability for the four parameters analyzed.

the surface charges [20,26,27]. On the contrary, addition of a positive charge may result to the addition of a charge repulsion which decreases stability [28]. To test if introduction of a new positive charge may be responsible for the change of stability observed, we estimated the electrostatic stabilization expected for the addition of a charge using Coulomb's Law (Fig. 5). The stabilization provided by some mutations such as I548R may originate from the formation of new coulombic interaction, in that case between Arginine 548 and Glutamate 546.

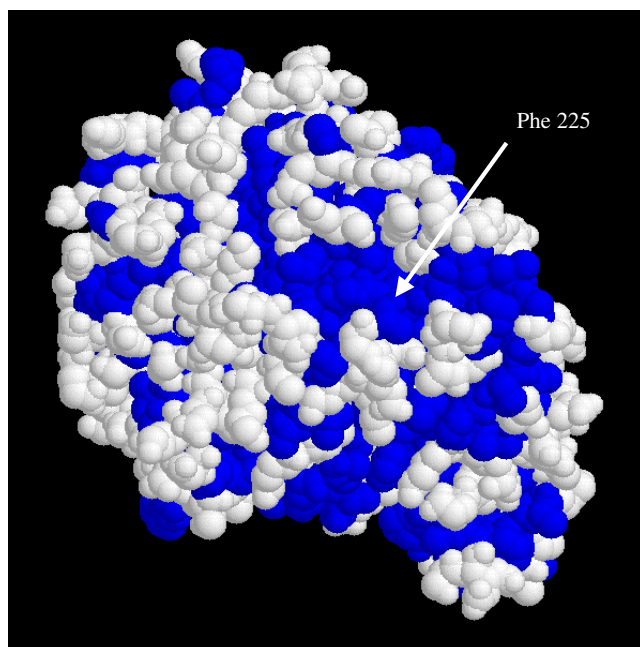


Figure 4
Position of Phe 225 inside an hydrophobic area at the surface of the protein. (hydrophobic residues have been colored in blue).

Methods

Protein production and purification

cDNA encoding wild type drosophila AChE and mutant were expressed with the baculovirus system [29]. This enzyme is a dimer anchor to the membrane via a glycolipid [30]. We expressed a soluble dimeric form obtained by deletion of the hydrophobic peptide, precursor of the glycolipid anchor at the C-terminal end [31]. Furthermore, the enzyme was deleted of the loop from aminoacids 103 to 136 which has been replaced by 3 histidines. Secreted AChE was purified to homogeneity using the following steps: ammonium sulfate precipitation, ultrafiltration with a 10 kDa cutoff membrane, NTA-nickel chromatography, affinity chromatography with procainamide as ligand and gel filtration [6]. 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. [32]. Mutations did not alter the specific activity of the proteins. Molecular modeling of mutated residues was performed from the structure solved by Harel *et al.* [[33], ref 1QO9] by homology using Swiss model server available at <http://expasy.org/swissmod/>. Residue numbering followed that of the mature protein.

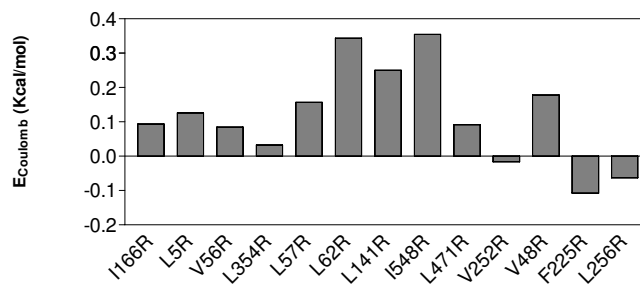


Figure 5
Electrostatic stabilization expected for the charge addition according to the Coulomb's Law ($E = q_1q_2/Dr$) with a dielectric constant of 80 by summing the interactions of the new charge with all the other charges of the protein.

Production

One liter containing 10^6 Sf9 cells was infected with more than 10^7 virus. After four days incubation at 28°C, the cells were lysed by adding Triton X-100 (0.1%). Amount of AChE was estimated by active site titration using an irreversible inhibitor, chlorpyrifos oxon [34]. The mean of at least five independent productions was compared to the production of the wild type enzyme.

Denaturation

All denaturation experiments were performed with 10 picomoles of enzyme in one ml 25 mM phosphate buffer pH7 at 25°C. AChE was incubated in denaturing conditions. Aliquots were taken at regular intervals, diluted 20 x to stop the action of the denaturing agent and the remaining activity was measured.

To analyze thermosensitivity, enzymes were incubated at 50°C with 1 mg/ml Bovine Serum Albumin in the buffer. Before recording the remaining activity, aliquots were mixed with cold buffer chilled on ice and the solution was incubated at 25°C for ten minutes to eliminate the reversible component [35]. For urea denaturation, unfolding of AChE was induced by adding 4 M urea into the incubation buffer. 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.

Three to nine batches of each mutant were produced and purified. Three repeats were performed for each batch and each denaturing agent. Significance of difference observed in stability was tested using the Mann Whitney test.

Coulombic interaction estimation

In order to estimate the effects of charge distribution on each of the mutants, the contributions from electrostatic interactions were extracted from the total energy of the minimized structures of the molecules. The minimization was performed with the GROMACS software [36], using the simulated annealing protocol. Position restraints were imposed on the whole molecule with the exception of the mutated residue, which was allowed to adopt the energetically favorable configuration. The Coulomb's interactions were normalized by subtracting the value obtained for the wild type protein and by rescaling the numbers by 80, to take into account the dielectric constant of water.

List of abbreviations

AChE, acetylcholinesterase

Authors' contributions

CS and CA purified the protein and performed biochemical analysis, AL performed *in vitro* mutagenesis, CA produced proteins, JC performed estimations of electrostatic interactions and DF conceived and coordinated the study. All authors read and approved the final manuscript.

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