The effect of engineered disulfide bonds on the stability of Drosophila melanogaster acetylcholinesterase

Omid Ranaei Siadat1,2, Andrée Lougarre1, Lucille Lamouroux1, Caroline Ladurantie1 and Didier Fournier*1

Address: 1IPBS-CNRS 205 route de Narbonne, Toulouse, France and 2New Ideas Research Group (NIRG), #11, Proshat Alley, Motahhari Street, Tehran, Iran

Email: Omid Ranaei Siadat - ranaei@irnewideas.ir; Andrée Lougarre - Andree.Lougarre@ipbs.fr; Lucille Lamouroux - Lucille.Lamouroux@ipbs.fr; Caroline Ladurantie - Caroline.Ladurantie@ipbs.fr; Didier Fournier* - Didier.Fournier@ipbs.fr

* Corresponding author

Background:
Acetylcholinesterase (AChE, EC 3.1.1.7) is a serine hydro-lase, 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 depolarized and synaptic transmission cannot take place so the insect dies. These compounds are used to control proliferation of 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 to detect low levels of contaminants in crops, soil, water or food samples [1,2].

Results:
To create a disulfide bond that could increase the stability of the Drosophila melanogaster acetylcholinesterase, we selected seven positions taking into account first the distance between Cβ of two residues, in which newly introduced cysteines will form the new disulfide bond and second the conservation of the residues in the cholinesterase family. Most disulfide bonds tested did not increase and even decreased the stability of the protein. However, one engineered disulfide bridge, I327C/D375C showed significant stability increase toward denaturation by temperature (170 fold at 50°C), urea, organic solvent and provided resistance to protease degradation. The new disulfide bridge links the N-terminal domain (first 356 aa) to the C-terminal domain. The quantities produced by this mutant were the same as in wild-type flies.

Conclusion:
Addition of a disulfide bridge may either stabilize or unstabilize proteins. One bond out of the 7 tested provided significant stabilisation.
Drosophila AChE (DmAChE) was found to be the most sensitive enzyme when compared to enzymes of non-insect origin and in-vitro-mutagenesis has permitted the selection of enzymes up to 300-fold more sensitive [3,4]. But like most enzymes from mesophilic organisms, DmAChE is not stable, and this instability precludes its utilization in biosensors. It can be stabilized by additives: proteins such as bovine serum albumin, reversible inhibitor, polyethylene glycol or 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. Elimination of a free cysteine and mutation of the hydrophobic residues at the protein surface into hydrophilic residues have been used to increase the stability of DmAChE [9,10]. Here we focused on another method: engineering new disulfide bridges.

Disulfide bonds are present in most extracellular proteins, where they presumably stabilize the native conformation by lowering the entropy of the unfolded form [11] or by decreasing the unfolding rate of irreversibly denatured proteins [12,13]. This stabilizing property makes disulfide bond cross-linking an attractive strategy for engineering additional conformational stability into proteins by site-directed mutagenesis [14].

DmAChE is a dimer linked to membrane via a GPI anchor. There are eight cysteines in each monomer [15]. Six are involved in intrachain disulfide bonds, they are highly conserved in the protein family and their mutations result in inactivation of the protein. One cysteine is involved in an interchain disulfide bond and one, at position 290 (328 using precursor numbering) remains free [16,17]. The aim of this work was to stabilize DmAChE by introducing new disulfide bonds.

**Results**

**Mutation**

There are 35 potential disulfide bridges in DmAChE if we consider that every distance between two Cβ of 3.6 to 4 Å is suitable to form a disulfide bridge following the mutation of the two residues in cysteines. Among them, we selected 7 using two criteria: the two amino-acids involved should not be conserved in the cholinesterase family and a serine at these positions is present in one of the available sequences [18]. All these 7 disulfide bonds were predicted by MODIP, automated software for modeling disulfide bonds in proteins [19] with grades A (ideal stereochemistry), B (geometrically suitable but with distorted stereochemistry) and C (sites close enough to allow the formation of a disulfide bond) [20]. We verified that the engineered disulfide bonds were formed by assaying free sulfhydryl groups with the Ellman reagent in the presence of 6 M urea. The results were consistent with the expected disulfide bonds. We verified that the new cysteines did not promote a higher degree of polymerization. SDS-gel electrophoresis performed in non-reducing conditions showed that all mutants were dimeric proteins like the wild type: introduction of cysteines did not provide additional intersubunit interactions in the mutants.

In our conditions, production of wild type DmAChE in insect cells via the secretory network is 52 nmoles per liter, five bridges did not significantly affect this protein production; two, m3 and m4 decreased production and no mutation increased production (Table 1).

**Heat denaturation**

We first analyzed denaturation with the most common method used to study protein denaturation: incubation at high temperature. The stability of the mutated protein was estimated by studying irreversible thermal inactivation at several temperatures (from 35 to 65°C) and plotted the first-order denaturation rate constant (kd) against the reciprocal of the absolute temperature (K⁻¹). It appeared that one bridge (m2) increased thermostability while one (m6) decreased it (Fig. 1).

**Urea and organic solvent denaturation, protease sensitivity**

Stability was assayed with three denaturing agents. In all cases, denaturation was irreversible and followed appar-

---

**Table 1: Production ratio of mutant in baculovirus compared to wild type enzyme. Reference is the wild type DmAChE which was produced at 52 nanomoles per liter of culture. #: significant difference, n: number of batches analyzed**

| Mutant code | Mutated amino-acids | Grade (MODIP) | distance (Cβ) in tertiary structure (Å) | n | mean | Standard error |
|-------------|---------------------|---------------|----------------------------------------|---|------|----------------|
| m1          | R24/A169            | B             | 3.65                                   | 5 | 1.43 | 0.71           |
| m2          | I327/D375           | C             | 3.77                                   | 20 | 1.06 | 0.73           |
| m3          | L354/A456           | C             | 3.56                                   | 19 | 0.22 | 0.26           |
| m4          | T369/M476           | A             | 3.62                                   | 15 | 0.04 | 0.07           |
| m5          | L388/Q427           | C             | 3.70                                   | 16 | 0.96 | 0.63           |
| m6          | A452/S533           | B             | 3.91                                   | 7  | 0.73 | 0.45           |
| m7          | T464/S543           | D             | 3.96                                   | 8  | 0.77 | 0.47           |
Figure 1
Arrhenius plots of thermal inactivation rate constants of mutated DmAChE (in red) compared to wild type (in blue). $k$: denaturation first order rate constant (in min$^{-1}$).
ent first order kinetics. Stability was 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 was 13.6 min. in 4 M urea. Protease was used as a denaturant because a protein’s resistance to proteolysis increases with its conformational stability due to the fact that the susceptibility to proteolysis reflects the rate of local unfolding [21,22]. The half life of wild type DmAChE was 13.9 min in 0.1 mg/mL pronase. 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. The half life of the wild type protein was 1.7 min in 20% acetonitrile. The thermostability provided by bridge m2 is conserved for other denaturing agents (Table 2). Identically, the low stability provided by bridge m6 is found again. In addition, low stability was found for bridges m1 and m5.

**Table 2**: Relative stability of mutated AChEs. For each mutation, the t50 ratio (t50 mutant/t50 wild type) was calculated for each denaturation agent (*: significant difference. n: number of independent batches analyzed)

| mutation | n   | 50°C | 20% acetonitrile | 4 M urea. | 0.1 mg/mL pronase. |
|----------|-----|------|-----------------|-----------|-------------------|
| m1       | 1   | 2    | 0.43*           | 0.40*     | 0.27*             |
| m2       | 10  | 170* | 2.11*           | 12.35*    | 1.60*             |
| m3       | 9   | 1.4  | 0.85            | 1.87*     | 2.37*             |
| m5       | 6   | 0.47*| 0.13*           | 0.05*     | 0.17*             |
| m6       | 2   | 0.05*| 0.33*           | 0.26*     | 0.71              |
| m7       | 2   | 0.73 | 0.94            | 0.71      | 0.79              |

**Specific activity**

The specific activity of the mutants, and the patterns of the pS curves, were not significantly changed with the introduction of new bridges (Fig. 2). This suggests that entrance of the substrate into the active site as well as the catalytic efficiency was not affected by the mutations.

**Discussion**

From the first works of Villafranca et al. [23] and Perry and Wetzel [24], introduction of non-native disulfide bonds has been used to stabilize proteins [25-34]. These successes pushed us to use this technique to stabilize DmAChE.

**The effect of addition of disulfide bridges was either stabilization or destabilization**

Most new disulfide bonds introduced in DmAChE did not affect protein stability, one decreased stability. Destabilization has sometimes been reported [35,36]. This instability has been interpreted as the result of atypical sets of dihedral angles in newly formed disulfide bridges [37], from stabilization of the denatured state [38] or from reduction of disulfide bonds followed by disulfide exchange or chemical reaction of the SH groups formed [39,40]. Attempts to predict destabilization by modeling using MODIP failed, suggesting that selected positions were too flexible for a fulfilling prediction.

We found one mutation which stabilizes the protein (m2). Two subdomains forming the active site may be distinguished in cholinesterases and mutations decreasing interactions between them decrease protein stability [41]. Disulfide bridge m2 links the two subdomains of the enzyme (Fig. 3), strengthens subdomain interactions and increases overall stability. This suggests that the contact area of the two subdomains is the weakest site of the protein, taking into account the hypothesis that unfolding of a protein molecule starts at its weakest site, and local stabilization of this fragile region results in global stabilization of the whole molecule [42].
Addition of new disulfide bonds may impair protein production

Production is a key issue for application of the stable enzymes in biosensors. We found that addition of a disulfide bond may result in a decrease of protein production since two mutations out of the seven studied, affected protein production. Most probably, increasing the number of sulfhydryl groups in a protein decreases the

Figure 2
Effect of mutations on acetylthiocholine hydrolysis versus substrate concentration (log scale). (blue dots): wild type; (red dots): mutant, Acetylthiocholine concentration in micromoles per liter; v/[E] specific activity in s⁻¹.
folding efficiency by increasing the number undesirable disulfide bonds which results in a misfolded protein.

Conclusion
Addition of a disulfide bridge may either stabilize or unstabilize proteins.

Methods
Protein engineering
Possible sites for the introduction of disulfide bonds were located according to Wakarchuk et al. [23], by searching for pairs of residues for which the inter- Cβ distance was between 3.6 and 4 Å in the structure of DmAChE [17].

Protein production and purification
cDNA encoding DmAChE and mutants were expressed with the baculovirus system [43]. We expressed a soluble dimeric form deleted of the hydrophobic peptide at the C-terminal end which is exchanged for a glycolipid anchor. A 3 × histidine tag replaced the loop from amino-acids 103 to 136 to facilitate purification. This external loop is at the other side of the molecule with respect to the active site entrance and its deletion affects neither the activity nor 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]. Residue numbering followed that of the mature protein.

Enzyme activity
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, an analogue of the neurotransmitter allowing easy detection of the remaining activity was measured spectrophotometrically at 25°C for ten minutes before recording the remaining activity. For urea denaturation, unfolding of DmAChE was induced by adding 4 M urea to 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.

Denaturation
DmAChE is denatured irreversibly, ΔGd cannot be determined. Instead, the changes in the stability relative to a wild-type protein may be defined as the rate of enzymatic activity decrease [46]. 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, aliquots were taken out at regular times, diluted 10-fold in enzyme reaction mixture and remaining activity was measured, since residual enzymatic activity is related to the proportion of non-denatured protein. To analyze heat sensitivity, enzymes were incubated at 50°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.

Abbreviations
DmAChE: Drosophila acetylcholinesterase, ATCh: acetyltiocoline, BSA Bovine Serum Albumin.

Authors’ contributions
Al and LL performed in vitro mutagenesis, ORS and CL produced the protein and performed biochemical analysis. DF conceived and coordinated the study. All authors participated in the interpretation of the results, in the writing and revising of the manuscript, read and approved the final manuscript. This work has been supported by grants from CRSSA (Centre de recherche du Service de Santé de l’Armée) and the European contract n° QLK3-CT-2000-00650.

References
1. Marty JL, Sode K, Karube I: Biosensor for detection of organophosphate and carbamate insecticides. Electroanalysis 1992, 4:891-893.
2. Bachmann TT, Leca B, Vilatte F, Marty JL, Fournier D, Schmid RD: Improved multianalyte detection of organophosphates and carbamates with disposable multielectrode biosensors using recombinant mutants of Drosophila acetylcholinesterase and artificial neural networks. Biosens Bioelectron 2000, 15(3-4):193-201.
3. Vilatte F, Marcel V, Estrada-Mondaca S, Fournier D: Engineering sensitive acetylcholinesterase for detection of organophosphate and carbamate insecticides. Biosens Bioelectron 1998, 13(2):157-164.
4. Boublik Y, Saint-Aguet P, Lougarre A, Arnaud M, Vilatte F, Estrada-Mondaca S, Fournier D: Acetylcholinesterase engineering for detection of insecticide residues. Protein Eng 2002, 15(1):43-50.
5. Payne GS, Saeed M, Wolfe AD: Ligand stabilization of cholinesterases. Biochim Biophys Acta 1989, 999(1):46-51.
6. Wilson EJ, Massoulie J, Bon S, Rosenberry TL: The rate of thermal inactivation of Torpedo acetylcholinesterase is not reduced in the C231S mutant. FEBS Lett 1996, 379(2):161-164.
7. Estrada-Mondaca S, Fournier D: Stabilization of recombinant Drosophila acetylcholinesterase. Protein Expr Purif 1998, 12(2):166-172.
8. Nasseau M, Boublik Y, Meier W, Winterhalter M, Fournier D: Substrate-permeable encapsulation of enzymes maintains effective activity, stabilizes against denaturation, and protects against proteolytic degradation. Biotechnol Bioeng 2001, 75(5):615-618.
9. Fremaux I, Mazeres S, Brisson-Lougarre A, Arnaud M, Ladurantie C, Fournier D: Improvement of Drosophila acetylcholinesterase stability by elimination of a free cysteine. BMC Biochem 2002, 3(1):21.
10. Strub C, Alies C, Lougarre A, Ladurantie C, Czaplicki J, Fournier D: Mutation of exposed hydrophobic amino acids to arginine to increase protein stability. BMC Biochem 2004, 5(1):9.

Page 6 of 7 (page number not for citation purposes)
11. Anfinsen CB, Scheraga HA: Experimental and theoretical aspects of protein folding. Adv Protein Chem 1975, 29:205-300.

12. Plaza del Rio I, Ibarrat-Molero B, Sanchez-Ruiz JH: Lower limits to protein thermal stability: a proposal regarding protein stability in vivo and its relation with misfolding diseases. Proteins 2000, 40(1):58-70.

13. Clarke J, Fersht AR: Engineered disulfide bonds as probes of the folding pathway of barnase: increasing the stability of proteins against the rate of denaturation. Biochemistry 1993, 32(16):4322-4329.

14. Eijsink VG, Bjork A, Gaseidnes S, Sirevag R, Synstad B, van den Burg B, Vriend G: Rational engineering of enzyme stability. J Biotechnol 2004, 113(1-3):105-120.

15. Hall LM, Spierer P: The Ace locus of Drosophila melanogaster: structural gene for acetylcholinesterase with an unusual 5' leader. Emb J 1986, 5(11):2949-2954.

16. Mutero A, Fourdier D: Post-translational modifications of Drosophila. Wetzel R: Disulfides. In vitro mutagenesis and expression in Xenopus oocytes. J Biol Chem 1992, 267(3):1695-1700.

17. Harel M, Kryger G, Rosenberry TL, Mallender WD, Lewis T, Fletcher RJ, Guss JM, Silman I, Sussman JL: Three-dimensional structures of Drosophila melanogaster acetylcholinesterase and of its complexes with two potent inhibitors. Protein Sci 2000, 9(6):1063-1072.

18. [http://bioweb.ensam.inra.fr/ESTHER/general/whatindex].

19. [http://www.ncbi.nlm.nih.gov/faculty/minic/dsdbase/]

20. Davis JL, Ramakrishnan C, Varadarajan R: MODIP revisited: re-evaluation and refinement of an automated procedure for modeling of disulfide bonds in proteins. Protein Eng 2003, 16(3):187-193.

21. Ogasahara K, Tsunawasa S, Suda Y, Yuzani K, Sugino Y: Effect of single amino acid substitutions on the protease susceptibility of tryptophan synthesize alpha subunit. Eur J Biochem 1985, 150(1):17-21.

22. Braxton S, Wells JA: Incorporation of a stabilizing Ca(2+)-binding loop into subtilisin BPN'. Biochemistry 1992, 31(34):7796-7801.

23. Villarranca JE, Howell EE, Voet DH, Strobel MS, Ogden RC, Abelson JN, Kraut J: Directed mutagenesis of dihydrofolate reductase. Science 1983, 222(4625):782-788.

24. Perry LJ: Disulfide bond engineered into T4 lysozyme: stabilization of the protein toward thermal inactivation. Science 1984, 226(4674):555-557.

25. Wakarchuk WW, Sung WL, Campbell RL, Cunningham A, Watson DC, Yaguchi M: Thermostabilization of the Bacillus circulans xylanase by the introduction of disulfide bonds. Protein Eng 1994, 7(11):1379-1386.

26. Villarranca JE, Howell EE, Oatley SJ, Xuong NH, Kraut J: An engineered disulfide bond in dihydrofolate reductase. Biochemistry 1987, 26(6):2182-2189.

27. Matsumura M, Signor G, Matthews BW: Substantial increase of protein stability by multiple disulfide bonds. Nature 1989, 342(6247):291-293.

28. Kanaya S, Katsuda C, Kimura S, Nakai T, Kitakuni E, Nakamura H, Katayanagi K, Morikawa K, Ikehara M: Engineering disulfide bond in dihydrofolate reductase. J Biol Chem 1991, 266(10):6038-6044.

29. Mitchison C, Wells JA: Protein engineering of disulfide bonds in subtilisin BPN'. Biochemistry 1989, 28(11):4807-4815.

30. Eder J, Wilmanns M: Protein engineering of a disulfide bond in a beta/alpha-barrel protein. Biochemistry 1992, 31(18):4437-4444.

31. Mansfeld J, Vriend G, Dijkstra BW, Veltman OR, Van den Burg B, Vennema G, Ulbrich-Hofmann R, Eijssink VG: Extreme stabilization of a thermolysin-like protease by an engineered disulfide bond. J Biol Chem 1997, 272(17):11521-11526.

32. Ivens A, Mayans O, Sadowski H, Jurgens C, Wilmanns M, Kirschner K: Stabilization of a (betaalpha)8-barrel protein by an engineered disulfide bridge. Eur J Biochem 2002, 269(4):1145-1153.

33. Clarke J, Heinrich K, Fersht AR: Disulfide mutants of barnase. I. Changes in stability and structure assessed by biophysical methods and X-ray crystallography. J Mol Biol 1995, 253(3):493-504.

34. van den Akker F, Fell IK, Roach C, Platas AA, Merritt EA, Hol WG: Crystal structure of heat-labile enterotoxin from Escherichia coli with increased thermostability introduced by an engineered disulfide bond in the A subunit. Protein Sci 1997, 6(12):2644-2649.

35. Wells JA, Powers DB: In vivo formation and stability of engineered disulfide bonds in subtilisin. J Biol Chem 1986, 261(14):6564-6570.

36. Betz SF: Disulfide bonds and the stability of globular proteins. Protein Sci 1993, 2(10):1551-1558.

37. Katz BA, Kossiakoff A: The crystallographically determined structures of atypical strained disulfides engineered into subtilisin. J Biol Chem 1986, 261(33):15480-15485.

38. Betz SF, Marmorigo JL, Saunders AJ, Doyle DF, Young GB, Pielak GJ: Unusual effects of an engineered disulfide on global and local protein stability. Biochemistry 1996, 35(23):7422-7428.

39. Mozhaev VV: Mechanism-based strategies for protein thermostabilization. Trends Biotechnol 1993, 11(3):88-95.

40. Volklin DB, Kilbanov AM: Thermal destruction processes in proteins involving cystine residues. J Biol Chem 1987, 262(7):2945-2950.

41. Morel N, Bon S, Greenblatt HM, Van Belle D, Wodak SJ, Sussman JL, Massouli J, Silman I: Effect of mutations within the peripheral anionic site on the stability of acetylcholinesterase. Mol Pharmacol 1999, 55(6):982-992.

42. Ulbrich-Hofmann R, Arnold U, Mansfeld J: The concept of the unfolding region of approaching the mechanism of enzyme stabilization. J Mol Catalysis B: Enzymatic 1999, 7:125-131.

43. Chabbi H, Fourdier D, Fedon Y, Bossy JP, Ravalac M, Devauchelle G, Cerutzi M: Biochemical characterization of Drosophila melanogaster acetylcholinesterase expressed by recombinant baculoviruses. Biochem Biophys Res Commun 1994, 203(1):734-742.

44. Elman GL, Courtney KD, Andrews VJ Jr, Feather-Stone RM: A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961, 7:88-95.

45. Charpentier A, Menozzi P, Marcel V, Villatte F, Fourdier D: A method to estimate acetylcholinesterase-active sites and turnover in insects. Anal Biochem 2000, 285(1):76-81.

46. Betz SF, Pielak GJ: Introduction of a disulfide bond into cytochrome c stabilizes a compact denatured state. Biochemistry 1992, 31(49):12337-12344.

Submit your manuscript here: http://www.biomedcentral.com/info/publishing_adv.asp

Publish with BioMed Central and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime." Sir Paul Nurse, Cancer Research UK

Your research papers will be:
* available free of charge to the entire biomedical community
* peer reviewed and published immediately upon acceptance
* cited in PubMed and archived on PubMed Central
* yours — you keep the copyright