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Thermal denaturation of wild type and mutant recombinant acetylcholinesterase from amphioxus: effects of the temperature of *in vitro* expression and of reversible inhibitors

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Abstract

We have studied the thermal inactivation at 37°C of wild type and mutant ChE2 (C310A, F312I, C466A, C310A/F312I, and C310A/C466A) from amphioxus (*Branchiostoma floridae*) expressed *in vitro* in COS-7 monkey cells under three sets of conditions: 30°C for 48 h, 30°C for 24 h and 37°C for 24 h, and 37°C for 48 h. We found biphasic denaturation curves for all enzymes and conditions, except wild type and C310A ChE2 expressed at 30°C for 48 h. Generally, single mutants are more unstable than wild type, and the double mutants are even more unstable. We propose a model involving stable and unstable conformations of the enzymes to explain these results, and we discuss the implications of the model. We also found a correlation between the melting temperature of the ChEs and the rates at which they denature at 37°C, with the denaturation of the unstable conformation dominating the relationship. Reversible cholinergic inhibitors protect the ChEs from thermal denaturation, and in some cases produce monophasic denaturation curves; we also propose a model to explain this stabilization.

Keywords

Acetylcholinesterase; thermal denaturation; ligand stabilization; amphioxus

Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7) is a serine hydrolase that hydrolyzes the neurotransmitter acetylcholine at cholinergic synapses throughout the animal kingdom (Massoulié et al., 1993). An acetylcholinesterase (AChE) from the invertebrate *Branchiostoma*

floridae, cholinesterase 2 (ChE2), has two free cysteines present in its catalytic gorge: Cys310, near the top of the gorge and the acyl pocket, which is partially composed of Phe312; and Cys466, near the bottom of the gorge and Trp109 of the choline-binding site of the enzyme (Sutherland et al., 1997; McClellan et al., 1998; Pezzementi et al., 2003). The sulfhydryl reagents 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and *N*-ethylmaleimide (NEM) inactivate ChE2 (Pezzeменти et al., 2003). To study the involvement of the two Cys residues in the sulfhydryl inactivation we made a number of mutants: C310A, F312I, C466A, C310A/F312I, and C310A/C466A, and expressed them in COS-7 cells *in vitro* (Pezzeменти et al., 2006). We found that although both Cys residues could be involved in the inactivation, Cys310, near the top of the gorge was more accessible and more easily reduced by the disulfide reagents, resulting in enzyme inactivation. Many aphids, mosquitoes, and other insects have Cys residues homologous to Cys310 (Pezzeменти et al., 2006; Pang, 2006a; Pang, 2006b; Rowland et al., 2008), and are sensitive to reduction by sulfhydryl reagents (Zahavi et al., 1972; Smissart, 1976), raising the possibility of this residue as a target for specific insecticides.

Site-directed mutation of AChE from *Torpedo californica* and *Drosophila melanogaster* alters the thermal stability of the enzymes (Wilson et al., 1996; Morel et al., 1999; Estrada-Mondaca and Fournier, 1998; Fremaux et al., 2002; Strub et al., 2004), with some mutations stabilizing the enzymes, while others resulted in destabilization. Indeed, to isolate the inactivation rates of wild type and mutant ChE2s due to sulfhydryl reagents at 22°C from any differences in thermal denaturation rates among the enzymes, we controlled for the small differences (no more than 8 fold) in the slow denaturation rates among the enzymes at that temperature (Pezzeменти et al., 2006). Nevertheless, these small differences suggested that there might be substantial differences in thermostability at higher temperatures. Here we present a detailed study of the thermal denaturation of wild type and mutant ChE2s at 37°C, including the effects of different *in vitro* expression conditions for the enzymes, and the effects of various reversible inhibitors on the denaturation rate.

Materials and Methods

Materials

Dulbecco's modified Eagle medium, fetal calf serum, and OptiMEM medium were purchased from Invitrogen. FuGene was obtained from Roche. QuikChange site-directed mutagenesis kits were purchased from Stratagene. Acetylthiocholine (ATCh), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), *N*-ethylmaleimide (NEM), 1:5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284c51), ethyl (m-hydroxyphenyl) dimethylammonium bromide, 10-(2-diethylaminopropyl) phenothiazine hydrochloride (ethopropazine), 3,8-diamino-5[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide (propidium), and (3-hydroxyphenyl) dimethylethylammonium bromide (edrophonium) were purchased from Sigma.

Site-directed mutagenesis and *in vitro* expression

Site-directed mutagenesis of a ChE2 expression clone (McClellan et al., 1998) was performed with QuikChange II site-directed mutagenesis kits and mutagenic primers. Mutations were confirmed by DNA sequencing.

COS-7 monkey cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle medium containing 10% fetal calf serum. Cells were plated at a density of 2×10^6 cells/75 cm² culture flask, incubated overnight, and transferred to OptiMEM medium. FuGene was then used to transfect the cells with 7.8 µg of DNA purified with a Qiagen MaxiPrep Kit. The cells were then incubated for either 48h at 30°C, 24h at 37°C and 24h at 30°C, or 37°C for 48h before being extracted in 10 mM NaHPO₄, pH 7, 1 M NaCl, 1 mM EDTA, 1% Triton X-100.

Previously, we found that the first two *in vitro* expression conditions produced more enzyme than the third regimen (Pezzementi et al., 2003). Extracts were centrifuged at $30,000 \times g$ for 20 min and the supernatants were assayed for AChE activity.

Measurement and analysis of AChE activity and inhibition

AChE activity was measured in 100 mM NaHPO₄, pH 7, 0.3 mM DTNB according to the method of Ellman et al. (1961) as modified by Doctor et al. (1987); ATCh was used as the substrate.

Values of IC₅₀ for the inhibitors used were determined by pre-incubating wild type and mutant enzymes with various concentrations of the inhibitors for 20 min and then assaying for enzyme activity in the presence of ATCh. SigmaPlot was then used to fit the data to a three-parameter logistic function, yielding IC₅₀.

Melting point (T_m) determination assay

ChE2 extracts were incubated for 20 minutes at various temperatures in preheated microcentrifuge tubes, quenched on ice, and then assayed for residual activity. SigmaPlot was used to fit the data to a 3-parameter sigmoidal function, yielding the melting point (T_m).

Inactivation of AChE in the absence and presence of inhibitors

Inactivation was measured by incubating enzyme for various periods at 37°C and then determining residual enzyme activity. When the experiments were performed in the presence of inhibitors, the inhibitors were added to enzyme at the beginning of the incubation, and then the same procedure was followed. The total dilution of inhibitor was at least 10-fold. In both cases, inactivation followed first-order kinetics. Data were plotted as $\ln v/v_o$ as a function of time. To determine k_1 (min⁻¹), the first-order inactivation rate constant, for monophasic thermal denaturation, SigmaPlot was used to fit the data to the equation for a single exponential decay (Equation 1):

$$v/v_o = \exp(-k_1 t) \quad (1)$$

where v represents enzyme velocity at time t , v_o is the initial velocity, and v/v_o is the fractional activity at time t . For biphasic thermal denaturation curves, SigmaPlot was used to fit the data to the equation for a double exponential decay (Equation 2):

$$v/v_o = f_1 \exp(-k_1 t) + f_2 \exp(-k_2 t) \quad (2)$$

where k_1 (min⁻¹) and k_2 (min⁻¹) represent the fast and slow rate constants responsible for the fast (f_1) and slow (f_2) denaturing fractions of the enzyme; the other parameters remain the same.

Molecular Modelling

Molecular modelling was performed on a Silicon Graphics Indigo O2 computer with the Discover and Insight II programs of Accelrys (San Diego, 2005). The 3D structure of amphioxus ChE2 was built by using the Homology module of Insight II and the crystal structure of *Torpedo* AChE (pdb index 1EA5) as a template. The structure was minimized for 10,000 iterations of steepest descent *in vacuo* with the distance-dependent dielectric constant by the program Discover (Accelrys, 2005).

Results

As one measure of thermal inactivation, we determined T_m values for wild type and mutant AChEs expressed under different conditions (Fig. 1; Table 1). Generally, wild type enzyme

was the most stable, single mutants showed intermediate stability, and double mutants were the most unstable. There are, however, exceptions: the C310A mutant expressed at 30°C for 48 h is slightly more stable than wild type enzyme, and the C310A/F312I mutant is just as stable as the wild type.

To obtain additional information about the thermal stability of the AChEs, we determined thermal inactivation rates (Fig. 2: Table 2). In all cases, except two, biphasic thermal denaturation curves were observed; the exceptions are wild type and C310A enzyme expressed at 30°C for 48 h. The data for thermal stability are complex because four parameters result from the biphasic curves: k_1 and k_2 , the thermal inactivation rates for the fast and slow phases of inactivation; and f_1 and f_2 , the fractional amounts of the total enzyme activity in the two phases. All four of these parameters can and do change. Again, in general, the mutants have faster k_1 values than wild type enzyme; there are two notable exceptions, C310A expressed at 30°C for 48 h, and at 37°C for 24 h and 30°C for 24 h. Additionally, the double mutants, except for C310A/F312I expressed at 37°C for 48 h, denature faster than the single mutants, as measured by k_1 values. The fraction of enzyme distributed between fast and slow denaturation rates does not appear to show a pattern.

Since both the rate and fraction of enzyme inactivated should influence T_m , we plotted T_m as a function of f_1k_1 , f_2k_2 , and $f_1k_1 + f_2k_2$ (Fig. 3). T_m shows a strong negative correlation with f_1k_1 and $f_1k_1 + f_2k_2$.

Ligands can stabilize enzymes to thermal denaturation. To determine if various reversible inhibitors could stabilize AChE, we determined IC_{50} values for BW284c51, edrophonium, propidium, and ethopropazine to find appropriate concentrations of the ligands (Table 3). We then conducted thermal denaturation experiments with wild type and mutant enzymes in the presence of these ligands. In 29 out of 30 cases, the inhibitors stabilized the enzymes; the one exception is the lack of effect of BW284c51 on the F312I mutant (when the SE is taken into account) (Table 4). In nine instances, the inhibitors actually converted the denaturation curves from biphasic to monophasic.

A molecular model shows the positions of the mutated and other important residues (Fig. 4). Cys310 is near to both the top of the catalytic gorge and the acyl pocket, which is partially composed of Phe312. The F312I mutant opens up the catalytic gorge and the acyl pocket. Cys466 is near the bottom of the gorge, close to Trp109 of the choline-binding site (Pezzeменти et al., 2006).

Discussion

We have studied the thermal inactivation at 37°C of wild type and mutant recombinant ChE2s from amphioxus expressed *in vitro* under three sets of conditions: 30°C for 48 h, 30° for 24 h and C37°C for 24 h, and 37°C for 48 h. We found biphasic denaturation curves for all enzymes, except wild type and C310A ChE2 expressed at 30°C. Generally, single mutants are more unstable than wild type, and the double mutants are even more unstable. There is a correlation between the melting temperature of the ChEs and the rates at which they denature at 37°C. Reversible inhibitors protect the ChEs from thermal denaturation, and in some cases produce monophasic denaturation curves.

There are many examples in the literature of biphasic thermal denaturation curves for ChEs produced *in vivo* and *in vitro* (Vigny et al., 1978; Edwards and Brimjoin, 1983; Johnson and Russell, 1983; Yague-Guirao et al., 1986; Cauet et al., 1987; Duval et al., 1992; Weingand-Ziade et al., 2001). In rat superior cervical ganglia, both AChE and BuChE showed biphasic, but different, thermal inactivation rates, and the fact that the inactivation did not follow first-order kinetics was attributed to a possible molecular heterogeneity of the two enzymes (Vigny

et al., 1978). Edwards and Brimijoin (1983) found that the different molecular forms of AChE and BuChE from various species inactivated at different rates, resulting in biphasic inactivation curves. They proposed three features that might cause the molecular forms of ChEs to differ in their thermostability: intersubunit disulfide bonds in multimers, hydrophobic or ionic interactions in multimers, and covalent interactions with collagen in asymmetric forms (Edwards and Brimijoin, 1982). In the AChEs from *C. elegans*, some molecular forms were inactivated rapidly, while other forms were more stable. The rapidly inactivated forms also showed biphasic kinetics, which was attributed to possible molecular heterogeneity (Johnson and Russell, 1983). The thermal denaturation of the molecular forms of AChE from rabbit muscle microsomes has also been studied (Yague et al., 1986). Both the monomeric and tetrameric forms of the AChE showed biphasic denaturation curves. The authors speculate that the two phases could be due to a rapid change in protein conformation that impairs but does not inactivate the enzyme, followed by a slow change in protein structure that results in complete inactivation, which is a modification of the classic Lumry and Eyring (1954) model of protein denaturation. However, the alternative of heterogeneous enzymes with the same sedimentation coefficients could not be ruled out. The biphasic thermal denaturation of native horse serum BuChE has also been attributed to structural heterogeneity (Cauet et al., 1987). In contrast, Weingand-Ziade et al. (2001) propose a complex series-inactivation process based on the Lumry-Eyring (1954) model of thermal denaturation of native horse serum BuChE, involving multiple intermediates.

Initially we expressed ChE2 in COS-7 cells at 37°C for 48 h (McClellan et al., 1998). Subsequently, we serendipitously found that expression of enzyme could be increased up to 50-fold by incubating the cells at either 30°C for 48h or at 37°C for 24 h and then at 30°C for 24 h; we adopted the latter protocol because it produced more enzyme (Pezzemanti et al., 2003). A similar temperature dependence was reported for *in vitro* expression of AChE from *T. californica* and *T. marmorata*, where efficient protein folding does not occur at 37°C but only at lower temperatures (Gibney et al., 1990; Gibney and Taylor, 1990; Duval et al., 1992). Additionally, Duval et al. (1992) found that some of the molecular forms of AChE from *T. marmorata* expressed *in vitro* showed monophasic thermal denaturation, while other forms showed biphasic kinetics, even at the lower temperature, and they speculated that these kinetics might be due to the active conformation failing to be stabilized by glycosylation, disulfide bonding, or oligomerization. In the course of this study, we wondered if the biphasic thermal denaturation curves for wild type and mutant ChE2 could be the result of two different tertiary structures being present because of the two-temperature expression conditions that we had adapted. To investigate this possibility, we performed our thermal denaturation experiments with enzyme produced under all three regimens. For all three expression conditions, we found that the ChE2s produced showed biphasic thermal denaturation kinetics, except for wild type and C310A ChE2 expressed at 30°C for 48 h, where we saw monophasic curves. The results cannot be explained by the differential stability of different molecular forms, since only the G₂ form of the enzyme is produced (McClellan et al., 1998). Additionally, it seems unlikely that the biphasic rates are due to the coexistence among the extracted AChE of ligand-free and ligand-bound enzyme, since COS-7 cells do not express cholinergic proteins. Thus, it seems to us that the simplest explanation for the biphasic curves is that there are two different folded, active forms of the enzymes produced in different proportions (*f*₁ and *f*₂) in most cases: a stable conformation (*S*) and an unstable conformation (*U*), which then denature to inactive enzyme (*I*) at different rates (*k*₁ and *k*₂). Although denaturation can be described as occurring through an intermediate molten globule (*MG*) state or analogous state (Lumry and Eyring, 1954; Eichler et al., 1994; Kreimer et al., 1995; Kreimer et al., 1996; Shin et al., 1997; Morel et al., 1999), we did not use techniques that would have allowed us to identify such a state; thus, we do not include the *MG*, or analogous, state in our model (although they could be easily incorporated), which is shown below.

These results of AChE expression *in vitro* in heterologous systems suggest that it is difficult to produce homogeneous enzyme preparations. Rather, most preparations may consist of heterogeneous enzymes, and this factor should be taken into account in the interpretation of kinetic and pharmacological experiments. Nevertheless, we have not seen biphasic kinetics for inactivation of ChE2 by sulfhydryl reagents, carbamates, organophosphates, or at 22°C (Pezzeменти et al., 2003; Patel et al., 2006; Pezzeменти et al., 2006). We should also note that we do not know if the inactivation is reversible or irreversible; both cases have been reported (Cauet et al., 1987; Masson and Laurentie, 1988; Burgess and Osendine, 1993; Weingand-Ziade et al., 2001; Cengiz et al., 2002).

It is noteworthy that the C310A ChE2 mutant is the only mutant that stabilizes the enzyme to thermal denaturation, implying that Cys310 may play a role in the thermal inactivation process. The C466A mutation does not promote thermal stability and C310A does not counteract the destabilizing effects of either the C466A or F312I mutations, with the possible exception of the C310A/F312I double mutant expressed at 37°C for 48 h. The elimination of a free cysteine in *D. melanogaster* AChE (a C290V mutation) resulted in an increase in thermostability, which was proposed to be a result of decreased thiol-disulfide interchanges (Fremaux et al., 2002). The fact that the C310A mutant, which is easily accessible to reducing agents, can stabilize the enzyme; while C466A, which is deep in the catalytic gorge, does not (Pezzeменти et al., 2006), suggests that modification of Cys310 by endogenous reducing agents could also contribute to the denaturation. It should be noted that our results are in marked contrast with Wilson et al. (1996) who found that in *Torpedo* AChE a C231S mutation, which eliminated a free cysteine, decreased the thermal stability of the enzyme. However, a general destabilization of the enzyme by the mutants may also be involved, as mutations of residues in the catalytic gorge of AChE have been proposed to disrupt cross-gorge interactions and, ultimately, the entire structure (Morel et al., 1999).

The correlation between T_m and thermal denaturation rate is interesting, if not surprising. We, however, have not seen this type of analysis for AChE in the literature. Clearly, the fraction of enzyme with the fast rate constant (f_1 and k_1) dominates the T_m , with f_2k_2 contributing little, if any, to the observed melting temperature.

Virtually all the inhibitors: BW284c51, a bisquaternary ligand that binds to the choline-binding site and the peripheral site; edrophonium, which binds to the choline-binding site; propidium, which binds to the peripheral site; and ethopropazine, which binds in the catalytic gorge, stabilize the wild type and mutant enzymes to thermal denaturation. In nine cases the inactivation curves are shifted from biphasic to monophasic, suggesting that the ligands (L) convert the unstable enzyme conformation (U) to the stable structure (S), as shown in the model below.

Seven of the nine cases where the denaturation kinetics became monophasic involved C310A ChE2, further implicating Cys310 in the thermal inactivation process.

Ligand stabilization of ChEs has been reported in the literature (Payne et al., 1989; Burgess and Oxendine, 1993; Estrada-Mondaca and Fournier, 1998). Fetal bovine serum AChE is stabilized, in order of potency, by edrophonium, decamethonium (a bisquaternary inhibitor), pralidoxime chloride (an oxime reactivator of organophosphate-inhibited enzyme), and procainamide; likewise, human BuChE was stabilized by decamethonium, procainamide, edrophonium, and pralidoxime, with the ligands apparently maintaining the integrity of the active site (Payne et al., 1989). Human BuChE and eel AChE are stabilized by choline chloride and acetylcholine iodide, which is accompanied by a decrease in absorbance at 280 nm, implying the involvement of a conformational change in protein structure in the stabilization (Burgess and Oxendine, 1993). Decamethonium also stabilizes AChE from *D. melanogaster* (Estrada-Mondaca and Fournier, 1998). The osmolyte trimethylamine-*N*-oxide, and the divalent cation Mg^{2+} can promote thermal stability of AChE from *T. californica* (Millard et al., 2003). Perhaps these agents could also produce the stable state of ChE2 resulting in a homogenous sample of enzyme.

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References

- Burgess SK, Oxendine SL. Thermal inactivation of butyrylcholinesterase and acetylcholinesterase. *J Prot Chem* 1993;12:651–658.
- Cauet G, Friboulet A, Thomas D. Substrate activation and thermal denaturation kinetics of the tetrameric and the trypsin-generated monomeric forms of horse serum butyrylcholinesterase. *Biochim Biophys Acta* 1987;912:338–342. [PubMed: 3567204]
- Duval N, Massoulié J, Bon S. H and T subunits of acetylcholinesterase from *Torpedo*, expressed in COS cells, generate all types of globular forms. *J Cell Biol* 1992;118:641–653. [PubMed: 1639848]
- Edwards JA, Brimijoin S. Thermal inactivation of the molecular forms of acetylcholinesterase and butyrylcholinesterase. *Biochim Biophys Acta* 1983;742:509–516. [PubMed: 6838885]
- Eichler J, Kreimer DI, Varon L, Siman I, Weiner L. A “molten globule” of *Torpedo* acetylcholinesterase undergoes thiol-disulfide exchange. *J Biol Chem* 1994;269:30093–30096. [PubMed: 7982909]
- Estrada-Mondaca S, Fournier D. Stabilization of recombinant *Drosophila* acetylcholinesterase. *Prot Exp Pur* 1998;12:166–172.
- Fremaux I, Mazère S, Brisson-Lougarre A, Arnaud M, Ladurantie C, Fournier D. Improvement of *Drosophila* acetylcholinesterase stability by elimination of a free cysteine. *BMC Biochem* 2002;3:21. [PubMed: 12149129]
- Gibney G, Camp S, Dionne M, MacPhee-Quigley K, Tayler P. Mutagenesis of essential functional residues in acetylcholinesterase. *Proc Natl Acad Sci USA* 1990;87:7546–7550. [PubMed: 2217185]
- Gibney G, Taylor P. Biosynthesis of *Torpedo* acetylcholinesterase in mammalian cells: functional expression and mutagenesis of the glycopospholipid-anchored form. *J Biol Chem* 1990;265:12576–12583. [PubMed: 2165068]
- Johnson CD, Russell RL. Multiple molecular forms of acetylcholinesterase in the nematode *Caenorhabditis elegans*. *J Neurochem* 1983;41:30–46. [PubMed: 6864228]
- Kreimer DI, Shnyrov VL, Villar E, Silman I, Weiner L. Irreversible thermal denaturation of *Torpedo californica* acetylcholinesterase. *Prot Sci* 1995;4:2349–2357.
- Kreimer DI, Shin I, Shnyrov VL, Villar E, Silman I, Weiner L. Two partially unfolded states of *Torpedo californica* acetylcholinesterase. *Prot Sci* 1996;5:1852–1864.
- Lumry R, Eyring H. Conformation changes of proteins. *J. Phys Chem* 1954;58:110–120.
- McClellan JS, Coblenz WB, Sapp M, Rulewicz G, Gaines DI, Hawkins A, Ozment C, Bearden A, Merritt S, Cunningham J, Palmer E, Contractor A, Pezzementi L. cDNA cloning, *in vitro* expression, and biochemical characterization of cholinesterase 1 and cholinesterase 2 from amphioxus: comparison with cholinesterase 1 and cholinesterase 2 produced *in vivo*. *Eur J Biochem* 1998;258:419–429. [PubMed: 9874207]
- Millard CB, Shnyrov VL, Newstead S, Shin I, Roth E, Silman I, Weiner L. Stabilization of a metastable state of *Torpedo californica* acetylcholinesterase by chemical chaperones. *Prot Sci* 2003;12:2337–2347.
- Morel N, Bon S, Greenblatt HM, Van Velle D, Wodak SJ, Sussman JL, Massoulié J, Silman I. Effect of mutations within the peripheral anionic site on the stability of acetylcholinesterase. *Mol Pharm* 1999;55:982–992.
- Pang YP. Species marker for developing novel and safe pesticides. *Med Chem Lett* 2006a;17:197–199.
- Pang YP. Novel acetylcholinesterase target site for malaria mosquito control. *PLoS ONE* 2006b;1(e58): 1–8.
- Patel R, Sanders R, Brown L, Baker S, Tsigelny I, Pezzementi L. A tryptophan in the bottleneck of the catalytic gorge of an invertebrate acetylcholinesterase confers relative resistance to carbamate and organophosphate inhibitors. *Cell Biochem Biophys* 2006;46:253–264. [PubMed: 17272851]
- Payne CS, Aseed M, Wolfe AD. Ligand stabilization of cholinesterases. *Biochim Biophys Acta* 1989;999:46–51. [PubMed: 2804138]
- Pezzementi L, Johnson K, Tsigelny I, Cotney J, Manning E, Barker A, Merritt S. Amino acids defining the acyl pocket of an invertebrate cholinesterase. *Comp Biochem Physiol* 2003;136B:813–832.
- Pezzementi L, Rowland M, Wolfe M, Tsigelny I. Inactivation of an invertebrate acetylcholinesterase by sulfhydryl reagents: the roles of two cysteines in the catalytic gorge of the enzyme. *Invert Neurosci* 2006;6:47–55. [PubMed: 16586114]

- Rowland M, Tsigelny I, Wolfe M, Pezzementi L. Inactivation of an invertebrate acetylcholinesterase by sulfhydryl reagents: a reconsideration of the implications for insecticide design. *Chem Biol Interact.* 2008in press
- Shin I, Kreimer D, Silman I, Weiner L. Membrane-promoted unfolding of acetylcholinesterase: a possible mechanism for insertion into the lipid bilayer. *Proc Natl Acad Sci USA* 1997;94:2848–2852. [PubMed: 9096309]
- Smissart HR. Reactivity of a critical sulfhydryl group of the acetylcholinesterase from aphids (*Myzus persicae*). *Pest Biochem Physiol* 1976;6:215–222.
- 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;5:9. [PubMed: 15251041]
- Sutherland D, McClellan JS, Milner D, Soong W, Axon N, Sanders M, Hester A, Kao YH, Poczek T, Routt S, Pezzementi L. Two cholinesterase activities and genes are present in amphioxus. *J Exp Zool* 1997;277:213–229. [PubMed: 9062997]
- Vigny M, Gisiger V, Massoulie J. “Nonspecific” cholinesterase and acetylcholinesterase in rat tissues: molecular forms, structural and catalytic properties, and significance of the two enzyme systems. *Proc Natl Acad Sci USA* 1978;75:2588–2592. [PubMed: 78492]
- Weingand-Ziade A, Ribes F, Renault F, Masson P. Pressure- and heat-induced inactivation of butyrylcholinesterase: evidence for multiple intermediates and the remnant inactivation process. *Biochem J* 2001;356:487–493. [PubMed: 11368776]
- Wilson EJ, Massoulié J, Bon S, Rosenberry TL. The rate of thermal inactivation of *Torpedo* acetylcholinesterase is not reduced in the C231S mutant. *FEBS Lett* 1996;379:161–164. [PubMed: 8635584]
- Yague-Guirao A, Munoz-Delgado E, Vidal CJ. Thermal stability of molecular forms of acetylcholinesterase from muscle microsomes. *Biochem Int* 1986;5:685–692. [PubMed: 3729965]
- Zahavi M, Tahori AS, Klimer F. An acetylcholinesterase sensitive to sulfhydryl inhibitors. *Biochim Biophys Acta* 1972;276:577–583.

Fig. 1.

Fractional ChE2 activity remaining as a function of incubation temperature after 20 minutes. *Top:* wild type and mutant ChE2 expressed at 48 hours at 30°C. *Middle:* ChE2 WT and mutants expressed at 24 hours at 37°C and 24 hours at 30°C. *Bottom:* wild type and mutant ChE2 expressed at 48 hours at 37°C. Wild type (filled circle), C310A (empty circle), F312I (filled triangle), C466A (empty triangle), C310A/F312I (filled square), C310A/C466A (empty square).

Fig. 2.

Fractional ChE2 activity as a function of incubation time at 37°C. *Top:* wild type and mutant ChE2 expressed at 48 hours at 30°C. *Middle:* wild type and mutant ChE2 expressed at 24 hours at 37°C and 24 hours at 30°C. *Bottom:* wild type and mutant ChE2 expressed at 48 hours at 37°C. Wild type (filled circle), C310A (empty circle), F312I (filled triangle), C466A (empty triangle), C310A/F312I (filled square), C310A/C466A (empty square).

Fig. 3.

Correlation of melting point (T_m) and thermal denaturation rates. (*Top*): T_m as a function of f_1k_1 product. (*Middle*): T_m as a function of f_2k_2 product. (*Bottom*): T_m as a function of $f_1k_1 + f_2k_2$. The solid line is the regression line and the dashed lines show the 95% confidence limits.

Fig. 4.

Location of relevant amino acid residues in the catalytic gorge of ChE2. The Cys310 at the top of the gorge and the Cys466 at the bottom of the gorge are shown as CPK-colored space-filling residues. The entrance to the gorge is indicated at the top by an arrow. Aromatic amino acids that line the gorge and other amino acids that define the gorge are shown. The acyl pocket consists of Phe312 (*right*) and Phe422 (*left*), and are shown as orange space-filling residues. Cys310 is near the acyl pocket. The major amino acid of the choline-binding site, Trp109, is located at the bottom of the gorge and is represented as a green space-filling residue. Cys466 is in close proximity to Trp109. Ser224 is the active site serine and is indicated as a red space-filling residue.

Table 1
Melting point (T_m , °C; temperature for 50% inactivation) of wild type and mutant ChE2 expressed under various conditions

Conditions	WT	C310A	F312I	C466A	C310A/F312I	C310A/C466A
30°C 48h	34.3±0.12	35.8±0.93	32.3±0.46	30.5±0.30	34.0±0.14	29.0±0.13
37°C 24h, 30°C 24h	36.4±0.17	33.6±0.05	ND	31.9±0.06	ND	30.0±0.04
37°C 48h	36.1±0.09	34.3±0.01	ND	33.8±0.17	ND	31.3±0.05

Data are mean ± SE of three determinations. ND, not determined.

Table 2
Thermal inactivation rates (k ; min^{-1}) at 37°C for wild type and mutant ChE2 expressed under various conditions

Expression Conditions	WT	C310A	F312I	C466A	C310A/F312I	C310A/C466A
30°C 48h						
k_1	0.09±0.003	0.03±0.004	1.09±0.162	0.46±0.082	1.12±0.348	1.11±0.305
k_2	-	-	0.10±0.007	0.14±0.078	0.04±0.007	0.08±0.020
f_1	1	1	0.42±0.048	0.75±0.082	0.29±0.042	0.80±0.062
f_2	-	-	0.58±0.048	0.29±0.108	0.72±0.035	0.21±0.059
37°C 24h ,30°C 24h						
k_1	0.41±0.065	0.31±0.064	0.73±0.141	0.69±0.103	1.42±0.189	0.87±0.136
k_2	0.06±0.017	0.01±0.003	0.19±0.040	0.10±0.011	0.06±0.005	0.07±0.008
f_1	0.61±0.056	0.51±0.088	0.75±0.031	0.52±0.079	0.44±0.045	0.58±0.058
f_2	0.42±0.055	0.51±0.084	0.25±0.030	0.49±0.081	0.56±0.036	0.43±0.054
37°C 48h						
k_1	0.22±0.0298	0.38±0.054	0.63±0.104	0.79±0.093	0.48±0.029	1.28±0.135
k_2	0.01±0.006	0.04±0.003	0.01±0.001	0.05±0.017	0.02±0.001	0.11±0.005
f_1	0.74±0.059	0.50±0.044	0.37±0.025	0.90±0.027	0.37±0.014	0.69±0.082
F_2	0.27±0.052	0.51±0.042	0.62±0.027	0.11±0.024	0.62±0.015	0.31±0.082

k_1 and k_2 are the fast and slow first-order rate constants for thermal inactivation of enzyme; f_1 and f_2 are the fractions of enzyme inactivated at the two rates. Where data are missing, the thermal inactivation rate became monophasic. Data are mean ± SE of 3 or more determinations.

Table 3
IC₅₀ Values (μM) of Wild Type and Mutant ChE 2 for Various Inhibitors

<i>Inhibitor/Enzyme</i>	<i>WT</i>	<i>C310A</i>	<i>F312I</i>	<i>C466A</i>	<i>C310A/F312I</i>	<i>C310A/C466A</i>
BW284c51	1.28±0.35	1.50±0.59	2.40±0.08	0.93±0.62	1.13±0.22	0.70±0.09
Edrophonium	2.73±0.06	5.86±0.45	10.4±0.05	8.12±0.24	15.1±2.39	7.68±0.17
Propidium	21.8±3.67	13.7±5.62	6.03±1.43	7.10±0.17	1.52±0.10	5.49±0.27
Ethopropazine	69.7±25.3	27.8±16.9	5.47±2.78	11.1±0.42	1.75±0.02	16.8±1.80

Data are mean ± SE of 3 or more determinations.

Table 4 Thermal inactivation rates (k ; min^{-1}) at 37°C for wild type and mutant ChE2 in the presence and absence of inhibitors

Inhibitor	WT	C310A	F312I	C466A	C310A/F312I	C310A/C466A
Control						
k_1	0.41±0.065	0.31±0.064	0.73±0.141	0.69±0.103	1.42±0.189	0.87±0.14
k_2	0.06±0.017	0.01±0.003	0.19±0.040	0.10±0.011	0.06±0.005	0.07±0.01
f_1	0.61±0.056	0.51±0.088	0.75±0.031	0.52±0.079	0.44±0.045	0.58±0.06
f_2	0.42±0.055	0.51±0.084	0.25±0.030	0.49±0.081	0.56±0.036	0.43±0.05
BW284c51						
	1×10 ⁻⁶ M	1×10 ⁻⁶ M	1×10 ⁻⁶ M	3×10 ⁻⁶ M	1×10 ⁻⁶ M	3×10 ⁻⁶ M
k_1	0.18±0.061	0.03±0.001	0.93±0.108	0.50±0.024	0.03±0.001	0.24±0.03
k_2	0.03±0.007	-	0.09±0.001	0.06±0.003	-	0.03±0.01
f_1	0.41±0.100	1	0.27±0.009	0.24±0.010	1	0.65±0.08
f_2	0.60±0.101	-	0.73±0.008	0.77±0.008	-	0.38±0.08
Edrophonium						
	1×10 ⁻⁵ M	1×10 ⁻⁵ M	1×10 ⁻⁵ M	1×10 ⁻⁵ M	1×10 ⁻⁵ M	1×10 ⁻⁵ M
k_1	0.14±0.065	0.20±0.001	0.05±0.004	0.03±0.003	0.03±0.002	0.05±0.03
k_2	0.01±0.002	-	0.01±0.006	-	-	0.02±0.01
f_1	0.45±0.077	1	0.77±0.071	1	1	0.41±0.07
f_2	0.57±0.077	-	0.23±0.072	-	-	0.61±0.08
Propidium						
	1×10 ⁻⁵ M	1×10 ⁻⁵ M	1×10 ⁻⁵ M	3×10 ⁻⁵ M	1×10 ⁻⁶ M	3×10 ⁻⁵ M
k_1	0.18±0.050	0.10±0.060	0.14±0.02	0.31±0.10	0.03±0.002	0.37±0.03
k_2	0.01±0.004	0.01±0.003	0.01±0.01	0.02±0.01	-	0.04±0.01
f_1	0.58±0.080	0.36±0.070	0.77±0.09	0.34±0.10	1	0.71±0.03
f_2	0.44±0.077	0.66±0.068	0.24±0.09	0.68±0.10	-	0.32±0.03
Ethopropazine						
	1×10 ⁻⁴ M	3×10 ⁻⁵ M	1×10 ⁻⁴ M	3×10 ⁻⁵ M	1×10 ⁻⁶ M	3×10 ⁻⁵ M
k_1	0.05±0.014	0.03±0.001	0.08±0.073	0.03±0.003	0.02±0.002	0.24±0.046
k_2	0.02±0.006	-	0.01±0.002	-	-	0.03±0.013
f_1	0.43±0.038	1	0.41±0.048	1	1	0.62±0.128
f_2	0.60±0.031	-	0.63±0.047	-	-	0.41±0.119

ChE2 was expressed at 37°C for 24 h and 30°C for 24 h. k_1 and k_2 are the fast and slow first-order rate constants for thermal inactivation of enzyme; f_1 and f_2 are the fractions of enzyme inactivated at the two rates. Concentrations of inhibitors used are indicated. Where data are missing, the thermal inactivation rate became monophasic. Data are mean ± SE of 4 or more determinations.