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INACTIVATION OF AN INVERTEBRATE ACETYLCHOLINESTERASE BY SULFHYDRYL REAGENTS: A RECONSIDERATION OF THE IMPLICATIONS FOR INSECTICIDE DESIGN

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Abstract

Previously we used site-directed mutagenesis, *in vitro* expression, and molecular modeling to investigate the inactivation of an invertebrate acetylcholinesterase, cholinesterase 2 from amphioxus, by the sulfhydryl reagents 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and N-ethylmaleimide (NEM). We created the mutants C310A, C466A, C310A/C466A and C310A/F312I to assess the roles of the two cysteines and a proposal that the increased rate of inactivation previously found in an F312I mutant was due to increased access of sulfhydryl reagents to Cys310. Our results indicated that both of the cysteines could be involved in inactivation by sulfhydryl reagents, but that the cysteine near the acyl pocket was more accessible. We speculated that the inactivation of aphid AChEs by sulfhydryl reagents was due to the presence of a cysteine homologous to Cys310 and proposed that this residue could be a target for a specific insecticide. Here we reconsider this proposal.

Keywords

acetylcholinesterase; sulfhydryl reagents; aphids; insecticides

Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7) is a serine hydrolase whose primary function is the very efficient and specific hydrolysis of the neurotransmitter acetylcholine (ACh) at cholinergic synapses throughout the animal kingdom (Massoulié et al., 1993). It has been known for many years that sulfhydryl reagents inactivate the AChEs of aphids (Zahavi et al., 1972; Smissart, 1976; Manulis et al., 1981; Brestkin et al., 1985; Novoshilov et al., 1989); however, the molecular basis of this inactivation was not known. Recent sequence data indicated that there is a free cysteine in the acyl pocket of AChEs in the aphids, *Myzus persicae*, *Aphis gossypii*, *Schizapus graminum*, *Sitobion avenae*, and *Rhopalosiphum padi*, and the mosquitoes *Anopheles gambia*, *Culex pipiens*, and *Culex tritaeniorhynchus*, the honeybee *Apis*

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mellifera (Shapira et al., 2001; Gao et al., 2002; Li and Han, 2002; Weill et al., 2002; Nabeshima et al., 2003, 2004; Chen and Han, 2006), and a number of other insects. It is not known if the mosquito and honeybee AChEs are inhibited by sulfhydryl reagents. However, Moralev and Rozengart (2006) proposed that the acyl pocket cysteine is responsible for the sensitivity of aphid AChEs to sulfhydryl reagents and that mosquito AChEs possessing the residue should be sensitive also.

The cephalochordate amphioxus (*Branchiostoma* spp.) has two AChEs, cholinesterase 1 (ChE1) and cholinesterase 2 (ChE2) (McClellan et al., 1998). ChE2 has two free cysteines in the catalytic gorge: one in the acyl pocket near the top of the catalytic gorge, Cys310, homologous to the free cysteine in aphids and mosquitoes; and another, Cys466, adjacent to the choline-binding site near the bottom of the gorge. We thought this enzyme could serve as a model system to investigate the inactivation of AChE by sulfhydryl reagents. In a study examining the nature of the acyl pocket and the substrate specificity of ChE2, we created an F312I acyl pocket mutation and found that 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) rapidly inactivated the mutant AChE during the Ellman esterase assay (Ellman *et al.*, 1961). The mutation of the aromatic amino acid Phe312 to the smaller aliphatic amino acid Ile312 increased the reactivity of Cys310. Notably, a C310A mutant was less susceptible to inactivation by DTNB than was wild type and much less susceptible than ChE2 F312I (Pezzementi et al., 2003).

We then conducted a more detailed study of the inactivation of ChE2 by the sulfhydryl reagents DTNB and N-ethylmaleimide (NEM), using site-directed mutagenesis, *in vitro* expression, and molecular modeling to investigate the inactivation of wild type ChE2, the site-directed mutants C310A, C466A, and F312I, and the double mutants C310A/C466A and C310A/F312I, to assess the relative roles of the two cysteines (Pezzementi et al., 2006). Our results suggested that both cysteines were involved in inactivation by sulfhydryl reagents, but that the cysteine in the acyl pocket was more accessible.

In 1972, Zahavi et al. (1972) found that DTNB inhibited the AChE from the green peach aphid *Myzus persicae*, and increasing concentrations of substrate decreased the inhibition. They concluded, "acetylcholinesterase from aphids contains an essential SH group located at the active site serine or at an area greatly influencing this site." We proposed that the sensitivity of aphid AChE to inactivation by sulfhydryl reagents was due to the presence of a cysteine homologous to Cys310 in the acyl pocket of ChE2 (Pezzementi et al., 2006). At that time, we also proposed that the aphid AChEs containing the conserved cysteine, *Myzus persicae*, *Aphis gossypii*, and *Schizapus graminum*, (Gao et al., 2002; Li and Han, 2002; Nabeshima et al., 2003), as well as the AChEs from the mosquitoes *Anopheles gambia* and *Culex pipiens* (Weill et al., 2002), and the honeybee *Apis mellifera* (Shapira et al., 2001), which also possess this cysteine, should be sensitive to sulfhydryl reagents. Given the sensitivity of aphid AChE to inactivation by sulfhydryl reagents, Smissaert (1976) raised the possibility of whether one could design "specific aphidical thiol reagents." Indeed, we proposed that a high affinity bi-functional cholinergic reagent that reacts transiently with the active site serine, and thus becomes inactivated and non-toxic as a traditional covalent AChE inhibitor, but reacts irreversibly with the acyl pocket cysteine could be a candidate for a selective aphidicide or insecticide (Pezzementi et al., 2006). Pang has more recently made similar proposals, but with a reversible inhibitor, and extending the approach to other invertebrates (2006, 2006). The structure of the inhibitor we proposed would be quite similar to those that he has proposed, except that in our case the tetrahedral intermediate of the covalent inhibitor transiently bound to the active site serine would position the sulfhydryl-reactive group to react with the acyl pocket cysteine.

Results and Discussion

On further consideration, however, we have some reservations about the value of this approach. The k_i values of DTNB and NEM for inactivation of wild type ChE2 are 19.77 and 11.08 $\text{M}^{-1} \text{min}^{-1}$, respectively (Pezzementi et al., 2006). In contrast, the k_i values for the carbamate and organophosphate insecticides propoxur, aldicarb, paraoxon, and dichlorvos range from $1.03\text{--}4.89 \times 10^5$, approximately 5,000 to 50,000 times greater (Pezzementi et al., 2003). Even in the F312I acyl pocket mutation, where the rates for inactivation of ChE2 by DTNB and NEM increase about 50–100 fold, to 1149 and 935 $\text{M}^{-1} \text{min}^{-1}$, respectively, the rates of inactivation are still approximately 100–500 times slower than for the carbamates and organophosphates. We propose that the inactivation rates of wild type amphioxus ChE2 by sulfhydryl reagents are so slow because it is difficult for the reagents to gain access to the acyl pocket, where the susceptible cysteine (Cys310) is located. In our original alignment, we did not show both of the phenylalanines of the acyl pockets of the susceptible invertebrate AChEs, but only one, corresponding to Phe290 in *Torpedo* AChE (Pezzementi et al., 2006). Such a presentation could have been interpreted to mean that invertebrate acyl pockets are defined by a single phenylalanine, making the cysteine accessible to reduction by sulfhydryl reagents. Pang (2006a, 2006b) presented similar alignments with additional AChEs possessing the acyl pocket cysteine. Here we present a more complete analysis, showing that two phenylalanines are present in the acyl pockets of all these enzymes (Table 1). It is just that in the invertebrates, the acyl pocket is constructed in a fundamentally different way than it is in the vertebrates (Pezzementi et al., 2003). In vertebrate AChEs, phenylalanines corresponding to Phe288 and Phe290 in *Torpedo* AChE define the acyl pocket; in contrast, in invertebrates AChEs, phenylalanine residues corresponding to positions Phe290 and Val400 in *Torpedo* AChE usually define the pocket (Pezzementi et al., 2003). Based on our results with ChE2 from amphioxus, the two phenylalanines, which are 3.9 Å apart in our molecular model, appear to restrict access of the sulfhydryl reagents NEM and DTNB to the invertebrate acyl pocket cysteine, despite the favorable pre-binding energies, accounting for the slow inactivation rates. In contrast, Smissaert (1976) reported the k_i of DTNB for the AChE from the aphid *Myzus persicae* is $1.7 \times 10^3 \text{ M}^{-1} \text{min}^{-1}$, which is comparable to our F312I mutant. The structure of AChE from *Myzus persicae* is not known, so we do not know the width of the pocket in the enzyme, and we only have information on the width of the acyl pocket for one other invertebrate AChE, that of *Drosophila melanogaster*, where it is 4.5 Å (Harel et al., 2000). The difference in width of the acyl pocket between that of *Drosophila* AChE and amphioxus ChE2 appears to be significant, since ChE2 does not hydrolyze butyrylthiocholine appreciably, while *Drosophila* AChE hydrolyzes the substrate at about 50% of the rate of acetylthiocholine hydrolysis. Thus, it may be that the sensitivity to sulfhydryl reagents of an AChE containing a cysteine in the acyl pocket is dependent on the width of the pocket, i.e., the distance between the two phenylalanines, and that this is an important consideration for the feasibility of this approach and in the design of effective insecticides targeting the residue.

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Amino acids corresponding to positions 288, 290, and 400 of AChE from *Torpedo californica* in human AChE and various invertebrate AChEs.¹

Table 1

Common Species Name	Taxonomic Species Name	GenBank Accession Number	Amino acid 288	Amino acid 290	Amino acid 400	Amino acid 466
Electric Ray	<i>Torpedo californica</i>	X03439	Phe	Phe	Val	Ile
Human	<i>Homo sapiens</i>	M55040	Phe	Phe	Val	Ile
Fruit Fly	<i>Drosophila melanogaster</i>	X17572	Leu	Phe	Phe	Ile
Amphioxus	<i>Branchiostoma floridae</i>	U74318	Cys	Phe	Phe	Cys
Cotton Aphid	<i>Aphis gossypii</i>	AF505082	Cys	Phe	Phe	Ile
Green Peach Aphid	<i>Myzus persicae</i>	AF287291	Cys	Phe	Phe	Met
Greenbug	<i>Schizaphis graminum</i>	AF321547	Cys	Phe	Phe	Ile
Grain Aphid	<i>Stiabion avenae</i>	AY819704	Cys	Phe	Phe	Ile
Wheat Aphid	<i>Rhopalosiphum padi</i>	AY667435	Cys	Phe	Phe	Ile
Silk Moth	<i>Bombyx mori</i>	DQ186606	Cys	Phe	Phe	Met
Diamondback Moth	<i>Plutella xylostella</i>	AY970293	Cys	Phe	Phe	Ile
Codling Moth	<i>Cydia pomonella</i>	DQ267977	Cys	Phe	Phe	Ile
House Mosquito	<i>Culex pipiens</i>	AY762905	Cys	Phe	Phe	Ile
Japanese Encephalitis Mosquito	<i>Culex tritaeniorhynchus</i>	AB122152	Cys	Phe	Phe	Ile
African Malaria Mosquito	<i>Anopheles gambiae</i>	AI88492	Cys	Phe	Phe	Ile
Rice Leaf Beetle	<i>Oulema oryzae</i>	AY147799	Cys	Phe	Phe	?
Cotton Bollworm	<i>Helicoverpa armigera</i>	DQ064790	Cys	Phe	Phe	Met
Beet Armyworm	<i>Spodoptera exigua</i>	DQ280488	Cys	Phe	Phe	?
Honey Bee	<i>Apis mellifera</i>	XM_393751	Cys	Phe	Phe	?
German Cockroach	<i>Blattella germanica</i>	DQ288847	Cys	Phe	Phe	Ile

¹ **Methods.** Sequences were aligned with ClustalW. The AChE from *Torpedo californica* is the reference enzyme for amino acid position number. The AChE from humans is included as another typical vertebrate AChE and for comparison with the invertebrate AChE sequences. The fruit fly sequence is included as an invertebrate that does not have the acyl pocket cysteine. The ? indicates that the partial sequence does not include this residue.