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Probing the role of amino acids in oxime-mediated reactivation of nerve agent-inhibited human acetylcholinesterase

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ABSTRACT

In this study, we employed site-directed mutagenesis to understand the role of amino acids in the gorge in oxime-induced reactivation of nerve agent-inhibited human (Hu) acetylcholinesterase (AChE). The organophosphorus (OP) nerve agents studied included GA (tabun), GB (sarin), GF (cyclosarin), VX, and VR. The kinetics of reactivation were examined using both the mono-pyridinium oxime 2-PAM and bis-pyridinium oximes MMB4, HI-6, and HLö-7. The second-order reactivation rate constants were used to compare reactivation of nerve agent-inhibited wild-type (WT) and mutant enzymes. Residues including Y72, Y124 and W286 were found to play important roles in reactivation by bis-pyridinium, but not by mono-pyridinium oximes. Residue Y124 also was found to play a key role in reactivation by HI-6 and HLö-7, while E202 was important for reactivation by all oximes. Residue substitutions of F295 by Leu and Y337 by Ala showed enhanced reactivation by bis-pyridinium oximes MMB4, HI-6, and HLö-7, possibly by providing more accessibility of the OP moiety associated at the active-site serine to the oxime. These results are similar to those observed previously with bovine AChE and demonstrate that there is significant similarity between human and bovine AChEs with regard to oxime reactivation.

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1. Introduction

Organophosphorus (OP) chemical nerve agents are potent and irreversible inhibitors of acetylcholinesterase (AChE; EC 3.1.1.7), an enzyme which catalyzes the hydrolysis of neurotransmitter acetylcholine (ACh). In the presence of a strong nucleophile such as an oxime, OP-inhibited AChE can be reactivated (Wong et al., 2000). However, oximes are not equally effective in reactivating nerve agent-inhibited AChE. The reactivation of OP-inhibited AChE by oximes depends on the structures of OP and oxime compounds and the source of the enzyme (Luo et al., 2007, 2010a). Extensive studies with current oximes have been conducted previously in an effort to find more potent and broad-spectrum nerve agent antidotes (Kassa, 2002; Worek et al., 2002, 2004, 2007; Luo et al., 2010b). The catalytic site of AChE is located near the floor of a deep (~20 Å) and narrow gorge lined predominantly with aromatic amino acid residues (Sussman et al., 1991). The catalytic triad (S203-H447-E334) is surrounded by three sub-sites that are important for catalytic activity: (1) the esteratic sub-site or choline binding site (ES) consists of aromatic residues (W86, Y133, Y337, and F338), which bind to the quaternary trimethylammonium moiety of the choline group of the substrate for optimal positioning of the carbonyl carbon of the ester at the acylation site. The catalytic process is facilitated by orientation of the substrate's carbonyl oxygen toward the oxyanion hole formed by the hydrogens from G120 and G121 and A204; (2) the anionic sub-site or acyl pocket (AS) of AChE formed by the side-chains of F295 and F297 is the binding pocket for the acyl group of the substrate or

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; rHu AChE, recombinant human acetylcholinesterase; WT, wild-type; OP, organophosphorus compounds; GA, tabun; GB, sarin; GD, soman; GF, cyclosarin; VX, O-ethyl-S-[2-(diisopropylamino) ethyl] methyl phosphonothioate; VR, O-isobutyl-S-[2-(diethylamino) ethyl] methylphosphonothioate; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); ATCh, acetylthiocholine; MMB4, 1,1′-methylenebis [4-[(hydroxyimino) methyl]-pyridinium] dimethanesulfonate; 2-PAM, 2-[hydroxyimino methyl]-1-methylpyridinium chloride; HI-6, 1-(2-hydroxy-iminomethyl-1-pyridinium)-1-(4-carboxy-aminopyridinium)-dimethyl ether hydrochloride; HLö7, (1-[[[4-(aminocarbonyl) pyridinio] methoxy]methyl]-2,4-bis [(hydroxyimino) methyl] pyridinium dimethanesulfonate); PAS, peripheral anionic site; AS, anionic sub-site or acyl pocket; ES, esteratic sub-site or choline binding site.

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the methyl group of methylphosphonate (Artursson et al., 2013); and (3) the peripheral anionic site (PAS), which plays a significant role in excess substrate concentration kinetics, is located at the rim of the gorge and consists of residues (Y72, D74, Y124, W286, and Y341).

The use of site-directed mutagenesis technology to introduce single or multiple changes in amino acid residues in different regions of AChE has improved our understanding of the inhibition of AChE by OPs and the aging and oxime reactivation of OP-inhibited AChE (Ashani et al., 1995; Wong et al., 2000; Kovarik et al., 2004, 2006, 2007; Taylor et al., 2007; Artursson et al., 2009; Küçükkilinç et al., 2010; Luo et al., 2010a, 2010b; Cochran et al., 2011). For example, it was first observed that amino acid residues W286, Y124, Y72 and D74 located at the PAS of mouse AChE, influenced reactivation kinetics of HI-6 (Ashani et al., 1995). In studies with mouse AChE, reactivation of the double mutant F295L/Y337A AChE inhibited with the OP compound cycloheptyl methylphosphonyl thiocholine, was enhanced by HI-6 (Kovarik et al., 2004, 2007). Taylor et al. (1999) found that by mutating Phe to smaller aliphatic residues at positions 295 and 297, reactivation was enhanced 10- to 20-fold because this allowed greater access of the oxime. Küçükkilinç et al. (2010) showed that double mutants of Hu AChE, which contain substitution of an active center residue besides the peripheral site residue D134 had 20 times slower inhibition by paraoxon and similar magnitude of slowdown in 2-PAM reactivation of the inhibited enzyme. Cochran et al. (2011) designed a Hu AChE Y337A/F338A double mutant that showed enhanced reactivation rates over wild-type AChE and displayed no apparent aging when analogs of soman, sarin and VX were used as OP inhibitors. In fact, Cochran et al. suggested that the Hu AChE double mutant Y337A/F338A in the presence of an oxime could be considered as a viable bioscavenger candidate instead of butyrylcholinesterase (BChE) because the AChE-OP conjugate with this double mutant does not display 'aging'.

X-ray crystallographic studies of various oxime-AChE and oxime-AChE•OP complexes have also provided a deeper insight into the interaction of oxime with OP-inhibited AChE (Ekström et al., 2006, 2009; Sanson et al., 2009). Ekström et al. (2009) investigated the interaction of mouse AChE•sarin conjugate (non-aged) with HI-6 and showed that the amide containing ring in HI-6 has sandwich stacking between W286 and Y124 of AChE. Using *Torpedo californica* (*Tc*) AChE conjugated with soman, Sanson et al. showed that the mutation of F331 to Ala decreased the rate of aging by 160-fold, whereas the mutation of F330 to Ala did not show a similar effect (Sanson et al., 2009). Residues F330 and F331 in *Tc* AChE correspond to residues Y337 and F338 in Hu AChE, respectively. The F331 to Ala mutation (which is in the acyl pocket) widens the cavity into which the imidazole ring of the catalytic His residue can move, thereby showing the potential role that amino acid residues in the acyl pocket can play in 'aging' and reactivation of OP-inhibited AChE (Sanson et al., 2009). However, it must be noted that most of these studies were conducted with AChEs from animal species such as *Tc*, bovine, and mouse.

To aid the development of treatment for nerve agent poisoning for humans, a thorough understanding of oxime reactivation of OP-inhibited Hu AChE is needed. Knowledge must also be gained using OP nerve agents, as well as their analogs. And as Worek et al. (2012) noted, kinetic investigations of oxime-AChE•OP interactions need to be pursued. Toward this effort, we generated Hu AChEs containing the following mutations: at the PAS, which includes mutants-Y72N, D74G, Y124Q, Y124F, W286A, and Y72N/Y124Q/W286A; at the AS, which includes mutants-F295L and F297V; and at the ES, which includes mutants-E202Q and Y337A (see Fig. 1 and Table 1). These mutant enzymes were used to study the effect of changes on the reactivation potency for each oxime. The results of our study highlight the importance of PAS residues in the reactivation of OP-inhibited AChE by bis-pyridinium oximes and Y124 for HI-6 and HLö-7 and substantiate the similarity of Hu AChE to bovine and mouse AChEs.

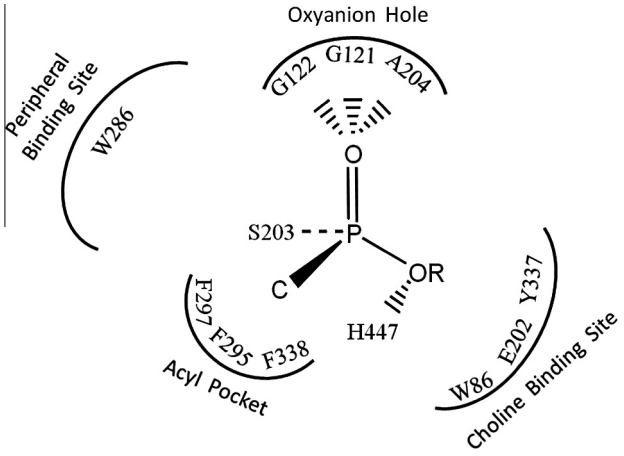

Fig. 1. A schematic representation of AChE showing the active site, which comprises two sub-sites: the anionic and the esteratic sub-sites. Shown are the acyl pocket (also referred to as the catalytic anionic sub-site), the peripheral anionic site and the choline binding site (also referred to as the esteratic sub-site). The oxyanion hole is also indicated.

Table 1
Amino acid residues mutated in this study.

Amino acid	Location	Role	Substitution
F295	AS	Substrate specificity	L
F297	AS	Substrate specificity	V
E202	ES	Excess substrate inhibition	Q
Y337	ES	Orientation and binding of substrate	A
Y72	PAS	Excess substrate inhibition, allosteric modulation of activity	N
D74	PAS	Excess substrate inhibition, allosteric modulation of activity	G
Y124	PAS	Excess substrate inhibition, allosteric modulation of activity	Q, F
W286	PAS	Excess substrate inhibition, allosteric modulation of activity	A

2. Materials and methods

2.1. Cloning of wild-type Hu AChE

A plasmid construct (graciously provided by Dr. Oksana Lockridge of the University of Nebraska) containing the coding sequence for Hu AChE, as described by Soreq et al. (1990), was cloned into the pGS plasmid vector. The pGS clone was used as the DNA template for subsequent PCR amplification of the Hu AChE coding sequences, using the Phusion High-Fidelity PCR kit protocol (New England Biolabs, Beverly, MA). The PCR fragments were isolated and sub-cloned into the mammalian expression vector pTriEx4-neo (EMD Bioscience/Novagen, San Diego, CA) using the manufacturer's specifications.

2.2. Sequencing

For sequence determination, the ABI PRISM Big Dye version 3.1 Ready Reaction Terminator Cycle Sequencing Kit from Life Technologies/Applied Biosystems (Foster City, CA) was utilized according to the manufacturer's instructions and run on the ABI