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Investigating the structural influence of surface mutations on acetylcholinesterase inhibition by organophosphorus compounds and oxime reactivation

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ABSTRACT

Organophosphates (OPs) exert their toxicity by inhibiting primarily acetylcholinesterase (AChE) and to a lesser extent butyrylcholinesterase (BChE). Binary mixtures of mammalian AChE and oximes of varying structure have been recently considered for treatment of OP poisoning as catalytic bioscavengers. In this study wild type human AChE and human AChE with residue mutations D134H, D134H_E202Q and D134H_F338A were characterized and investigated for inhibition by OPs and consequent oxime reactivation of phosphylated enzymes. The rationale for selecting these substitution positions was based on D134H being a naturally occurring single nucleotide polymorphism (SNP) in humans and that E202Q and F338A mutations slow aging of OP inhibited AChEs.

Inhibition of D134H by paraoxon and analogues of cyclosarin was 2–8 times slower than inhibition of wild type (wt), while reactivation of the paraoxon inhibited enzyme by 2PAM was 6 times faster. Both inhibition and reactivation of D134H_E202Q and D134H_F338A double mutants were up to two orders of magnitude slower than the wt indicating that introduction of the active center substitutions abolished fully the effect of the peripherally located D134H. These results indicate that selected residues outside the active center influence inhibition, reactivation and catalysis rates through longer range interactions.

1. Introduction

To minimize the toxicity of organophosphate (OP) inhibitors, binary mixtures of mammalian AChE and oximes of varying structure were recently considered as catalytic bioscavengers promoting the catalysis of the OP in plasma before it reacts with the target site in skeletal muscle or the nervous system [1]. Our goal is to generate site-specific mutations in AChE protein sequence in order to convert human AChE into catalytic scavenger that, when coupled with an oxime, will inactivate multiple OP molecules per one molecule of scavenger.

Human AChE wild type and D132H, D134H_E202Q, and D134H_F338A mutants were characterized and investigated for inhibition by OPs (an insecticide and nerve agent analogues; Fig. 1) and reactivation of the phosphylated enzymes with 2PAM and HI6. The rationale for selecting these substitution positions was based on D134H being a naturally occurring SNP in humans and that E202Q and F338A mutations slow aging of OP inhibited AChEs [2,3].

2. Materials and methods

Nerve agent analogues of cyclosarin (methylphosphonic acid 3-cyano-4-methyl-2-oxo-2H-coumarin-7-yl ester cyclohexyl ester), VX (methylphosphonic acid 3-cyano-4-methyl-2-oxo-2H-coumarin-7-yl ester ethyl ester), soman (methylphosphonic acid 3-cyano-4-methyl-2-oxo-2H-coumarin-7-yl ester pinacolyl ester) were synthesized as previously described [4], as well as S_P-Cycloheptyl methyl phosphonyl thiocholine (S_PCHMP) and R_P-Isopropyl methyl phosphonyl thiocholine (R_PIPMP) [5]. O,O-Diethyl O-(4-nitrophenyl) phosphate (paraoxon) and the oxime 2PAM were purchased from Sigma–Aldrich (St. Louis, MO). The oxime

Abbreviations: OPs, organophosphates; AChE, acetylcholinesterase; hAChE, human AChE; BChE, butyrylcholinesterase; SNP, single nucleotide polymorphism; wt, wild type; ATCh, acetylthiocholine; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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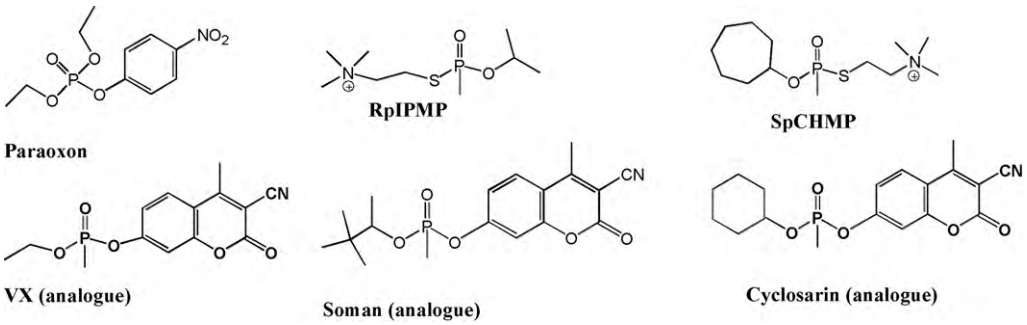

Fig. 1. Structures of OP inhibitors used in this study.

HI6 was purchased from US Biological (Swampscott, MA). Oximes were used in a range of 0.05–10 mM. Final concentrations of organic solvents were less than 1% in enzyme assays.

HEK-293 cells were transfected with the hAChE D134H DNA construct, prepared as described for the mouse AChE [6], with exception that hAChE constructs had FLAG peptide sequence encoded at the 5′ end of the construct. The stable cell clones were selected by treatment with G-418. The protein was purified in mg quantities by adsorption and desorption from an antiFLAG peptide antibody resin.

In reactivation experiments wild type and mutant enzymes (40 nM) were inhibited for 10–30 min until inhibition was greater than 95%. Inhibited enzyme and the control were passed through two consecutive Sephadex G-50 spin columns to remove excess unreacted inhibitor. Inhibited and control enzymes were incubated with oxime and time courses of recovery of hAChE activity were measured in aliquots of reactivation and control reaction mixtures by spectrophotometric Ellman assay (at 22 °C, in 100 mM phosphate buffer, pH 7.4, containing 1 mM ATCh as substrate and 333 μM DTNB as final concentrations) Detailed description of experimental procedures and calculations is given elsewhere [6]. Inhibition kinetic experiments were conducted as described before [7] using final OP concentrations between 10 nM and 50 μM.

Table 1
Relative inhibition rates of hD134H wt; hD134H_E202Q and hD134H_F338A.

Enzyme	Paraoxon	VX analogue	Cyclosarin analogue	Soman analogue
hAChE wt[a]	1	1	1	1
hD134H	0.4	1	0.1	1
hD134H_E202Q	0.5	0.2	0.01	0.04
hD134H_F338A	0.1	1	0.2	0.3

The second-order inhibition rate constants of hAChE wt with paraoxon and analogues of VX, cyclosarin and soman were 330×10^4, 290×10^4, 580×10^4 and 60×10^4 M^{-1} min^{-1}, respectively. Inhibition by S_PCHMPTCh, R_PIPMPTCh was not measured.

3. Results and discussion

Inhibition of human D134H with paraoxon and an analogue of cyclosarin was 2–8 times slower than that of human wt (Table 1), whereas inhibition by soman and analogues of VX was not affected by D134H substitution. Introduction of additional substitutions in two double mutants further slowed inhibition up to two orders of magnitude.

2PAM reactivation of paraoxon inhibited human D134H is, on the other hand, 6 times faster than human wt (Table 2). Our previous study indicated that differential occupation of the acyl pocket

Table 2
Relative reactivation rates of OP inhibited hD134H; hD134H_E202Q and hD134H_F338A.

hAChE	OP	OXIME	Relative reactivation rate
wt[a]	Paraoxon	2PAM	1
D134H			6
D134H_E202Q			0.1
D134H_F338A			1
wt[b]		HI6	1
D134H			3
wt[c]	S_PCHMPTCh	2PAM	1
D134H			2
wt[d]		HI6	1
D134H			0.3
D134H_E202Q			0.02
D134H_F338A			0.002
wt[e]	VX analogue	2PAM	1
D134H			1
wt[f]	R_PIPMPTCh	2PAM	1
D134H			1

The second-order rate constants for reactivation of OP inhibited wt hAChE by oximes were as follows.
[a] 120 M^{-1} min^{-1} (paraoxon inhibited wt hAChE reactivated by 2PAM).
[b] 45 M^{-1} min^{-1} (paraoxon inhibited wt hAChE reactivated by HI6).
[c] 275 M^{-1} min^{-1} (S_PCHMPTCh inhibited wt hAChE reactivated by 2PAM).
[d] 13,000 M^{-1} min^{-1} (S_PCHMPTCh inhibited wt hAChE reactivated by HI6).
[e] 490 M^{-1} min^{-1} (VX inhibited wt hAChE reactivated by 2PAM).
[f] 270 M^{-1} min^{-1} (R_PIPMPTCh inhibited wt hAChE reactivated by 2PAM). Reactivation of cyclosarin and soman inhibited hAChE was not measured.

in mouse AChE, by covalent ligands of varying geometry, had distinct effects on spectra of fluorophores covalently attached on the AChE surface in the general spatial vicinity of residue D134 [8]. In order to determine whether the acyl pocket stabilized ethoxy substituent on phosphorus in diethylphosphorylated hAChE was primarily responsible for the observed enhancement of reactivation rates, the enzymes were inhibited with an excess of racemic VX analogue. Inhibition was assumed to yield wt and D134H AChEs conjugated primarily by the S_P VX enantiomer with methylphosphonyl substituent of the conjugate stabilized in the acyl pocket and the ethoxy substituent oriented towards the choline binding site. Reactivation of both wt and D134H hAChE S_P VX conjugates with 2PAM showed similar reactivation rates. This suggests that the 2PAM reactivation rate enhancement observed in the diethylphosphorylated (paraoxon inhibited) D134H mutant may be linked with improved stabilization of the ethoxy substituent in the mutant acyl pocket and is consistent with the observed three-fold enhancement of the HI6 reactivation rate (Table 2). To verify this hypothesis further 2PAM reactivation of the R_PIPMP inhibited enzyme conjugates (identical to R_P Sarin conjugated AChE) was studied but yielded no effect of D134H mutation. Thus, our experiments implicate differential flexibility of the acyl pocket in D134H substituted hAChE, but the actual mechanism of the 2PAM reactivation enhancement remains unclear.

Human D134H_E202Q and D134H_F338A non-aging double mutants did not reactivate any faster than the wt or the hD134H mutant (Table 2). In fact, of all tested enzymes the one most compromised with mutations was D134H_E202Q mutant. It showed both inhibition and reactivation rates 20 times slower in comparison to wt and D134H (Tables 1 and 2). The characteristics of double mutants mostly arose from E202Q mutation that has slow phosphonylation and reactivation rates. We were thus unable to combine resistance to aging (coming from E202Q or F338A substitutions) and enhanced reactivation (coming from D134H substitution) in the same double mutant hAChE protein.

In summary, our initial study shows that selected surface residue substitutions in the AChE molecule located remotely from the active center affect its catalytic parameters, OP inhibition and oxime reactivation and may be useful in designing oxime-hAChE couples for catalytic OP hydrolysis. However, combining diverse properties of single site mutations to form multiple site hAChE mutants does not result in a simple summation of ΔG values, rather there is a coupling of energy values that appear linked.

Conflict of interest statement

None declared.

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