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Engineering resistance to 'aging' of phosphylated human acetylcholinesterase

Role of hydrogen bond network in the active center

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Recombinant human acetylcholinesterase (HuAChE) and selected mutants (E202Q, Y337A, E450A) were studied with respect to catalytic activity towards charged and noncharged substrates, phosphylation with organophosphorus (OP) inhibitors and subsequent aging of the OP-conjugates. Amino acid E450, unlike residues E202 and Y337, is not within interaction distance from the active center. Yet, the bimolecular rates of catalysis and phosphylation are 30–100 fold lower for both E450A and E202Q compared to Y337A or the wild type and in both mutants the resulting OP-conjugates show striking resistance to aging. It is proposed that a hydrogen bond network, that maintains the functional architecture of the active center, involving water molecules and residues E202 and E450, is responsible for the observed behaviour.

Organophosphorus inhibitor; Mutagenesis; Serine hydrolase

1. INTRODUCTION

Cholinesterases (ChE's) are readily phosphylated**, at the active site serine, by a variety of organophosphonates and organophosphates (OP) [1,2]. The OP-ChE conjugates then undergo postinhibitory processes, the nature and extent of which depend upon the structure of the inhibitor and the particular enzyme studied. Spontaneous reactivation, through displacement of the phosphyl moiety (Scheme 1) from the active site, is usually very slow [3]; however, the enzyme can be reactivated by various oxime nucleophiles ('Re' in Scheme 1) [1,4]. In certain cases such reactivation is thwarted due to a concomitant unimolecular process termed aging [1,5]. It was proposed that this process consists of dealkylation through alkyl–oxygen bond scission resulting in a formal negative charge in the OP–ChE conjugate (see Scheme 1) [6,7]. The aged OP-conjugate is refractive to reactivation [6,8] and thus renders treatment, following intoxication with certain OP insecticides or nerve gas agents, extremely difficult [9].

Although the aging process is understood on the basis of a carbonium ion mechanism [10] and depends on the phosphorus stereochemistry in the conjugates [11], the role of the enzyme environment in facilitating the process is not well understood. In general, the catalysis of aging is particularly efficient in ChE's compared to OP-conjugates with other serine hydrolases [12,13], indicating a specific involvement of residues vicinal to the phosphyl moiety in the ChE active center.

Recently we have generated a large number of recombinant human acetylcholinesterase (HuAChE) mutants and identified amino acids important for maintaining the structural integrity of the enzyme [14,15] as well as residues involved in catalysis and in interactions with various substrates and reversible inhibitors [16–18]. In these studies, active center residues comprising the catalytic triad, the anionic subsite and the acyl pocket, as well as residues of the peripheral anionic subsites were identified. Here we report on the extension of this approach to gain insight into reactions with irreversible OP inhibitors and the subsequent postinhibitory processes.

Comparison of reaction rates of inhibition and aging of selected active center HuAChE mutants and the wild type enzyme reveals key residues involved in the generation and stabilization of the negatively charged E–P–O^- conjugate.

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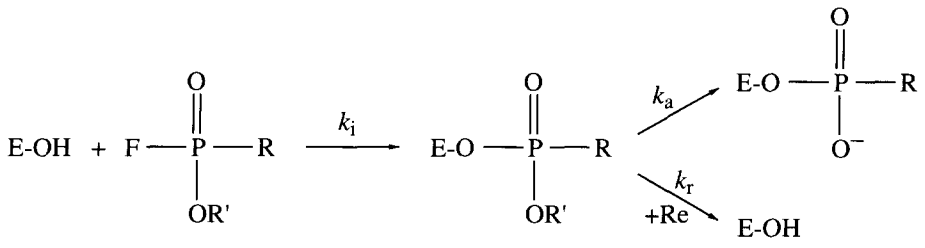
** The comprehensive term 'phosphyl' is adopted from Bourne and Williams [37] for all tetravalent P electrophilic groups.



Scheme 1

2. MATERIALS AND METHODS

2.1. *Substrates and inhibitors*

Acetylthiocholine iodide (ATC), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), diisopropyl phosphorofluoridate (DFP) were purchased from Sigma. *S*-3,3-dimethylbutyl thioacetate ($CH_3C(O)SC_2H_4C(CH_3)_3$-TB) was synthesized and purified as described previously [18]. Purified pinacolyl methylphosphonofluoridate (soman) was a gift from Dr. Y. Segall; 7-(methylethoxyphosphophinylloxy)-1-methylquinolinium iodide (MEPQ) a gift from Dr. Y. Ashani; and 1-(2-hydroxyiminomethylpyridinium)-1-(4-carboxyiminopyridinium)dimethylether dichloride (HI-6) was a gift from Dr. G. Amitai.

2.2. *Recombinant HuAChE and its mutants*

Expression of recombinant HuAChE and its mutants in a human embryonal kidney derived 293 cell line, was described previously [15,19,20]. Generation of mutants E202Q(*E199*)* and Y337A(*F330*) was described previously [18]. Substitution of residue E450(*E443*) was carried out by replacement of the *Bst*BI-*Bam*HI DNA fragment of the AChE-w3 variant [15] with synthetic DNA duplexes carrying the GCC(Ala) codon. Stable recombinant cell clones expressing high levels of each of the mutants were established according to the procedure described previously [20]. The resulting recombinant clones were propagated in multitray systems [21]. The secreted enzymes in the cell supernatant (2–6 liters) were purified (over 90% purity) by affinity chromatography as described previously [20].

2.3. *Kinetic studies and analysis of data*

AChE activity was assayed according to Ellman et al. [22] (in the presence of 0.1 mg/ml BSA, 0.3 mM DTNB, 50 mM sodium phosphate buffer pH 8.0), carried out at 27°C and monitored by a Thermomax microplate reader (Molecular Devices). Michaelis-Menten constant (K_m) values were obtained from the double-reciprocal Lineweaver-Burk plots and k_{cat} calculations were based on ELISA quantitations [17] as well as on determination of active site concentration by MEPQ titrations [14,23] (see Fig. 2). Phosphylation experiments were carried out using at least 4 different concentrations of OP-inhibitor (I) and two concentrations of enzyme, and enzyme residual activity (E) at various times was monitored. The apparent bimolecular phosphylation rate constants (k_i in Scheme 1) determined under pseudo-first-order conditions were computed from the plot of slopes of ln(E) vs. time at different inhibitor concentrations. Rate constants under second-order conditions were determined from plots of $\ln\{E/[I_o - (E_o - E)]\}$ vs. time. In aging experiments the initial OP-conjugates were obtained under conditions where $k_i[I_o] \geqslant k_a$ (see Scheme 1) and with over 98% inhibition of the initial enzyme activity. The reactivatable (non-aged OP-conjugate) fraction was determined by reactivation with HI-6 under conditions where $k_r[Re] > k_a$ (Scheme 1). The excess OP-inhibitor was removed either by column filtration (Sephadex G15) or by 100-fold dilution, prior to reactivation. The activity of the reactivated enzyme (E_r) was routinely corrected for the inhibitory effect of the reactivator [24]. The first-order rate constants of aging, k_a, were determined from the slopes of $\ln(E_r)$ vs. time.

3. RESULTS

3.1. *Selection of OP inhibitors and HuAChE mutants for study*

The OP inhibitors chosen for this study represent two important classes of extensively studied agents [3]: diisopropyl phosphorofluoridate (DFP) and pinacolyl methylphosphonofluoridate (soman). Both of these OP inhibitors result in OP–ChE conjugates that undergo aging [1]. For the selection of mutants we were guided by molecular models of OP–HuAChE adducts [25], based on the X-ray structure of TcAChE [26] and on structures of OP-conjugates of chymotrypsin [27]. Initially, residues Y337 and E202 were selected since the main focus of this study is the evaluation of interactions of the active center residues with the initial phosphyl moiety and in particular with the alkyl leaving group and the resulting P-O^- moiety in the aged conjugate

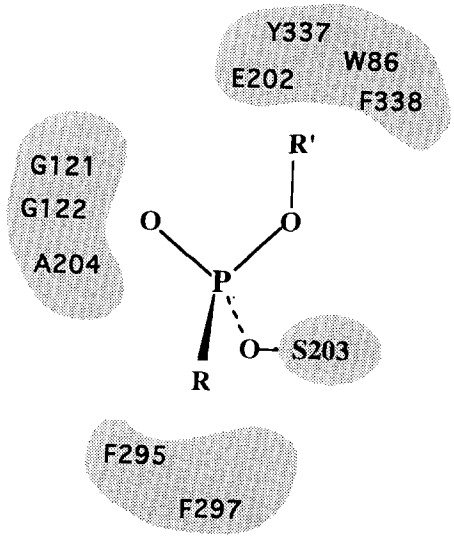


Fig. 1. Schematic presentation of the OP–HuAChE conjugate. Residue domains within interaction distances [25] from the phosphorus substituents are displayed, as well as the catalytic site serine 203.

* Amino acids and numbers in parentheses refer to the position of analogous residues in *Torpedo* AChE (TcAChE) according to the recommended nomenclature [38].

(Fig. 1). The models suggest that amino acid Y337 is vicinal to the isopropyl (DFP) or the pinacolyl (soman) groups of the phosphyl moieties of the corresponding OP–HuAChE conjugates and residue E202 is proximal to the scissible O–alkyl bond. Indeed, Qian and Kovach [28] proposed recently that E202 may be involved in stabilization of the evolving carbonium ion during the dealkylation process. In addition to mutation in these two residues, we included in the study HuAChE mutated at position 450. Residue E450, is remote from the phosphyl moiety but may affect the positioning of E202 in the active center (see Fig. 3 and discussion).

3.2. *Effect of mutation on hydrolysis of charged and uncharged substrates*

The three HuAChE mutants Y337A, E202Q and E450A were compared with respect to their efficiency in catalyzing the hydrolysis of acetylthiocholine (ATC) and its uncharged isoester *S*-3,3-dimethylbutyl thioacetate (TB). As shown previously [18], replacement of the aromatic residue Y337 had only a marginal effect on the kinetic parameters (K_m and k_{cat}) for the two substrates (Table I) while the corresponding values for hydrolysis of ATC catalyzed by E202Q HuAChE [16], or of the corresponding mutation E199Q in TcAChE [29], differ significantly from those of the wild type enzyme. We now demonstrate that this decrease in catalytic efficiency of E202Q is observed also for the noncharged substrate TB (Table I). This indicates that the effect of replacement of E202 of HuAChE on catalysis is not solely due to electrostatic interactions with the substrate. Interestingly, a similar pattern of reduction of the catalytic parameters, for both ATC and TB, is observed when residue E450, which is remote from the active site, is replaced by alanine (Table I).

3.3. *Interaction of HuAChE mutants with irreversible OP inhibitors*

To determine the effects of mutations on the stoichiometry of the inhibition reaction of the various AChEs by the OP inhibitors the recombinant enzymes were titrated with different concentrations of soman and MEPQ. The organophosphonate MEPQ is a potent

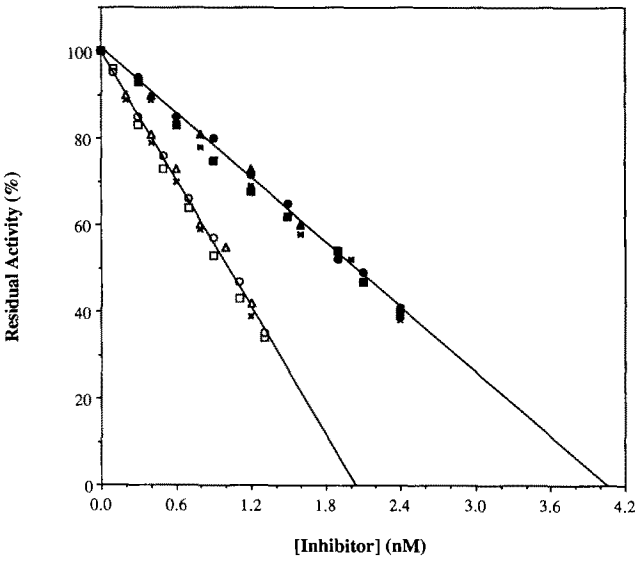


Fig. 2. Active site titration of HuAChE and its mutants with soman and MEPQ. In all titration experiments the concentration of the catalytic subunits of HuAChE and its mutants was approximately 0.12 μg/ml as determined by ELISA. The residual activity was determined after 30 and 60 min incubation (27°C) with soman (WT (■), Y337A (●), E202Q (▲) and E450A (+)) and MEPQ (WT (□), Y337A (○), E202Q (△) and E450A (×)). The intercepts for zero activity were determined by extrapolation and the values of active site concentration were approximately 2.05×10^{-12} mol/ml for MEPQ and twice as high for soman titration (see text).

OP-inhibitor of AChEs, commonly used as titrant for standardization of concentration of AChE active sites [23]. As shown in Fig. 2, the wild type enzyme and all the mutants examined show the expected 1:1 stoichiometry in reactions with MEPQ. The concentrations of the active site subunits, determined by this method, are in good agreement (± 10%) with results obtained from the quantitative immunological (ELISA) assays. Furthermore, under similar experimental conditions, the titration studies demonstrate that for soman 1:2 stoichiometry is obtained for the wild type enzyme as well as for the mutants examined. Due to the chirality of the phosphorous in soman, this OP-inhibitor is known to exhibit a marked stereoselectivity in reactions with AChE from

Table I

Compilation of kinetic constants for ATC and TB hydrolysis by HuAChE and its mutants

HuAChEs	ATC			TB		
	K_m (mM)	k_{cat} ($\times 10^{-5}$ min^{-1})	k_{app}[a] ($\times 10^{-8}$ M^{-1} min^{-1})	K_m (mM)	k_{cat} ($\times 10^{-5}$ min^{-1})	k_{app}[a] ($\times 10^{-8}$ M^{-1} min^{-1})
Wild type	0.13 ± 0.015	3.7 ± 0.3	28.5	0.28 ± 0.08	0.5 ± 0.1	1.8
Y337A	0.10 ± 0.01	1.0 ± 0.1	10.0	0.20 ± 0.03	0.2 ± 0.05	1.0
E202Q	0.35 ± 0.04	0.75 ± 0.15	2.1	0.23 ± 0.03	0.1 ± 0.04	0.43
E450A	0.35 ± 0.04	0.10 ± 0.02	0.3	0.20 ± 0.04	0.006 ± 0.001	0.03

[a]The apparent bimolecular rate constant (k_{app}) was calculated from the ratio of k_{cat}/K_m.

various sources [30], including recombinant HuAChE [14]. Thus, the HuAChE mutants studied appear to retain the stereoselectivity towards soman diastereomers.

The actual bimolecular reaction rates of phosphylation of HuAChE and its mutants, by DFP and soman, were determined under either pseudo-first-order or second-order conditions (Table II). For the wild type enzyme the rates obtained for DFP and soman are in agreement with values published previously for different AChEs [31–33]. It appears that replacement of Y337 by alanine had a minimal effect on the bimolecular rate constants of inhibition. On the other hand, replacement of E202 by glutamine resulted in a marked decrease of the phosphylation rates. Radic et al. have recently reported similar effects for the reactivity of an analogous mutant of TcAChE (E199Q) with DFP [29]. Again, as for the acylation process, we find that the effect of replacement of E450 on the rates of phosphylation (Table II), parallels that of E202 substitution. It is worth noting that despite the marked decrease in the rates of phosphylation of the HuAChE E202Q and E450A mutants, the relative reactivity of DFP and soman with each mutant resembles that observed for the wild type enzyme.

Examination of the process of aging through measurements of the residual reactivatable enzyme activity [24] was carried out with the potent oxime reactivator HI-6 [34]. Under the experimental conditions used, we were able to achieve substantial regeneration of enzymatic activity, even for the pinacolyl methylphosphonyl derivative of the wild type HuAChE which proceeds very rapidly towards the non-reactivatable (aged) enzyme. It appears that reactivation of OP conjugates with the wild type HuAChE and Y337A enzymes proceeds at comparable rates while for the E202Q and E450A mutants, reactivation is slower (data not shown).

Table II

Rate constants of phosphylation (k_i) and aging (k_a) of HuAChE and its mutants

	k_i ($\times 10^{-4}$ $M^{-1} \cdot min^{-1}$)		k_a ($\times 10^3$ min^{-1})
HuAChEs	DFP[a]	soman[b]	soman[c]
Wild type	9.6 ± 0.52 (1.0)	8600 ± 1970 (1.0)	91 ± 12 (1.0)
Y337A	3.1 ± 0.15 (0.32)	2950 ± 636 (0.34)	180 ± 64 (2.0)
E202Q	0.18 ± 0.06 (0.019)	312 ± 100 (0.036)	0.58 ± 0.06 (0.0064)
E450A	0.085 ± 0.03 (0.009)	120 ± 20 (0.014)	3.4 ± 0.18 (0.037)

The correlation coefficients of the corresponding linear plots were at least 0.95. Values represent mean of at least 3 independent experiments. Numbers in parentheses represent the values relative to the wild type.

[a] Concentration range of DFP: for wild type and Y337A 5×10^{-7}–3×10^{-6} M and for E202Q and E450A 5×10^{-6}–7.5×10^{-5} M.

[b] Concentration range of soman: for wild type and Y337A 4×10^{-10}–1.4×10^{-8} M and for E202Q and E450A 1.4×10^{-8}–2.8×10^{-7} M.

[c] All enzymes were 95–98% inhibited by soman and reactivated at various times by HI-6 ($1–5 \times 10^{-4}$ M).

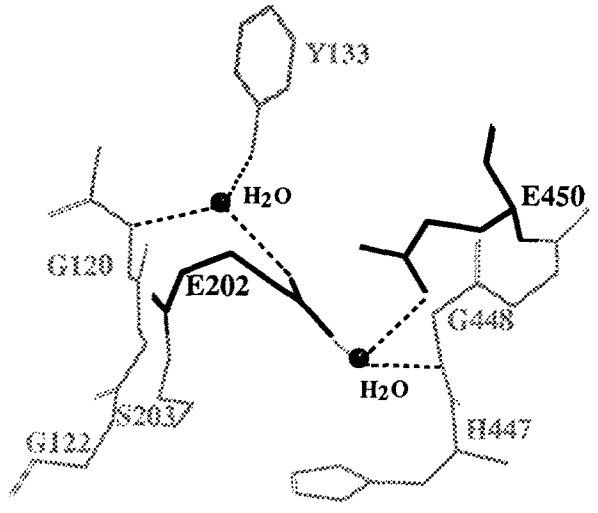


Fig. 3. Involvement of residues E202 and E450 in the hydrogen bond network across the active center of HuAChE. The positions of water molecules in the model of the HuAChE are assumed to be equivalent to those in the X-ray structure of TcAChE [26]. Segments of the two backbone stretches, spanned by the H-bond network, are displayed. The separation of the two backbone segments determines the relative orientation of the oxyanion hole (G121, G122) and the catalytic triad (H447). The distances within the network, marked by broken lines, are as follows: N(G121)–O(H_2O) 2.58 Å; O(Y133)–O(H_2O) 2.50 Å; O(E202)–O(H_2O) 3.01 Å; O(E450)–O(H_2O) 2.80 Å; O(E202)–O(H_2O) 2.36 Å; N(G441)–O(H_2O) 3.07 Å.

The first-order rate constant for aging of the diisopropyl phosphoryl derivative of HuAChE was determined to be 3.5×10^{-4} min^{-1}. This value is 260-fold lower than that for the corresponding pinacolyl methylphosphonyl derivative (Table II), and in good agreement with values published for the same OP-conjugates in other AChEs [24,32]. For the diisopropyl phosphoryl derivatives of E202Q and E450A HuAChE's the rates of aging appear to be very slow and not measurable under the experimental conditions used. Consequently, there is no experimental evidence that the aging process occurs in these cases. For the pinacolyl methylphosphonyl derivative of the Y337A, the first-order rate constant for aging is similar to that of the wild type enzyme while the corresponding values for E202Q and E450A are 150- and 30-fold lower, respectively. If we assume a similar decrease in the rate of aging for the diisopropyl phosphoryl derivatives of E202Q and E450A, then we could expect half-lives of 83 and 17 days, respectively, for these processes. It is therefore not surprising that no aging could be observed with DFP-conjugates of these mutants.

4. DISCUSSION

Molecular modeling studies of HuAChE and its phosphonylated conjugates implicated several residues, at the active center, in stabilizing the initial OP conjugate (Fig. 1) and in facilitating the subsequent aging process. These residues include the catalytic triad (S203, H447 and E334), the putative oxyanion hole (G121, G122 and A204), the pockets accommodating the alkoxy groups (W86, Y337 and F338), and residues which constitute the acyl pocket for ACh (F295 and F297) as well as E202 which is proximal to the P–O–C linkage. The participation of most of these residues in the analogous process of acylation was recently demonstrated experimentally, by site directed mutagenesis [15–17,29,35]. The kinetic studies reported here suggest that in phosphylation and even in the dealkylation of the OP conjugates of HuAChE, residue Y337 appears to have a minimal contribution. On the other hand replacement of E202 by a neutral residue results in 30–150-fold reduction in the bimolecular rate constant of phosphylation and in the first-order rate constant of aging.

Based on modeling studies of the soman-TcAChE adduct it was proposed by Qian and Kovach that the carboxylate of E199 (equivalent to E202 in HuAChE) would be within interaction distance of the positive charge on the chiral carbon, thereby facilitating the detachment of the alkyl group by means of electrostatic interactions [28]. However, it should be emphasized that substitution of E202 by glutamine has pleiotropic effects of comparable magnitude on catalytic activity towards both charged and non-charged substrates (Table I), on substrate inhibition [16,29], on interactions with reversible inhibitors [17,29] as well as on the rates of phosphylation by DFP and soman (Table II). These observations are therefore consistent with our previous suggestion [16,17] that replacement of E202 probably disrupts the *overall* spacial organization of the active center. Nevertheless this conformational change is not sufficient to abolish the stereoselectivity of the E202Q mutant for the soman diastereomers (Fig. 2).

Some clues as to how residue E202 could play such a role in maintaining the architecture of the active center is provided by the studies by Sussman et al. [26] on the X-ray structure of TcAChE. As shown in Fig. 3, the carboxyl group of E202 is a central element in a network of hydrogen bonds that spans the cross-section of the active center gorge. This array bridges two AChE backbone segments one of which contains the residues constituting the oxyanion hole while the other includes the catalytic triad histidine (H447). In this array, residue E202 is bound, via a water molecule, to residue E450. Therefore, although E450 is quite remote from the active site (distances of Oγ-S203 from Cδ of E202 and E450 are 5.29 Å and 9.72 Å, respectively), it is expected that substitution of E450 by alanine would be as disruptive to the hydrogen bond network as is the replacement of E202 by the neutral residue glutamine. In accordance with this prediction, the E450A HuAChE mutant exhibited reduced reactivity towards the substrates ATC and TB, or towards the OP inhibitors DFP and soman, in a similar manner to that observed for the mutant E202Q. Furthermore the OP conjugates of both E202Q and E450A mutants exhibit marked resistance to aging. A common mechanistic element, in these diverse processes, is a protonation step. This protonation can be mediated by the imidazole moiety of the catalytic triad histidine-447, and it is reasonable to assume that the appropriate positioning of H447 is achieved (Fig. 3) via the hydrogen bond network involving E202–water–E450 alignment in the active center.

Finally in addition to understanding some of the basic aspects of the mechanism of catalysis, phosphylation and aging, the findings reported here may have practical implications. Since aging is an irreversible process leading to a nonreactivatable enzyme, the demonstrated ability to engineer ChEs resistant to aging should be advantageous with respect to the proposed utility of such biomolecules for treatment [36] against OP insecticide poisoning or decontamination of environmentally harmful OP agents.

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