Aging of phosphorylated human acetylcholinesterase: catalytic processes mediated by aromatic and polar residues of the active centre

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We have examined the effects of 11 substitutions of active centre gorge residues of human acetylcholinesterase (HuAChE) on the rates of phosphorylation by 1,2,2-trimethylpropyl methylphosphonofluoridate (soman) and the aging of the resulting conjugates. The rates of phosphorylation were reduced to as little as one-seventieth, mainly in mutants of the hydrogen-bond network (Glu-202, Glu-450, Tyr-133). These recombinant enzymes as well as the F338A, W86A, W86F and D74N mutant HuAChEs varied in their resistance to aging (15–3300-fold relative to the wild type). The most dramatic resistance to aging was observed for the phosphonyl conjugate of the mutant W86A enzyme (1850–3300-fold relative to the wild type). It is proposed that Trp-86 contributes to the aging process by stabilizing the evolving carbonium ion on the 1,2,2-trimethylpropyl moiety, via charge– π interaction. The rate-enhancing effect of Trp-86

provides a rationale for the unique facility of aging in soman-inhibited cholinesterases, compared with the corresponding conjugates in other serine hydrolases. Replacements of Glu-202 by aspartic acid, glutamine or alanine residues resulted in a similar (1/130–1/300) decrease of the rates of aging. A comparable decrease was also observed for the conjugate of the F338A mutant. These results, and the similar pH dependence of aging rates for the wild-type and E202Q and F338A mutant HuAChEs, indicate that Glu-202 is not involved in proton transfer to the phosphonyl moiety. On the basis of these findings and of molecular modelling we suggest that Glu-202 and Phe-338 contribute to the aging process by stabilizing the imidazolium of the catalytic triad His-447 via charge–charge and charge– π interactions respectively, thereby facilitating an oxonium formation on the phosphonyl moiety.

INTRODUCTION

The inhibition of serine hydrolases such as cholinesterases (ChEs) by organophosphorus compounds involves phosphorylation of the active site serine and the formation of a stable conjugate [3,4]. Although spontaneous hydrolytic cleavage of such conjugates is usually very slow, the enzymes can still be effectively reactivated through reaction with nucleophilic agents such as quaternary oximes or fluoride ions [3,5]. In certain cases the ability to displace the phosphyl moiety decreases with time owing to a unimolecular process termed ‘aging’ (Scheme 1) [6,7]. This process, usually attributed to the loss of an alkyl group from the phosphyl alkoxy substituent, is most pronounced for branched alkyl groups [8]. The aging process of the phosphorylated acetylcholinesterase (AChE) prevents treatment and exacerbates the effects of human intoxication by organophosphorus agents, such as certain insecticides and nerve agents [4].

The rate-determining step of the aging process is probably the scission of the alkyl–oxygen bond, resulting in the formation of a carbonium ion and a formal negative charge (see Scheme 2) in the conjugate [9,10]. Although the involvement of the enzyme in facilitating this process was demonstrated long ago by the fact that aging does not occur in denatured phosphyl–AChE conjugate [11], the specific role of the enzyme is not well understood. Enzymic acceleration of C–O bond breaking through generation of a carbonium ion is not unique to ChEs; however, in other enzymes such as lysozyme, this reaction has a well-defined catalytic function in the glycosyl transfer processes [12]. Stabilization of charge separation, leading to the formation of a

carbonium ion, was recently proposed to involve the AChE active centre and in particular Glu-202(199) [13,14]. Such an involvement of Glu-202 could be consistent with the observed effects of its replacement by glutamine on the aging rate of the corresponding 1,2,2-trimethylpropyl methylphosphonyl conjugate [15,16]. However, Ordentlich et al. [15] have shown that the aging process was equally affected by replacement of Glu-450(443), which is remote from the phosphonyl moiety. Another unresolved issue related to aging is the profound resistance of the aged conjugates to reactivation. The proposed electrostatic barrier to a nucleophilic attack, due to the presence of additional negative charge [17], seems to be inconsistent with the reactivity properties of charged organophosphorus esters [18,19]. Resistance to reactivation has been explained on the basis of conformational changes taking place after aging [20,21], yet comparison of the refined X-ray structures of aged and non-aged organophosphoryl conjugates of γ -chymotrypsin showed virtually no conformational difference in the protein backbones of the two structures [22].

The role of the structure of the human acetylcholinesterase (HuAChE) active centre in facilitating reactions with organophosphate inhibitors was recently examined by a combination of site-directed mutagenesis and kinetic studies [23]. That study identified some of the residues interacting directly with the ligands and suggested that the functional architecture of the HuAChE active centre has a major role in the stabilization of the enzyme–phosphate Michaelis complexes. The same active centre residues might be involved in accelerating the aging process and could explain the more efficient aging of the phosphyl conjugates

Abbreviations used: AChE, acetylcholinesterase; ATC, acetylthiocholine; ChE, cholinesterase; DFP, di-isopropyl phosphorofluoridate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); HI-6, 1-(2-hydroxy-iminomethylpyridinium)-1-(4-carboxyimino-pyridinium) dimethylether dichloride; Hip, protonated histidine; HuAChE, human acetylcholinesterase; MEPQ, 7-[(ethoxymethylphosphinyl)oxy]-1-methylquinolinium iodide; soman, 1,2,2-trimethylpropyl methylphosphonofluoridate; TB, 3,3-dimethylbutyl thioacetate. Nomenclature: the comprehensive term ‘phosphyl’ is adopted from Bourne and Williams [1] for all tetravalent phosphorus electrophilic groups. Amino acids and numbers refer to HuAChE, the numbers in parentheses referring to the position of analogous residues in *Torpedo californica* AChE, in accordance with the recommended nomenclature [2].

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purity) either by ligand-affinity chromatography [28] or by fractionation on a monoclonal antibody affinity column [32].

Enzyme activity measurements and active site titrations

Activity of HuAChE enzymes was assayed at 27 °C (in the presence of 0.1 mg/ml BSA, 0.3 mM DTNB, 50 mM sodium phosphate buffer, pH 8.0, and various concentrations of ATC) by the method of Ellman et al. [33], and monitored by a Thermomax microplate reader (Molecular Devices).

Active site titration of enzyme solutions was performed in the presence of 0.1 mg/ml BSA in 50 mM sodium phosphate buffer, pH 8.0, by adding various amounts of phosphonate inhibitor (MEPQ or soman). Inhibition was allowed to proceed to completion and the residual activity was plotted against the concentration of inhibitor. MEPQ, a potent non-stereoselective inhibitor of ChEs [34] that reacts in a 1:1 ratio with various AChEs including the recombinant HuAChE [27], was used as a standard for the active site titrations. The concentrations of the active site subunits of all the enzymes, as determined by titration with MEPQ, were within $\pm 10\%$ of those measured by the specific ELISAs [26]. These values were used to determine the stoichiometry of the reactions of soman with the various HuAChE enzymes.

Measurements of phosphorylation rates

Kinetic measurements were made with at least four different concentrations of soman (I_0), and the enzyme residual activity (E) at various times was monitored. The apparent bimolecular phosphorylation rate constants (k_i) determined under pseudo-first order conditions were computed from the plot of slopes of $\ln E$ against time at different inhibitor concentrations. Rate constants under second-order conditions were determined from plots of $\ln\{E/[I_0 - (E_0 - E)]\}$ against time.

Measurements of aging rates

The initial soman-inhibited enzymes were obtained under conditions where the rate of phosphorylation was much greater than the rate of aging [$k_i(I_0) \gg k_a$] and with over 98% inhibition of the initial enzyme activity. The excess soman was rapidly removed either by column filtration (Sephadex G-15) or by dilution 1:1000 before reactivation. The reactivatable (non-aged soman conjugate) fraction was determined by reactivation with 0.5 mM HI-6 under conditions where the rate of reactivation was greater than the rate of aging [$k_r(\text{HI-6}) > k_a$]. The activity of the reactivated enzyme (E_r) was routinely corrected for the inhibitory effect of the reactivator [35]. The first-order rate constants of aging (k_a) were determined from the slopes of $\ln E_r$ against time.

The pH profiles of aging of HuAChE and of some of its aging-resistant mutants were examined by using the following buffer solutions (50 mM): 1,3-bis[tris(hydroxymethyl)methylamino]propane between pH 5.5 and 9.0, Tris between pH 7.0 and 9.0, and succinate between pH 4.6 and 6.0. The rates of aging could not be measured below pH 5.5 because of technical limitations (mainly enzyme stability).

Molecular modelling

Building and optimization of three-dimensional models of soman-HuAChE conjugates were performed on a Silicon Graphics workstation IRIS 70/GT with SYBYL modelling software (Tripos). The initial models were constructed as described previously for the 2-propyl analogues [36]. The models were optimized by molecular mechanics with the MAXMIN force field (and AMBER charge parameters for the enzyme) and

zone refined, including 127 amino acids (15 Å substructure sphere around O^γ-Ser-203). Optimization of the initial models included a restriction of the distances between the phosphonyl oxygen and the amide nitrogen atoms of residues Gly-121 and Gly-122 that were relieved in the subsequent refinement.

RESULTS AND DISCUSSION

Effect of selected HuAChE mutations on phosphorylation by soman

Several residues of the hydrophobic alkoxy subsite and of the hydrogen-bond network in the HuAChE active centre are involved in the phosphorylation process [23]. It is generally assumed that the electronic and steric requirements for the inhibitory processes in AChEs should be quite similar for phosphates and for phosphonates. This assumption was tested here by using selected HuAChE mutants and the phosphonylating agent soman, which yields conjugates that undergo rapid aging. Investigation of this reaction was also of interest because of the known stereoselectivity of AChE towards soman diastereomers [37]. Phosphorylation of different AChEs by the two P_s diastereoisomers was shown to be over 1000-fold faster than by the two P_r diastereoisomers [38,39]. Therefore, for all practical purposes, the stoichiometry of the reaction should be 1:2, provided that the HuAChE mutations do not affect the stereoselectivity toward soman. Titrations of the various HuAChE enzymes with MEPQ and soman resulted in stoichiometries of 1:1 and 1:2 respectively for all the enzymes examined. This result indicates that substitutions for residues of the hydrogen-bond network and at the hydrophobic alkoxy subsite do not affect the stereochemistry of the enzyme-soman interaction. This conclusion is consistent with the predictions from molecular modelling [36] and with the recent suggestion that stereoselectivity of AChE towards phosphonates results mainly from interactions in the acyl pocket of the enzyme [40].

The hydrophobic alkoxy pocket for substrate in the AChE active centre is defined by aromatic residues Trp-86, Tyr-337 and Phe-338 [30]. This pocket seems to have limited interaction with the alkoxy substituents of organophosphates such as di-isopropyl phosphorofluoridate (DFP) and diethyl phosphorofluoridate [23] (Table 1). A comparable lack of effect on the phosphorylation

Table 1 Apparent bimolecular rate constants (k_i) of phosphorylation and phosphorylation of HuAChE enzymes by soman and DFP

The values for k_i were calculated from the pseudo-first-order rate constants. Values are means $\pm$ S.D. for three or four independent measurements. The k_i values for DFP for most of the mutants were determined recently [23] and are cited for comparison.

HuAChE	k_i ($10^{-4} \text{ M}^{-1} \cdot \text{min}^{-1}$)	
	Soman	DFP
Wild-type	8600 $\pm$ 2000	14.0 $\pm$ 1.0
W86F	2510 $\pm$ 220	14.0 $\pm$ 1.3
W86A	540 $\pm$ 30	2.6 $\pm$ 0.1
Y337F	4800 $\pm$ 800	40 $\pm$ 10
Y337A	2950 $\pm$ 640	3.0 $\pm$ 0.2
F338A	9000 $\pm$ 3000	2.6 $\pm$ 0.2
Y133F	250 $\pm$ 30	1.4 $\pm$ 0.1
E202A	460 $\pm$ 250	0.9 $\pm$ 0.1
E202D	570 $\pm$ 100	0.8 $\pm$ 0.1
E202Q	310 $\pm$ 100	0.18 $\pm$ 0.06
E450A	120 $\pm$ 20	0.09 $\pm$ 0.03
D74N	3400 $\pm$ 600	19.7 $\pm$ 1.2

Figure 1 Energy-optimized models of the active centre in the unligated and in the 1,2,2-trimethylpropyl-methylphosphonylated HuAChE states

(A) In the unoccupied active centre the involvement of Glu-202, Glu-450 and Tyr-133 in the hydrogen-bond network, through water molecules w_1 and w_2 , is shown (for distances within the network see Ordentlich et al. [15]). (B) In the phosphonyl conjugate the relative orientations of the phosphorus substituents with Trp-86 and Phe-338 of the alkoxy pocket and residues of the hydrogen-bond network are shown. The shape complementarity is shown by the corresponding van der Waals volumes. Hydrogen atoms are omitted for clarity.

rates by soman has been observed for replacements of residues Tyr-337 and Phe-338 (Table 1). In contrast, replacement of Trp-86 by an alanine residue resulted in a 16-fold decrease of the phosphorylation rate whereas replacement by a phenylalanine residue led to a smaller decrease in the rate (1/3.4 relative to the wild type), implying a role for this aromatic residue in stabilizing the bulky 1,2,2-trimethylpropyl substituent.

The hydrogen-bond network [15,31], which has been suggested to contribute to the stabilization of the active centre's functional architecture, seems to be dependent on the precise positioning of Glu-202, Glu-450 and Tyr-133 (Figure 1A). Indeed, replacement of Glu-202 by an acidic or neutral residue, as well as replacements of Tyr-133 by phenylalanine and Glu-450 by alanine, caused a

decrease (1/15–1/28) in the respective phosphorylation rates relative to the wild type (Table 1). This reactivity pattern is analogous with that observed for the corresponding phosphorylation reactions with DFP.

The peripheral anionic-site residue Asp-74 was shown to be a key element involved in the allosteric interactions between the active centre and the remote enzyme exterior [26,29,41]. It was therefore of interest to monitor the effect of its replacement on phosphorylation and the aging processes. The phosphorylation rates of D74N mutant enzyme by soman and DFP were practically equivalent to those of the wild-type HuAChE (Table 1), indicating that the allosteric modulation of AChE activity might not contribute to the phosphorylation processes.

Thus examination of the phosphorylation reactions with soman seems to support the assumption about the similarity of the electronic and steric requirements for the phosphorylation and phosphorylation processes, as exemplified by the comparable inhibition patterns for the various mutants with soman and DFP (Table 1). Yet, as could be expected, the presence of a larger alkoxy substituent on the phosphyl moiety permits somewhat better mapping of the interactions of this moiety with the residues of the hydrophobic alkoxy subsite.

Aging of phosphonylated HuAChE and selected mutants

The aging processes of the 1,2,2-trimethylpropyl methylphosphonyl conjugates of HuAChE enzymes were monitored by measuring the reactivatable fraction of the conjugate in the presence of the potent oxime reactivator HI-6 (see Scheme 1) [42]. Under the experimental conditions used, a substantial regeneration of the enzymic activity was observed even for the conjugate of the wild-type HuAChE, for which the aging was fairly rapid. The exponential time courses, for aging of all the phosphonyl conjugates examined here, were consistent with first-order kinetics and with a single homogeneous population of reacting species.

Mutations of the hydrogen-bond network

The involvement of Glu-202 in the aging process was already demonstrated through the effect of its replacement by glutamine [15,16]. The observed decrease in the rate of aging to 1/150 relative to the wild type HuAChE, caused by this mutation [15], was attributed to the removal of the charged carboxylate moiety from the vicinity of the evolving carbonium ion [14]. However, the replacement of Glu-450, which is 9 Å away from the catalytic serine residue, by alanine (Table 2) also resulted in a significant decrease in the rate of aging. This was explained by a hydrogen-bond network involving Glu-202 and Glu-450 as well as Tyr-133 (see Figure 1A) that determines the orientation of the Glu-202 carboxylate [15]. Indeed we find that removal of the hydroxy group, through replacement of Tyr-133 by a phenylalanine residue, resulted in a decrease in the rate of aging that is remarkably similar to that observed for the E450A enzyme (Table 2). Because in the E450A or Y133F mutant enzymes the carboxylate of Glu-202 is still present within the active centre manifold and presumably only its position is affected, it seems that the role of Glu-202 in facilitating the hydrolytic activity as well as the aging process is strongly dependent on its exact location and orientation.

To examine the role of Glu-202 further we have tested the consequences of shortening the acidic side chain through substitution of the glutamic acid residue by the polar protic aspartate, or of its replacement by the non-polar residue alanine. The rates of aging for the two corresponding phosphonyl-conjugates are

Table 2 Rate constants of aging (k_a) of soman-inhibited HuAChE enzymes at pH 8.0

Values of the first-order rate constants are averages of at least three independent experiments at 24 °C.

HuAChE	k_a (10^3 min^{-1})	Ratio (wild-type/mutant)
Wild-type	110 ± 45	1
W86F	4.5 ± 1.5	24
W86A	$< 0.1^*$	$> 1000^*$
Y337F	40 ± 3.5	3
Y337A	160 ± 70	0.7
F338A	0.7 ± 0.4	157
Y133F	4.0 ± 0.9	28
E202A	0.33 ± 0.05	333
E202D	0.7 ± 0.1	155
E202Q	0.8 ± 0.3	138
E450A	4.6 ± 1.6	24
D74N	7.1 ± 2.1	15

* Aging reaction could not be detected after 96 h.

nearly equivalent to that of the E202Q mutant enzyme (Table 2). These results suggest that Glu-202 exerts its effect through either (1) a negative electric field, which is strongly dependent upon distance and stabilizes the positive charge on either the phosphonyl moiety or on the imidazolium moiety of His-447, or (2) involvement in either a proton transfer or a hydrogen-bonding interaction with the phosphonyl moiety.

The participation of Glu-199 in *Torpedo californica* acetylcholinesterase (equivalent to Glu-202 in HuAChE) in general acid catalysis has been previously suggested for the reactions of AChE with certain substrates [43]. A recent computational study has further suggested that Glu-202, rather than His-447, might be involved in the protonation necessary to induce the aging process [14]. To explore this possibility, the pH–rate profiles of aging were determined for the 1,2,2-trimethylpropyl methylphosphonyl conjugates of the wild-type and the E202Q mutant HuAChEs as well as of an additional mutant in which the hydrogen-bond network was not perturbed. As already reported for other AChEs [10,44,45], the rates of aging for the wild-type HuAChE and the two mutant enzymes increase with decreasing pH (Table 3 and Figure 2). Because of experimental limitations the rates of aging could not be measured below pH 5.5, precluding the completion of the titration curves for determination of pK_a values. We therefore compared the efficiency index of aging for each mutant as a function of pH (Figure 2). The efficiency index

Table 3 Enhancement of the rates of aging for soman-inhibited HuAChE enzymes with decrease in pH

Values for pH 8 are taken from Table 2. All first-order rate constants were determined at 24 °C; values are means $\pm$ S.D. for at least three independent experiments.

HuAChE	k_a (10^3 min^{-1})		
	pH 8	pH 7	pH 6
Wild-type	110 ± 45	370 ± 85	1660 ± 200
W86A	< 0.1	0.2 ± 0.05	0.5 ± 0.1
E202Q	0.8 ± 0.3	2.3 ± 0.9	6.4 ± 0.8
F338A	0.7 ± 0.4	2.2 ± 0.2	9.6 ± 1.6

Figure 2 pH-dependence of the efficiency indices of aging of the soman-inhibited HuAChE enzymes

The efficiency index for each enzyme is defined as the ratio between the first-order rate constant of aging at a given pH and the corresponding value at pH 5.5, for the wild-type HuAChE ($\square$) and mutants E202Q ($\circ$) and F338A (∇).

was calculated as the ratio of the rate constant at any given pH to the rate constant at pH 5.5. Although the actual rate of aging of the wild-type enzyme at a given pH is 100-fold those of the F338A and E202Q mutants (Table 3), all three enzymes showed a very similar dependence of their efficiency indices on pH (Figure 2). This similarity suggests that Glu-202 is not directly involved in proton transfer relevant to the aging process.

Mutants of the hydrophobic alkoxy pocket

In view of the hydrophobic nature of the 1,2,2-trimethylpropoxy substituent of the phosphonyl moiety and the plasticity of the HuAChE active centre, we examined the possible effect on the aging process of interactions with several aromatic residues (Trp-86, Tyr-337 and Phe-338) of the hydrophobic alkoxy subsite. Molecular models (Figure 1B) indicate that Tyr-337 is not involved in interactions with the phosphonyl moiety. This prediction is confirmed experimentally by the observation that replacements of Tyr-337 by an alanine or a phenylalanine residue resulted in only a marginal effect on the rates of phosphorylation and aging relative to the wild-type HuAChE (Tables 1 and 2).

As with Tyr-337, molecular models of the soman–HuAChE conjugate indicate that the aromatic side chain of Phe-338 is remote from the phosphonyl alkoxy group in both the Michaelis complexes (with the $P_S C_S$ and $P_S C_R$ soman diastereomers) and in the resulting covalent conjugates. This conclusion is supported by the finding that the phosphorylation rate of the F338A mutant by soman is equivalent to that of the wild-type enzyme. Nevertheless replacement of Phe-338 by an alanine residue resulted in a decrease to almost 1/160 in the rate of aging (Table 2), demonstrating the involvement of this aromatic amino acid in the aging process. Because this pronounced effect cannot be attributed to a direct interaction of Phe-338 with the alkoxy group, it might originate from a structural perturbation that induces a change in the mechanism of aging. This possibility seems unlikely in view of the similar pH–rate profiles of aging for the phosphonyl conjugates of the F338A mutant and wild-type HuAChEs (Figure 2). Alternatively the replacement of Phe-338

Figure 3 Stereo view of the proposed orientation of His-447 imidazolium group in relation to the alkoxy oxygen of the phosphonyl moiety

The 1,2,2-trimethylpropyl methylphosphonyl moiety is shown as balls and sticks; hydrogen atoms are not displayed. The interaction distances (Å) from the centroid of the protonated histidine (Hip-447) imidazolium ring to O^{ε1}(Glu-202) and the C^{δ2}(Phe-338) are shown as broken lines. These interactions contribute to the stabilization of both the charge of the imidazolium moiety and its positioning proximal to the alkoxy oxygen, as shown by the corresponding distance N^{ε1}(Hip-447)-O-alkyl.

might affect the process of proton transfer to the phosphonyl moiety through a local perturbation that affects the relative position of the proton-donating group (see below and Figure 3).

The potential participation of Trp-86 in the aging process of the soman-HuAChE conjugate was suggested both by the molecular models of the conjugates and by the observed differential effects of the replacement of Trp-86 by an alanine and a phenylalanine residue on the phosphorylation rates by soman (Table 1). The most marked effect on the rate of aging has been observed for the phosphonyl conjugate of W86A mutant HuAChE. For this conjugate at pH 8, no dealkylation reaction could be detected for several days, indicating that the rate of aging was 1/1000 of that of the wild-type enzyme, or less. In an attempt to determine whether the W86A conjugate was still capable of undergoing aging, measurements were made under conditions of low pH that were shown to accelerate aging in other mutants (Figure 2). Indeed, at pH 7 and pH 6 a slow dealkylation processes could be observed with rate constants 1/1850 and 1/3300 respectively of the corresponding values for the wild-type enzyme (Table 3). Assuming that this ratio is maintained at pH 8, then the calculated value of the half-life time for the 1,2,2-trimethylpropyl methylphosphonyl conjugate of the W86A enzyme should be 7.6–15 days compared with 6.3 min for the wild-type HuAChE. In contrast with the aging process for the W86A conjugate, that for the 1,2,2-trimethylpropyl methylphosphonyl conjugate of the W86F mutant could be monitored at pH 8, with a rate constant 1/25 of that of the wild-type enzyme. These findings underscore the crucial role of the aromatic residue at position 86 in the aging of the soman-AChE conjugates.

Mutant of the peripheral anionic site

Although Asp-74 is remote from the active centre, its replacement by asparagine has already been shown to affect the accommodation of the charged trimethylammonium group in the active centre because it lowered the affinity of HuAChE for ligands such as ATC or edrophonium [26,29,41]. However, this replacement had no effect on the affinity towards the uncharged substrate TB (A. Shafferman, A. Ordentlich, C. Kronman and B. Velan, unpublished work) or towards soman and DFP (Table 1).

As with the charged ligands, the mutation D74N resulted in a decrease (1/15) in the rate of aging of the D74N conjugate with soman, compared with the wild-type enzyme. These results might imply that interactions of the active centre with a positive charge are part of the aging process of 1,2,2-trimethylpropyl methylphosphonyl conjugates, operating by stabilization of the carbonium ion via the 'anionic' subsite in the active centre (Trp-86).

How does AChE catalyse the process of aging?

The generally accepted mechanism of aging involves protonation of the phosphonyl moiety at the alkoxy oxygen, followed by scission of the C–O bond and formation of a carbonium ion (Scheme 2). This view is supported by results from experiments with radiolabelled organophosphorus inhibitors [10,46], kinetic correlations with unimolecular solvolysis reactions [9] and crystallographic structures of the related aged conjugates of chymotrypsin [22] and by NMR studies of the aging products in AChE [19]. Although there is evidence that the aging process is facilitated by intermolecular interactions of the phosphonyl moiety with structural elements of the AChE active centre, the mechanism of the catalysis is not clear and many seemingly conflicting results can be found in the literature. In particular it is not clear whether the enzyme is catalytically involved in the step of protonation, in that of carbonium ion formation or in both steps.

The efficiency of the protonation step should depend mainly on the proper juxtaposition of the alkoxy group oxygen relative to the potential proton donors in the active centre. According to the molecular models of phosphonyl-HuAChE conjugates, the only acidic groups within hydrogen-bonding distance from the alkoxy oxygen are those of Glu-202 and the catalytic triad His-447 [26,47,48]. As already indicated, the pH-rate profile of the E202Q conjugate is not consistent with the action of Glu-202 as a proton donor. For Hip-447 (the protonated form of His-447), the models show (Figure 3) that formation of a short-distance N^{ε1}-O-alkyl hydrogen bond (2.81 Å) is possible through motion of the imidazolium moiety from the vicinity of the O^{ε1}(Ser-203) to a position near the alkoxy oxygen (Figure 4). This stable hydrogen bond might be essential for facilitating the protonation of the alkoxy oxygen and for maintaining a sufficient steady-state

Figure 4 Significance of conformational mobility of the side chain of His-447 to the catalysis of phosphonyl conjugates dealkylation: an analogy with substrate hydrolysis

The conformation of the protonated histidine (Hip-447) side chain ($\chi_1 = 167$; $\chi_2 = 89$; $\chi_3 = -179$), marked by heavy line, is probably involved in proton transfer to the phosphonyl moiety, maintaining a high steady-state concentration of the oxonium species. An analogous conformational state of Hip-447 is involved in the release of choline from the ACh–AChE tetrahedral intermediate. The alternative, less stable conformation in the phosphonylated enzyme (light line, $\chi_1 = -176$; $\chi_2 = 73$; $\chi_3 = -177$), where the imidazolium moiety forms a hydrogen bond with $O^-(Ser-203)$, is analogous with that involved in the release of the enzyme from the hydrated acetyl enzyme. In reaction with substrate, the motion towards $O^-(Ser-203)$ might be induced by the deprotonation of the imidazolium (through elimination of choline) whereas in phosphonate conjugates such motion is retarded because the alkoxy substituent is not a leaving group.

concentration of the oxonium reactive species for the dealkylation to take place. As shown in Figure 3, formation of the N^ϵ – O -alkyl hydrogen bond places the imidazolium ring within a cation– π interaction distance (4.06 Å) of the phenyl group of Phe-338, and within a short distance (3.15 Å) of the negative $O^\ominus$ of the Glu-202 carboxylate. The participation of Glu-202 and Phe-338 in the stabilization of the imidazolium positive charge and in its proper juxtaposition with the phosphonyl moiety is consistent with the effects of HuAChE mutagenesis of these residues on the rates of aging. Such preferential stabilization of the protonated form of histidine through cation– π interaction with an adjacent aromatic residue has been already established for barnase [49]. It should be noted that the motion of the imidazole moiety of His-447 between the two positions proximal to $O^-(Ser-203)$ and to the alkoxy oxygen (Figure 4) is actually an integral part of the catalytic machinery in serine hydrolases, operating in both the acylation and the deacylation steps respectively [50]. During the acylation of AChE by acetylcholine, this imidazole participates in proton transfer from $O^-(Ser-203)$ and the transition of the tetrahedral intermediate into the acyl enzyme is assisted by proton transfer from the same imidazolium to the choline oxygen. The latter is analogous with the protonation step of the alkoxy oxygen of phosphonyl conjugates and the formation of the oxonium ion during the aging process (Figure 4). Unlike the choline moiety in the substrate, the alkoxy substituent of phosphonates is not a leaving group. Therefore the stabilized N^ϵ – O -alkyl hydrogen bond in the phosphonyl conjugates might restrict the motion of the imidazolium, preventing the protonation of $O^-(Ser-203)$ [indeed in this conformation the distance $O^-(Ser-203)$ – N^ϵ (Hip-447) is 3.45 Å; see Figure 3]. Thus one of the reasons for the hydrolytic stability of phosphyl–AChE conjugates, and in particular that of their aged forms, might be the lack of protonation of $O^-(Ser-203)$, which is necessary for elimination of the enzyme from the conjugate [13,14]. The proposed localization of the imidazolium moiety relative to the

alkoxy oxygen is also consistent with the well-established [51] resistance of conjugates with the P_R phosphorus configuration to dealkylation. According to the corresponding molecular model (not shown), the N^ϵ -atom of the imidazolium is too remote from the alkoxy oxygen (4.3 Å) to form a stable hydrogen bond, precluding the formation of oxonium and consequently the dealkylation reaction of the P_R phosphonyl AChE conjugates.

The mechanistic hypothesis presented above suggests that AChE is catalytically involved in the aging process through one of its basic functional features, and consists mainly of accommodating the reactive oxonium species via the catalytic triad residue His-447. This mechanism should operate for all the P_S phosphonyl conjugates, irrespective of the nature of the alkyl group in the alkoxy moiety. Indeed the rate constants of aging for various alkyl methylphosphono-AChE conjugates have shown a good correlation with those for solvolysis of the corresponding alkyl tosylates [9,52], demonstrating an equivalent effect of the alkyl structure on both reactions. Furthermore the activation energy for the dealkylation reaction of cycloheptyl methylphosphono-AChE was found to be comparable to that for solvolysis of the cycloheptyl tosylate [45]. These results suggest that in all these cases the AChE is not involved in the step of charge separation and carbonium ion formation. However, the aging of two outstanding AChE phosphonyl conjugates, carrying the 1,2,2-trimethylpropyl [52] and the 2,2-dimethylcyclohexyl (H. P. Benschop, personal communication) alkyl groups, is much faster than could have been expected from the correlation between the rates of aging and solvolysis. We note that the two alkyl groups share a common branching pattern of a secondary C_α carbon atom and a tertiary C_β main-chain carbon atom (see Scheme 1). A clue to understanding the unique efficiency of AChEs in facilitating the dealkylation step of such branched alkoxy groups in the phosphonyl–HuAChE conjugates is provided in this study. We propose that the key element for the unique enzymic acceleration of dealkylation of the branched alkoxy substituents on the phosphonyl adducts might be due to the interaction of the 1,2,2-trimethylpropyl group with Trp-86. Stabilization of the carbonium ion on the C_α of the 1,2,2-trimethylpropyl moiety, through hyperconjugation with the adjacent alkyl groups, imparts a partial positive character to the C_β methyl substituents. The aromatic moiety of Trp-86 might contribute to the stabilization of this partial positive charge, thereby facilitating the charge separation process. This interaction is analogous with the well established cation– π interaction of Trp-86 with the trimethylammonium substituents of ligands such as edrophonium or the substrate acetylcholine [30,53]. The marked decrease (1/1850–1/3300) in the rate of aging for the 1,2,2-trimethylpropyl methylphosphonyl conjugate of the W86A mutant, compared with the conjugate of the wild-type HuAChE, can be rationalized by the absence of this stabilizing effect. A comparable decrease in affinity (1/4000–1/15000-fold) was observed for the W86A mutant enzyme towards the bis-quaternary ligands BW284C51 and decamethonium [31,41], whereas the affinities towards the uncharged bulky ligands such as *m*-*t*-butyl trifluoroacetophenone [54] and soman (Table 1) are only moderately affected (a decrease to approx. 1/20 in affinity) by this replacement. Moreover, the 25-fold decrease in the aging rate constant, compared with the wild-type, for the 1,2,2-trimethylpropylmethylphosphono W86F conjugate is quite similar to the effect of replacing the Trp-86 by phenylalanine on affinity towards charged ligands (edrophonium, decamethonium and BW284C51) [31]. These comparisons underscore the importance of the stabilization of the positive charge by Trp-86 to the reactivity of AChE towards substrates and other active-centre ligands. The greater capacity of tryptophan than phenyl-

alanine to interact with positively charged species is a result of the much larger negative electrostatic potential provided by the indole moiety [55]. The involvement of aromatic residues, revealed in this study, in the aging of soman-inhibited AChEs clarifies the absence of comparable acceleration effects on aging for soman-inhibited serine hydrolases such as chymotrypsin, where no aromatic residue adjacent to the phosphonyl alkoxy group can be found.

In conclusion, the enzymic involvement in the acceleration of both the protonation and the carbonium ion formation steps might account for the rapid aging of soman-inhibited AChEs compared with other phosphyl conjugates of ChEs. It is also consistent with the measured solvent isotope effects of aging for 2-propyl and 1,2,2-trimethylpropyl methylphosphono-AChE conjugates (k_H/k_D of 1.2 and 1.6 respectively [13,56]), which indicate that, for the latter, both steps (protonation and dealkylation) contribute to the overall rate of aging. According to this view it can be expected that for most AChE phosphonyl conjugates, where acceleration of the dealkylation step is limited, the substitution of residue equivalent to Trp-86 will have a less significant effect on the rates of aging.

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