Importance of aspartate-70 in organophosphate inhibition, oxime re-activation and aging of human butyrylcholinesterase

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Asp-70 is the defining amino acid in the peripheral anionic site of human butyrylcholinesterase (BuChE), whereas acetylcholinesterase has several additional amino acids, the most important one being Trp-277 (Trp-279 in *Torpedo* AChE). We studied mutants D70G, D70K and A277W to evaluate the role of Asp-70 and Trp-277 in reactions with organophosphates. We found that Asp-70 was important for binding positively charged echothiophate, but not neutral paraoxon and iso-OMPA. Asp-70 was also important for binding of positively charged pralidoxime (2-PAM) and for activation of re-activation by excess 2-PAM. Excess 2-PAM had an effect similar to substrate activation, suggesting the binding of 2 mol of 2-PAM to wild-type but not to the D70G mutant. A surprising result was that Asp-70 was important for irreversible aging, the D70G mutant having a 3- and 8-fold lower rate of aging for paraoxon-inhibited and di-isopropyl fluorophosphate-inhibited BuChE. Mutants of Asp-70 had the same rate constants for phosphorylation and re-activation by 2-PAM as wild-type. The A277W mutant behaved like wild-type in all assays. Our results predict that people with the atypical (D70G) variant of BuChE will be more sensitive to the toxic effects of echothiophate, but will be equally sensitive to paraoxon and di-isopropyl fluorophosphate. People with the D70G mutation will be resistant to re-activation of their inhibited BuChE by 2-PAM, but this will be offset by the lower rate of irreversible aging of inhibited BuChE, allowing some regeneration by spontaneous hydrolysis.

INTRODUCTION

Cholinesterases [acetylcholinesterase (AChE) EC 3.1.1.7 and butylcholinesterase (BuChE) EC 3.1.1.8] are inhibited by organophosphates through phosphylation (i.e. phosphorylation or phosphonylation) of their active-site serine [1,2]. Organophosphates such as paraoxon are currently used as pesticides and insecticides. Echothiophate is an anti-glaucoma drug. Other organophosphates such as soman, sarin and VX (*O*-ethyl-*S*[2]-di-isopropylaminoethyl methylphosphonothionate) are potent chemical warfare agents [3,4]. Phosphylated cholinesterases can be re-activated by nucleophilic agents, of which oximes are of pharmacological interest as antidotes against organophosphate poisoning [3]. Organophosphate adducts can, however, undergo a dealkylation reaction, called 'aging', which converts phosphylated enzymes into non-re-activatable aged species [1–3].

A considerable body of information is available on phosphylation, aging and re-activation of cholinesterases. However, the mechanisms that underlie these reactions are not fully understood. Resolution of the X-ray structure of *Torpedo californica* AChE showing that the catalytic site of cholinesterases is located near the bottom of a deep narrow gorge [5] has stimulated active research in the field. One of the most important issues is to identify the amino acids lining the gorge that are involved in the inhibition, re-activation and aging processes and eventually to determine how these reactions are controlled. Important contributions have recently been made [6–8]. However, kinetic complexities in re-activation of phosphylated AChE have suggested that the classical two-step re-activation kinetic model [9] may be inadequate to describe the re-activation process, in particular at high oxime concentrations [6,8].

The present work focuses on the role of Asp-70 in controlling the kinetic behaviour of oxime-mediated re-activation of phosphorylated human BuChE. This residue is located at the rim of the active-site gorge, where it has been found to be a part of the peripheral anionic site (PAS) of BuChE [10]. Moreover, our recent results indicate that, after initial interaction with Asp-70, positively charged substrates are correctly oriented to slide on to Trp-82, the actual 'anionic site' [54]. Asp-70 is also involved in activation by excess substrate [10]. The present results show that Asp-70 is involved in the complex dependence of re-activation on pralidoxime iodide (2-PAM) concentration. The D70G mutant has a lower rate of aging, suggesting that Asp-70 exerts indirect control of the rate of aging.

MATERIALS AND METHODS

Chemicals

The organophosphates used were commercially available compounds (Figure 1): iso-OMPA (tetramonoisopropylpyrophosphortetramide), paraoxon (*O*,*O*′-diethyl *O*″-*p*-nitrophenyl phosphate) (approx. 90 % pure) and DFP (di-isopropyl fluorophosphate) were purchased from Sigma (St. Louis, MO, U.S.A.), and echothiophate (*O*,*O*′-diethyl-*S*-ethyltrimethylaminephosphorothiolate iodide) was a gift from Promedica (La Chaussée Saint-Victor, France). Gold-label-grade 2-PAM was from Ega-Chemie (Steinheim, Germany). Other chemicals were of biochemical grade.

Natural and recombinant enzymes

Site-directed mutagenesis of the BuChE gene was performed as

Abbreviations used: AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; BuSCh, butyrylthiocholine; iso-OMPA, tetramonoisopropylpyrophosphortetramide or bis-(isopropylamino)phosphonic anhydride; DFP, di-isopropyl fluorophosphate; 2-PAM, pralidoxime iodide (2-pyridinealdoxime methiodide); PAS, peripheral anionic site; VX, *O*-ethyl-*S*[2]-di-isopropylaminoethyl methylphosphonothionate.

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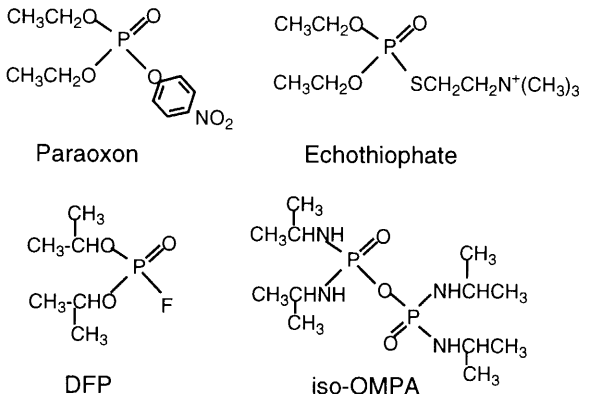
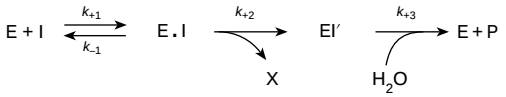


Figure 1 Chemical structures of organophosphates used in this study

described [10]. The mutated residues, Asp-70 and Ala-277, are located at the mouth of the active-site gorge [5]. In vertebrate AChE, the corresponding residues are aspartate and tryptophan. Both amino acids are important components of the PAS of AChE [11]. We made mutants D70G, D70K and A277W. Mutations on residues Asp-70 and Ala-277 were expected to alter affinity for positively charged ligands of BuChE. Recombinant wild-type and mutant human BuChE were expressed in stably transfected human embryonal 293 kidney cells [10]. Secreted recombinant enzymes were collected into Neuman & Tytell's serum-less medium (Gibco) and stored at 4 °C under sterile conditions. Recombinant wild-type and mutant BuChE were partially purified by affinity chromatography on procainamide gel [12]. This step was necessary because we found that 2-PAM strongly interacted with a product secreted by 293 cells. The nature of this secretion product was not determined, but it has been shown *in vivo* that oximes concentrate in kidney and proteoglycan-containing tisues [13] and *in vitro* that oximes bind via ionic interactions to proteoglycans secreted by cell cultures [14]. Natural BuChE (tetrameric form, G_4) was highly purified from pooled plasma by affinity chromatography and anion-exchange chromatography as described [15]. Enzyme samples were kept in 0.1 M sodium phosphate, pH 7.0.

Progressive inhibition by organophosphates

Progressive inhibition of wild-type and mutant BuChE by echothiophate, iso-OMPA and paraoxon was conducted in 0.1 M sodium phosphate, pH 7.0, at 25 °C. Stock solutions (10 mM) of organophosphates were prepared in ethanol/water (60 %, v/v) and stored at −70 °C. Volumes of working solutions of organophosphates (1 mM or 0.1 mM) in ethanol/water (10 %, v/v) were added to 70 μl of enzyme solution. The final echothiophate concentrations ranged from 0.1 to 50 μM, the iso-OMPA concentrations ranged from 2.5 to 50 μM and the concentrations of paraoxon ranged from 0.01 to 0.1 μM. Ethanol (10 %, v/v) was added to the enzyme samples so that the final ethanol concentration in the inhibition medium was 0.5 %. Such a low ethanol concentration has no effect on the activity of BuChE [16]. Residual activity at each organophosphate concentration was measured as a function of incubation time by the method of Ellman et al. [17] at 25 °C in 0.1 M sodium phosphate, pH 7.0, using butyrylthiocholine (BuSCh; 1 mM) as substrate, i.e., at V_{max}. Samples were 120-fold diluted in Ellman's medium. Inhibition kinetic experiments with eight different concentrations of organophosphate were performed in triplicate.



Scheme 1 Inhibition of cholinesterases by organophosphorus compounds

Inhibition of cholinesterases by organophosphorus compounds can be depicted by Scheme 1, where $K_i = k_{-1}/k_{+1}$ is the dissociation constant of the enzyme-inhibitor complex, k_{+2} the phosphylation rate constant and k_{+3} the spontaneous re-activation rate constant which is very sluggish. Thus Scheme 1 simplifies to $E + I \rightleftarrows E \cdot I \rightarrow EI'$. If [I] $\gg$ [E] and assuming that the equilibrium conditions ($k_{+2} \ll k_{-1}$) hold, the pseudo-first-order rate constant of phosphorylation (k_{obs}) can be derived [18] as:

$$k_{obs} = \frac{k_{+2}}{1 + K_i/[I]} \quad (1)$$

K_i and k_{+2} were determined from the reciprocal form of eqn. (1) using Kaleidagraph (Microsoft); the second-order inhibition constant is the ratio k_{+2}/K_i.

Reversible inhibition by 2-PAM

Reversible inhibition of BuChE by 2-PAM was carried out in 0.1 M sodium phosphate, pH 7.0, at 25 °C with butyrylthiocholine iodide (0.05, 0.075 and 0.1 mM) as substrate and concentrations of 2-PAM ranging from 0.3 to 3.5 mM. The type of inhibition and inhibition constants (K_i) were determined by graphical methods: Dixon plots (reciprocal of velocity against concentration of 2-PAM) and plots of substrate concentration/velocity against concentration of 2-PAM [19].

Re-activation of diethylphosphoryl-BuChE by 2-PAM

For the re-activation kinetic study of phosphorylated BuChE, the recombinant wild-type BuChE and the D70G and A277W mutants were diethylphosphorylated by paraoxon. Paraoxon at approx. 0.01 μM caused 90 % inhibition in less than 90 min. Unbound paraoxon was removed by centrifugation through a Millipore Ultrafree MC 10000 NMWL filter unit. Aging was not significant over the time course of the inhibition phase (under our experimental conditions < 8 % diethylphosphorylated wild-type BuChE and > 2.5 % diethylphosphorylated D70G mutant aged).

Re-activation of phosphorylated enzymes was carried out with the oxime 2-PAM. Concentrations of 2-PAM ranging from 0.5 to 19.5 mM in 0.1 M sodium phosphate, pH 7.0 and pH 8.2, at 20 °C were used. Aliquots (50 μl) of the reaction mixture were withdrawn at intervals within the 90 min period of re-activation. Activity of the re-activated enzyme was measured at pH 7.0 with 1 mM BuSCh. The dilution factor of the re-activated enzyme in the assay was 150 so that residual 2-PAM, which acts as a competitive inhibitor, did not interfere with cholinesterase activity measurements. Although reversible inhibition of BuChE by 2-PAM was carried out at 25 °C instead of 20 °C, the 5 °C difference between reversible inhibition and re-activation conditions does not alter the validity of the experimental conclusions.

The effect of buffer concentration on re-activation was determined by carrying out re-activation kinetics at 20 °C in pH 7.0 buffers of increasing sodium phosphate concentration (from 10 to 500 mM).

$$EI' + 2\text{-PAM} \underset{}{\overset{K_D}{\rightleftharpoons}} EI'\cdot 2\text{-PAM} \xrightarrow{k_r} E + P$$

Scheme 2

EI′ is the phosphorylated enzyme, 2-PAM is the re-activator, EI′·2-PAM is the intermediate complex, P represents the reaction product, i.e. phosphorylated 2-PAM and its breakdown products [20,21], and E is the re-activated enzyme. K_D and k_r are the dissociation and the rate constant for breakdown of the complex respectively.

Kinetic analysis of oxime-mediated re-activation

Oxime-mediated re-activation of phosphorylated cholinesterases can be depicted by the classical minimum Scheme 2 [9]. It should be mentioned that phosphylated oximes have been shown to be powerful irreversible inhibitors of cholinesterases [22].

Since $[EI']_0$, the initial concentration of inhibited enzyme, is equal to $[EI']+[EI'\cdot 2\text{-PAM}]+[E]$ and the rate of re-activation is $d[E]/dt = k_r[EI'\cdot 2\text{-PAM}]$, under the approximation of complete re-activation, i.e. $[EI']_0 = [E]_\infty$ and with $[2\text{-PAM}] \gg [EI']_0$, the re-activation reaction can be described by a simple pseudo-first-order rate equation:

$$\frac{d[E]}{dt} = k_{app}([E]_\infty - [E]) \quad (2)$$

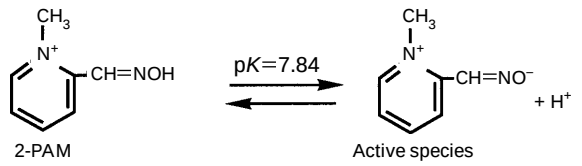
where

$$k_{app} = \frac{k_r}{1+\frac{K_D}{[2\text{-PAM}]}} \quad (3)$$

The time course of re-activation was followed for different concentrations of 2-PAM. Each re-activation curve reached a plateau corresponding to $[E]_\infty$ in less than 90 min. The pseudo-first-order rate constant k_{app} for the re-activation of phosphorylated enzyme by different concentrations of 2-PAM was calculated by linear regression analysis from the slope of the integrated form of eqn. (2):

$$\ln\left(\frac{[E]_\infty - [E]_t}{[E]_\infty}\right) = -k_{app}\,t \quad (4)$$

Apparent parameters of re-activation ($K_{D,app}$, k_r and $k_{II,app} = k_r/K_{D,app}$ the bimolecular rate constant of re-activation) were determined from the dependence of k_{app} on the total concentration of 2-PAM by plotting $1/k_{app}$ against 1/[2-PAM], the reciprocal of eqn. (3) and evaluating the intercepts.



Scheme 3 2-PAM undergoes deprotonation with a pK_a equal to 7.84 [23].

The reactive species of 2-PAM is the nucleophilic dipolar oximate ion (2-PAM$^{\pm}$) (Scheme 3). For calculation of the effective re-activation parameters ($K_{D,eff}$, k_r and $k_{II,eff} = k_r/K_{D,eff}$) the effective concentration of 2-PAM$^{\pm}$ at pH 7.0 and 8.2 was computed according to the classic Henderson–Hasselbalch equation:

$$pH - pK_a = \log[2\text{-PAM}^{\pm}]/([2\text{-PAM}^{+}]_0 - [2\text{-PAM}^{\pm}]) \quad (5)$$

where $[2\text{-PAM}^{+}]_0$ is the total oxime concentration and $[2\text{-PAM}^{\pm}]$ is the ionized oximate.

Aging of phosphorylated BuChE

Wild-type and D70G enzymes were 95 % inhibited by paraoxon or DFP in 50 mM sodium phosphate, pH 8.0, at 25 °C. Inhibition was performed at pH 8.0 to slow the rate of aging [24]. Re-activation by 2-PAM (1 and 10 mM) in 50 mM sodium phosphate buffer, pH 8.0, at 25 °C was carried out as follows: after inhibition, aliquots of inhibited enzyme were removed at various times (from 5 min to 70 h) and immediately subjected to the action of 2-PAM. The buffer and temperature conditions of re-activation for determination of aging rate constants were slightly different from the conditions used in the preceding section, but changing the conditions has no effect on the validity of the conclusions. For example, the results that we obtained for wild-type BuChE are similar to the literature values for wild-type even though the latter were obtained at a slightly different pH and temperature.

The activity of the re-activated enzymes was determined as described above. The rate constant of aging (k_a) was calculated using eqn. (6) [25]:

$$\log\left[\frac{(E_r - E_i)}{(E_o - E_i)}\times 100\right] = -k_a\,t \quad (6)$$

where E_o is the activity before inhibition, E_i the residual activity after inhibition and E_r the activity after re-activation. Results were expressed as half-times of aging, i.e. $t_{1/2} = \ln(2/k_a)$.

RESULTS

Progressive inhibition by organophosphates

Kinetic parameters for inhibition of recombinant wild-type and the mutants A277W and D70G of BuChE by paraoxon are shown in Table 1. There are no significant differences between the parameters for the wild-type and the two mutants. This agrees with results reported by Skrinjaric-Spoljar and Simeon

Table 1 Kinetic parameters for inhibition of wild-type and mutant BuChE by paraoxon in 0.1 M phosphate, pH 7.0, at 25 °C

Results are means ± S.E.M. for determination in triplicate.

	K_i (μM)	k_2 (min^{-1})	$10^{-6}\times k_2/K_i$ (M$^{-1}\cdot$min^{-1})
Wild-type*	0.26 ± 0.16	0.52 ± 0.28	2.26 ± 1.12†
A227W	0.13 ± 0.02	0.28 ± 0.1	2.22 ± 0.79
D70G	0.15 ± 0.02	0.57 ± 0.06	3.74 ± 0.38†

* Literature values were $K_i = 0.50 \pm 0.01$ μM; $k_2 = 1.0 \pm 0.1$ min^{-1}; $k_2/K_i = 2\times10^6$ M$^{-1}\cdot$min^{-1}, pH 8.0 at 27 °C, for the F295L/F297V mutant of human AChE, the reactivity of which toward paraoxon was found to be similar to that of BuChE [26].

† Literature values were 3.0×10^6 and 3.1×10^6 M$^{-1}\cdot$min^{-1} respectively for human serum BuChE phenotypes in 0.1 M phosphate buffer, pH 7.4 at 25 °C [27].

Table 2 Progressive inhibition by a neutral (iso-OMPA) and a charged (echothiophate) organophosphate

The experiment was carried out in 0.1 M sodium phosphate, pH 7.0, at 25 °C. G_4 stands for the highly purified tetrameric form of wild-type BuChE from human plasma and r for the recombinant enzymes. Values are means ± S.E.M. for three determinations.

	Iso-OMPA			Echothiophate		
	K_i (μM)	k_2 (min^{-1})	$10^{-3} \times k_2/K_i$ ($M^{-1} \cdot min^{-1}$)	K_i (μM)	k_2 (min^{-1})	$10^{-3} \times k_2/K_i$ ($M^{-1} \cdot min^{-1}$)
G_4	64 ± 32*	0.39 ± 0.11*	6.1 ± 4.8*	0.15 ± 0.06†	0.48 ± 0.10	3200 ± 1900
r wild-type	98 ± 22*	0.53 ± 0.25*	5.4 ± 3.7*	0.71 ± 0.32†	0.82 ± 0.26	1150 ± 880
4 D70G	75 ± 25	0.98 ± 0.31	13.2 ± 1.2	40.9 ± 15.0	0.66 ± 0.08	17.3 ± 4.8
r D70K	193 ± 171	1.85 ± 1.59	9.7 ± 1.1	114 ± 43	0.78 ± 0.36	6.8 ± 5.7

* Literature values were $K_i = 60 \pm 16$ μM; $k_2 = 0.53 \pm 0.06$ min^{-1}; $k_2/K_i = 8800 \pm 3300$ in 0.1 M phosphate, pH 7.0 at 25 °C [28].
† Literature values were $IC_{50} = 0.27$ μM, pH 7.4 at 22 °C [29].

[27] for paraoxon inhibition of 'usual' (wild-type) and 'atypical' (D70G) human serum cholinesterase phenotypes and by Schwarz et al. [7] for inhibition of recombinant wild-type BuChE and the D70G mutant by DFP. Thus mutations at the rim of the active-site forge of BuChE do not affect binding and phosphorylation by uncharged organophosphates. Moreover, progressive inhibition by the uncharged organophosphate iso-OMPA and the positively charged echothiophate (Table 2) did not show any significant difference in the first-order rate constants of phosphorylation, k_2, between wild-type BuChE and mutants D70G and D70K. However, because of the low affinity of Asp-70 mutants for echothiophate (K_i), the second-order rate constant ($k_i = k_2/K_i$) for phosphorylation by echothiophate was found to be about 100 times slower for the D70G and D70K mutants than for wild-type BuChE. A similar finding was previously observed for phosphorylation of 'usual' and 'atypical' (D70G) human BuChE phenotypes by the positively charged (protonated at pH 7.4) nerve agent VX [27]. In addition, affinity of the D70K mutant for the positively charged organophosphate MEPQ [30] was found to be very low (results not shown). Thus, regarding phosphylation of BuChE, it can be concluded that Asp-70 is only important for binding of positively charged organophosphates. Similar to its role in binding of positively charged substrates, Asp-70 may be regarded as the initial binding site of positively charged organophosphates.

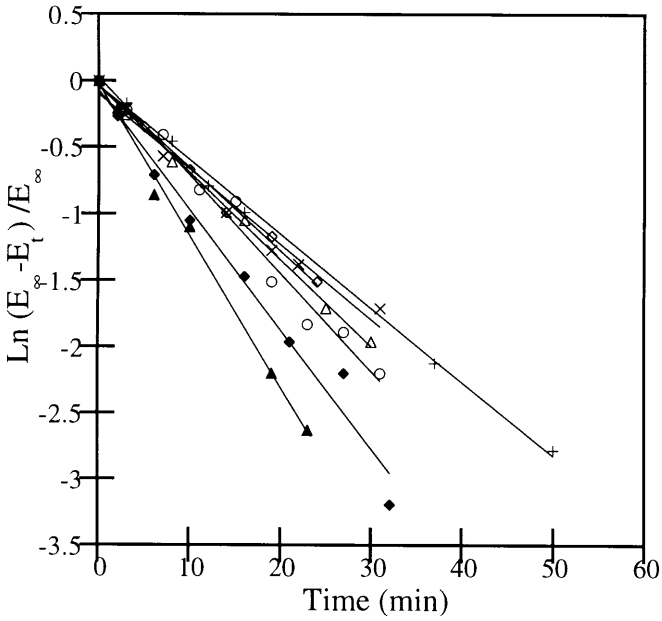


Figure 2 Semilogarithmic plot of the time course of re-activation of paraoxon-inhibited recombinant wild-type BuChE by 2-PAM in 0.1 M phosphate buffer, pH 7.0, at 20 °C

Concentrations of 2-PAM (mM) were: +, 1; ×, 1.25; ◇, 2.5; △, 5; ○, 7.5; ◆, 10; ▲, 15.

Re-activation of diethylphosphorylated enzymes

Only 75 % of paraoxon-phosphorylated BuChE was re-activated by 2-PAM at pH 7.0. A part of this loss may be attributed to aging during the time course of inhibition, but we cannot rule out the possible occurrence of other irreversible post-inhibition reactions [31]. These may include re-inhibition of re-activated BuChE by diethylphosphorylated 2-PAM formed *in situ*. However, it should be remembered that re-inhibition of AChE by diethylphosphorylated oxime has been found to be unimportant in determining the *in vitro* re-activation profile of *O*,*O*-diethylphosphorylated AChE [21]. The kinetices of re-activation of paraoxon-inhibited wild-type and mutant BuChE were found (Figure 2) to follow the simple first-order (mono-exponential) model described by eqn. (2), indicating homogeneity in the re-activatable enzyme species. However, re-activation of paraoxon-phosphorylated wild-type BuChE and mutant A277W showed a complex dependence of k_{app} on 2-PAM$^{\pm}$ concentration. The double-reciprocal plot for recombinant wild-type BuChE in Figure 3 was non-linear, curving downward at high 2-PAM$^{\pm}$ concentrations, indicative of activation. Mutant A277W behaved in an analogous manner (results not shown). On the other hand, the re-activation kinetics of the D70G mutant in the same range of 2-PAM$^{\pm}$ concentrations did not deviate from linearity (Figure 4). These results suggest that 2-PAM re-activation occurs by a mechanism more complex than that in Scheme 2.

At low 2-PAM concentrations (< 1.25 mM), the kinetics of re-activation of phosphorylated cholinesterases may be represented by Scheme 2. This fits with reported results from experiments performed over a 2-PAM concentration range of 0.1 to 1 mM [6,9,23]. At higher 2-PAM concentrations (> 1.25 mM), the rates of re-activation of paraoxon-phosphorylated recombinant wild-type BuChE and A277W mutant were accelerated. There was no evidence for multiple species of phosphorylated enzyme to account for this behaviour. Such a dependence of k_r on 2-PAM concentration is similar to the excess substrate activation

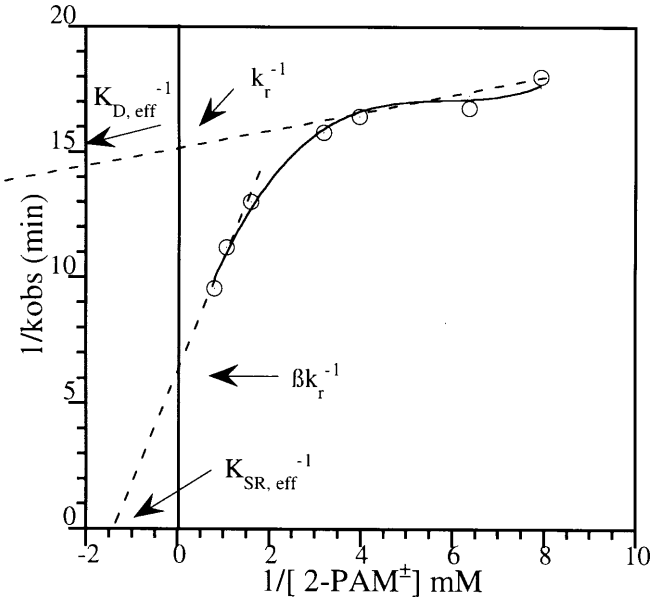


Figure 3 Re-activation of diethylphosphorylated (paraoxon-inhibited) recombinant wild-type BuChE by 2-PAM$^{\pm}$ in 10 mM phosphate buffer, pH 7.0, at 20 °C

Double-reciprocal plot of k_{app} against the calculated ionized 2-PAM$^{\pm}$ concentration. The kinetic parameters calculated from these data are different from those in Table 3 because the sodium phosphate concentration was lower: $k_r = 0.068$ min^{-1}; $\beta k_r = 0.155$ min^{-1}; $K_{D,eff} = 0.027$ mM ($K_{D,app} = 0.22$ mM); $K_{SR,eff} = 0.65$ mM ($K_{SR,app} = 5.22$ mM); $k_{II,eff} = 2450$ M$^{-1}\cdot$min^{-1}; $\beta k_{II,eff} = 5590$ M$^{-1}\cdot$min^{-1}.

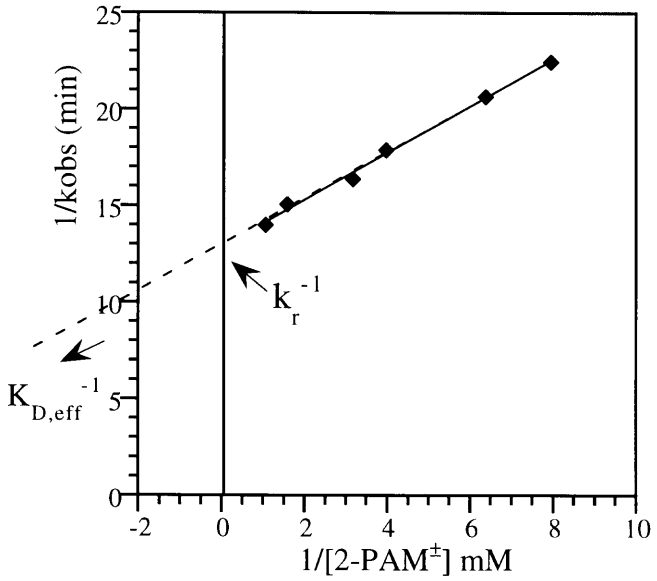
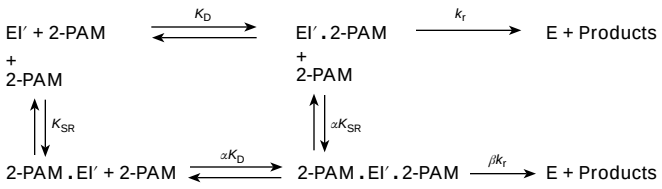


Figure 4 Re-activation of diethylphosphorylated (paraoxon-inhibited) recombinant D70G BuChE by 2-PAM$^{\pm}$ in 10 mM phosphate buffer, pH 7.0, at 20 °C

Double-reciprocal plot of k_{app} against the calculated ionized 2-PAM$^{\pm}$ concentration. The kinetic parameters calculated from these data are different from those in Table 3 because the sodium phosphate concentration was lower: $k_r = 0.078$ min^{-1}; $K_{D,eff} = 0.095$ mM ($K_{D,app} = 0.75$ mM); $k_{II,eff} = 825$ M$^{-1}\cdot$min^{-1}.



Scheme 4

observed for BuChE-catalysed hydrolysis of positively charged substrates [10,32–34]. Thus, by analogy, the 2-PAM re-activation pathway of phosporylated BuChE may be described by Scheme 4, according to which a second 2-PAM molecule binds to the phosphorylated enzyme and leads to acceleration of re-activation. Assuming $\alpha = 1$, the apparent rate constant of re-activation is:

$$k_{app} = \frac{k_r}{1+\frac{K_D}{[2\text{-PAM}]}} \times \frac{1+\frac{\beta[2\text{-PAM}]}{K_{SR}}}{1+\frac{[2\text{-PAM}]}{K_{SR}}} \quad (7)$$

where $\beta > 1$ is a parameter that reflects the extent of acceleration of re-activation by excess 2-PAM and K_{SR} is the dissociation constant of enzyme re-activator complexes for the second re-activator-molecule-binding site. In the present work, the parameter β was estimated graphically from curved plots of $1/k_{app}$ against 1/[2-PAM]. At low 2-PAM concentrations when [2-PAM] $\ll K_{SR}$, eqn. (7) reduces to eqn. (3), and intercepts on the reciprocal plot correspond to K_D^{-1} and k_r^{-1}. At high 2-PAM concentrations, $K_D/[2\text{-PAM}] \to 0$, the intercepts correspond to K_{SR}^{-1} and βk_r^{-1} (Figure 3). Re-activation parameters in 0.1 M sodium phosphate at 20 °C for wild-type BuChE (G_4 tetramer purified from plasma or recombinant enzyme) and recombinant mutants A277W and D70G are listed in Table 3. Although the concentration of oximate [2-PAM$^{\pm}$], the effective re-activator, is higher at pH 8.2 than at pH 7.0 (70% of the total 2-PAM concentration is ionized at pH 8.2 instead of 12.6% at pH 7.0), the rate constant for re-activation, k_r, did not change significantly with pH. However, $K_{D,eff}$ was found to increase with pH, so that the net effect of increasing pH was a decrease in the second-order rate constant $k_{II,eff}$. Since 2-PAM is more stable at pH 7, the re-activation parameters were generally measured at pH 7.0.

The behaviour of the A277W mutant was similar to that of the wild-type enzyme (k_r, k_{II} and β are of the same order), although it showed a slightly higher affinity (K_{SR}) for 2-PAM at high concentration (Table 3). The slightly lower K_{SR} of the A277W mutant may result from additive interactions of 2-PAM on Trp-277 and Asp-70. We have previously found that the A277W mutation did not significantly alter the behaviour of BuChE with BuSCh [10]. Moreover, regarding re-activation of paraoxon-phosphorylated AChE, it has been found that mutation of the homologous residue in human AChE (W289A) to alanine, the amino acid in BuChE at this position, leads to a decrease in k_r but does not affect K_D [8]. This behaviour difference between human BuChE and AChE supports the contention that residue 277 does not play a role in the PAS of BuChE.

Unlike re-activation of phosphorylated wild-type enzyme and A277W mutant, the kinetics of re-activation of the phosphorylated D70G mutant by 2-PAM was Michaelian with respect to oxime concentation (Figure 4). Consequently, the kinetic parameters of re-activation for D70G were the same at low and high 2-PAM concentrations. The re-activation rate

Table 3 Re-activation of diethylphosphorylated (paraoxon-inhibited) wild-type and mutant BuChEs by 2-PAM

The experiment was carried out in 0.1 M phosphate, pH 7.0 and 8.2, at 20 °C. Since pK_a (2-PAM) = 7.84 [23], re-activation kinetics were carried out at pH 7.0 and at pH 8.2, i.e. at two different oximate (2-PAM$^{\pm}$) concentrations.

		Plasma wild-type		rwild-type,	rD70G,	rA277W,
		pH 7.0	pH 8.2	pH 7.0	pH 7.0	pH 7.0
Low [2-PAM] (0.5–1.25 mM)	k_r (min^{-1})	0.054‡	0.092‡	0.055	0.11	0.097
	$K_{D,app}$ (mM)	0.17	0.39	0.26§	2.13	0.22
	$k_{II,app}$ ($M^{-1} \cdot min^{-1}$)	318	236	211	51	441
	$K_{D,eff}$ (mM)*	0.021	0.27‖	0.032	0.27	0.028
	$k_{II,eff}$ ($M^{-1} \cdot min^{-1}$)*	2570	340	1720	410	3460
High [2-PAM] (5–19.5 mM)	βk_r (min^{-1})	0.077	0.35	0.101	0.11	0.15
	$K_{SR,app}$ (mM)	2.20	15.50	4.11	–	1.07
	$\beta k_{II,app}$ ($M^{-1} \cdot min^{-1}$)	450	900	388	–	660
	$K_{SR,eff}$ (mM)*	0.28	10.80	0.51	–	0.12
	$\beta k_{II,eff}$ ($M^{-1} \cdot min^{-1}$)*	3660	1300	3160	410	5360
	β_{app}†	1.4	3.8	1.8	1	1.5

* Apparent parameters of re-activation k_r and K_D were determined from plots of $1/k_{app}$ against 1/[2-PAM]; $k_{II} = k_r/K_D$; effective parameters (denoted 'eff') of re-activation are corrected values taking into account the actual concentration of dipolar ion (2-PAM$^{\pm}$) as a function of pH [eqn. (5)].

† β is defined as the ratio k_r (high [2-PAM])/k_r (low [2-PAM]) [cf. eqn. (7)].

‡ Literature value: $k_r = 0.067$ min^{-1} at all pH values at 25 °C [23].

§ Literature value: $K_{D,app} = 0.30$ mM for di-isopropylphosphorylated BuChE at pH 7.4 at 22 °C [7].

‖ Literature value: $K_{D,eff} = 0.25$ mM in 40 mM boric acid/borax buffer, pH 8.2 at 25 °C [23].

constant, k_r, for D70G was similar to that of the wild-type enzyme at low 2-PAM concentrations, but the affinity for the nucleophilic oxime was reduced 10-fold compared with the wild-type enzyme (K_D in Table 3). The affinity of 2-PAM for D70G was lower as expected since the carboxylate group on residue 70 is missing. The difference in free energy for 2-PAM binding ($\Delta\Delta G$) between wild-type and D70G mutant is 6.68 kJ/mol, a value similar to those found for other ligands/substrates containing one positive charge [35]. The lower affinity of 2-PAM for D70G resulted in a lower k_{II} for the D70G mutant compared with the wild-type enzyme. Similar effects of mutation on residue 70 on 2-PAM re-activation parameters were recently reported for the human AChE mutant D74N [8].

Effect of buffer concentration on 2-PAM-mediated re-activation

As seen in Table 3, although the bimolecular rate constant (k_{II}) for re-activation of the D70G mutant was reduced 4–6-fold, the rate constant of re-activation (k_r) in 0.1 M sodium phosphate, pH 7.0, did not seem to be significantly altered by the mutation. Because we had previously found that k_{cat} for BuSCh hydrolysis depended on ionic strength [10], we wondered whether k_r was also affected by ionic strength. As shown in Figure 5 (top), k_r values for the wild-type enzyme were effectively independent of buffer concentration when the 2-PAM concentration was low. A similar result was reported for re-activation of phosphorylated wild-type BuChE by 2-PAM in 40 mM borate buffer in the presence and absence of 0.5 M NaCl [23]. Figure 5 (top) also shows that, although the k_r values for the D70G mutant are scattered, they are independent of phosphate buffer concentration. On the other hand, Figure 5 (bottom) shows that the K_D for the wild-type enzyme increased about 4-fold on increasing the buffer concentration, whereas there was no significant effect of the buffer concentration on K_D for the D70G mutant. This is consistent with the positively charged 2-PAM interacting first with the negatively charged Asp-70 residue during binding to wild-type enzyme before reaching its final binding position down the active-site gorge (molecular modelling and mutagenesis studies on AChE have shown that this binding site is the 'anionic' subsite [6,8], i.e. Trp-82 in BuChE). Increasing sodium phosphate concentration, i.e. ionic strength, would be expected to decrease the effectiveness of the initial solvent-exposed charge–charge interaction. Binding of 2-PAM to the D70G mutant does not include this coulombic interaction, consistent with the absence of an ionic strength effect on the K_D. As a result, the re-activation of wild-type BuChE at low concentration of 2-PAM was more susceptible to the ionic environment than re-activation of the D70G mutant. Unexpectedly, as seen in Figure 5, βk_r and K_{SR} for wild-type BuChE, and consequently the β factor (inset in the top panel of Figure 5), were strongly dependent on sodium phosphate concentration. These values decreased at high phosphate buffer concentration.

Reversible inhibition by 2-PAM

2-PAM served as a reversible inhibitor of BuSCh hydrolysis by BuChE. The inhibition was competitive with BuSCh at pH 7.0 for both the wild-type and D70G enzymes. Inhibition constants (0.20 mM for wild-type and 3.1 mM for D70G) were similar to the $K_{D,app}$ values for 2-PAM re-activation of phosphorylated BuChE (Table 3).

Aging of phosphorylated enzymes

The rate of aging of phosphylated cholinesterases is known to be dependent on the electronegativity of the alkoxy groups on the phosphorus atom [36]. Therefore the rates of aging (k_a) of the diethoxyphosphorylated conjugates (paraoxon-inhibited enzymes) were found to be 5 to 12 times lower than those of the di-isopropyloxyphosphorylated conjugates (DFP-inhibited enzyme) (Figure 6). Corresponding half-times of aging [$\ln(2/k_a)$] for paraoxon- and DFP-phosphorylated wild-type BuChE and D70G mutant are given in Table 4. The D70G mutation slowed the aging process, indicating that residue 70 exerts indirect control on the aging process.

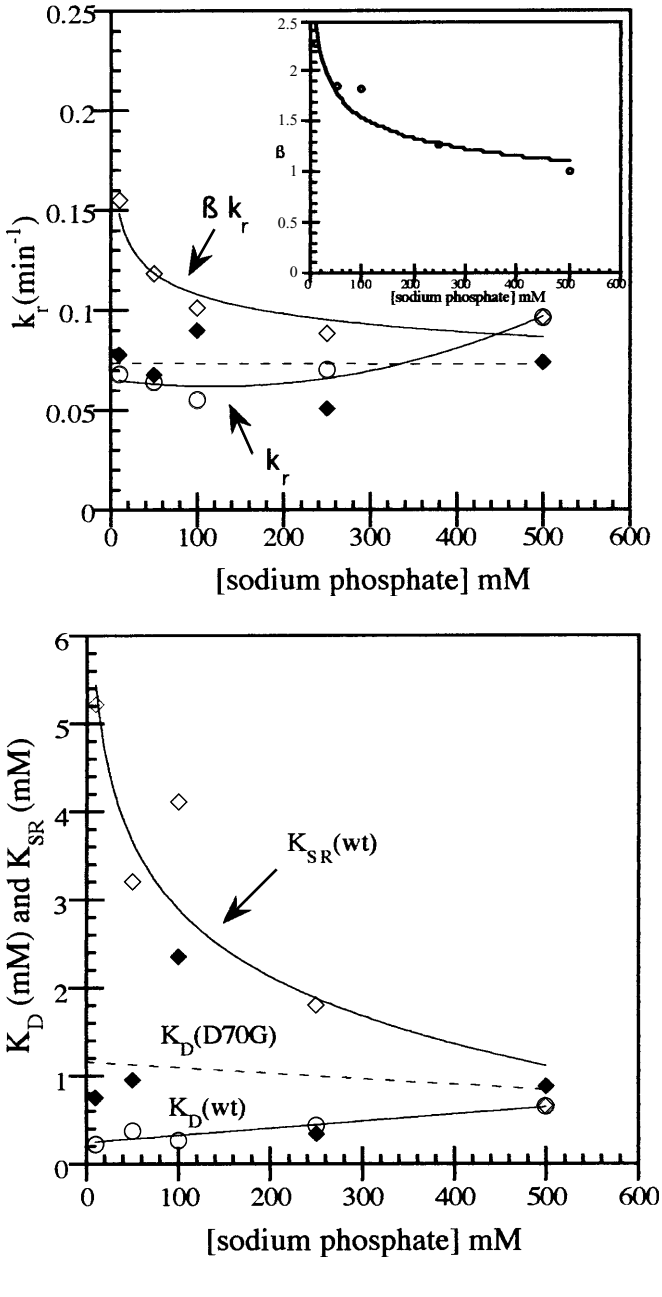


Figure 5 Dependence of the re-activation parameters (k_r, K_D, K_{SR} and β) on sodium phosphate concentration for paraoxon-phosphorylated recombinant wild-type BuChE and the D70G mutant at pH 7.0 and 20 °C

Solid lines and open symbols are for wild-type BuChE: ○, at low [2-PAM] (0.5–1.25 mM); ◇, at high [2-PAM] (> 1.25 mM). Dashed lines and filled symbols (◆) are for the D70G mutant. Top, k_r versus [PO_4] (inset β versus [PO_4]); bottom, K_D and K_{SR} versus [PO_4].

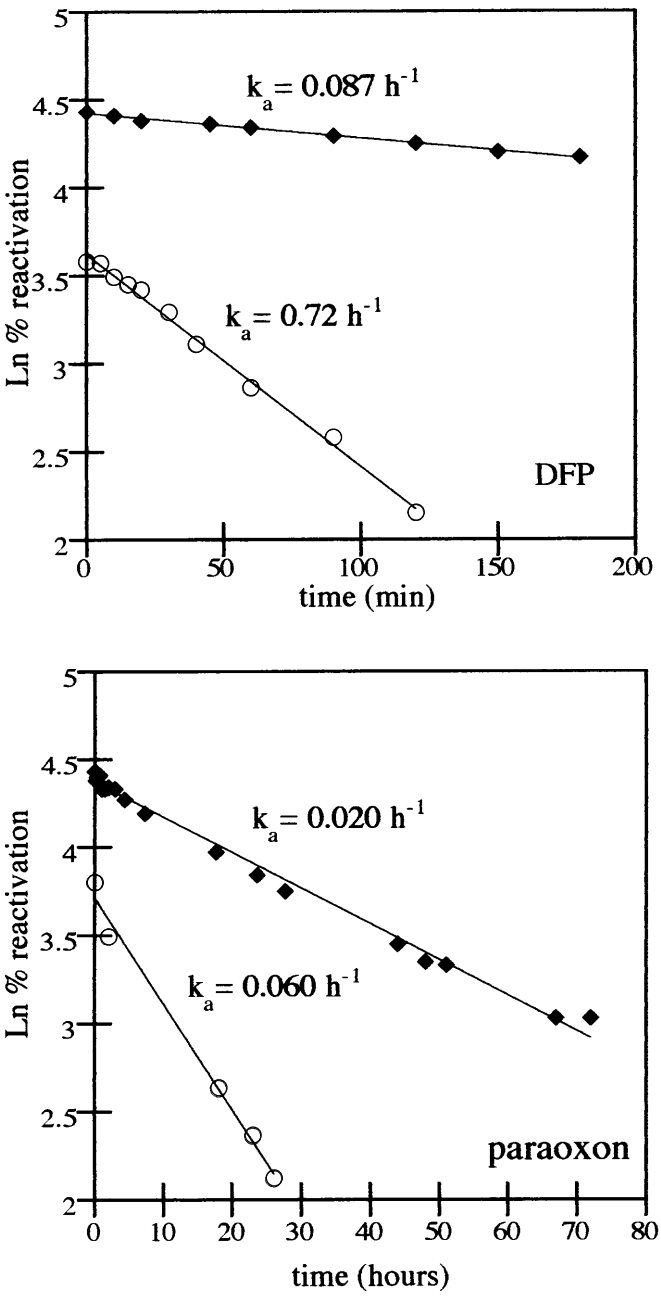


Figure 6 Decrease with time of the percentage of re-activation by 2-PAM (10 mM) of phosphorylated BuChE

DFP- (top) and paraoxon- (bottom) phosphorylated recombinant wild-type BuChE (○) and D70G mutant (◆) at pH 8.0 and 20 °C. The slope of the plots is the first-order rate constant of aging.

DISCUSSION

Organophosphate inhibition

Mutation at Asp-70 leads to enzymes with a lower affinity for positively charged organophosphates. We found that the K_i of the D70G mutant for echothiophate was 50–300-fold higher than that of wild-type BuChE (Table 2). This does not fit the general observation that the D70G mutant has a 10-fold lower affinity than wild-type enzyme for substrates/ligands containing one positive charge and a 100-fold lower affinity for compounds containing two positive charges [35]. According to data in Table 2, the mean difference in free energy of binding of echothiophate to the two enzymes is close to 12.5 kJ/mol, a value similar to those calculated for bulky substrates/ligands containing two positive charges which interact simultaneously with Asp-70 and Trp-82, whereas compounds having only one charge transiently bind to Asp-70 and then slide on to Trp-82 to form the Michaelis complex [54]. Thus the pronounced effect of the D70G mutation on the affinity for echothiophate cannot be explained simply.

Table 4 Half-times of aging [$\ln(2/k_a)$] of paraoxon- and DFP-inhibited wild-type BuChE and its D70G mutant in 50 mM sodium phosphate, pH 8.0, at 25 °C

Results are means ± S.E.M. for determinations in triplicate.

	Paraoxon		DFP	
	$t_{1/2}$ (h)	Ratio ($t_{1/2}$ paraoxon)	$t_{1/2}$ (h)	Ratio ($t_{1/2}$ DFP)
Wild-type	12 ± 2.5*	1	1.0 ± 0.1†	1
D70G	39 ± 6	3.2	8.2 ± 1.4	8.2

* Literature values for $t_{1/2}$ of diethoxyphosphorylated human BuChE (inhibited by the oxo form of triazophos) at pH 7.4 were found to be 12 and 39 h at 37 and 22 °C respectively [37].

† Literature values for $t_{1/2}$ of DFP-inhibited human BuChE at pH 7.8 and 37 °C was found to be 0.50 h [38].

From inhibition kinetic studies of *Torpedo* AChE [39] and computer modelling (docking) of thiocholine-containing organophosphates in wild-type mouse AChE and mutant F297I (F290 in *Torpedo* AChE) [40], it appears that, because of the tetrahedral configuration of the organophosphate molecule, the leaving group (thiocholine) must be directed out of the gorge to maximize phosphorylation rate. These results suggest that, in the echothiophate–BuChE Michaelis complex, the thiocholine group does not interact with Trp-82 as does thiocholine in thiocholine-ester–BuChE Michaelis complexes. Therefore it can be proposed that in the echothiophate–BuChE Michaelis complex, the thiocholine leaving group is oriented toward the mouth of the active-site gorge and interacts with Asp-70. By extension, mutations on Asp-70 may alter sensitivity to positively charged covalent inhibitors in general including carbamoylating agents. For example, the positively charged carbamate R02-0683, which is used to phenotype the atypical variant [35], reacts more slowly with atypical BuChE [41], having a second-order inhibition constant (carbamoylation rate divided by dissociation constant) that is lower by two orders of magnitude.

2-PAM re-activation

Re-activation of phosphorylated wild-type BuChE by 2-PAM showed a complex dependence of k_{app} on 2-PAM concentration. At high 2-PAM concentrations the re-activation process was accelerated. Re-activation of the phosphorylated D70G mutant did not show this complexity. The dependence of k_{app} on 2-PAM concentration followed a simple saturating (Michaelian) profile. These observations may be rationalized by analogy with the hydrolysis kinetics of BuSCh by BuChE. According to this analogy the activation of re-activation kinetics by high 2-PAM concentrations results from binding of a second 2-PAM molecule on a site affected by Asp-70. Asp-70 was previously identified as the PAS of BuChE and it has been shown to be involved in activation by excess substrate [10]. Thus we propose that 2-PAM can bind to phosphorylated wild-type BuChE at two sites: on Trp-82, the actual 'anionic site', at low concentration, and on both Asp-70 and Trp-82 at high concentrations. The present results support the proposal that residue Asp-70 is important in optimizing the conformation and reactivity of the active site. The remote effect of binding on Asp-70 may occur through a conformational change involving the Ω loop between Cys-65 and Cys-92. Indeed, the Ω loop has been shown to be involved in allosteric modulation of AChE by ligands of the PAS [42]. Moreover, a molecular-dynamics approach revealed that the Ω loop is highly mobile in AChE and that its movements affect the conformation of the side chain of Trp-84 (equivalent to Trp-82 in BuChE) [43].

Effects of buffer concentration on 2-PAM re-activation

Our results for the kinetics of oxime-mediated re-activation at low 2-PAM concentration (< 1.25 mM) support the hypothesis that Asp-70 is a peripheral sensor which buffers the catalytic machinery of BuChE against changes in the ionic strength of the medium. However, at high 2-PAM concentrations, where K_{SR} data are not consistent with K_D data, the effects of phosphate on oxime re-activation of phosphorylated BuChE cannot be explained in terms of ionic-strength-dependence. We should point out that, at high 2-PAM concentration, the observed effects of the sodium phosphate concentration on the re-activation parameters of diethoxyphosphorylated wild-type and D70G mutant BuChE are opposite to the effects of salts on hydrolysis at V_{max} of positively charged substrates (BuSCh and benzoylcholine) by BuChE phenotypes. For example, the activity of the 'atypical' enzyme (D70G) with BuSCh as substrate was found to be strongly inhibited by phosphate, whereas the 'usual' (wild-type) enzyme was found to be more resistant, showing slight activation up to 50 mM phosphate followed by weak inhibition at higher concentrations [44]. Hydrolysis of BuSCh by the D70G mutant was also found to be strongly inhibited by NaCl, whereas the maximum velocity, V'_{max}, at intermediate substrate concentrations of the wild-type enzyme was found to be slightly increased with NaCl concentrations up to 0.5 M and then inhibited at higher concentrations [10]. Similar effects of NaCl were observed on both phenotypes with benzoylcholine as substrate [45]. Moreover, we found that K_{SR} decreased with sodium phosphate concentration whereas in BuChE-catalysed hydrolysis of BuSCh the second substrate dissociation constant K_{SS} was found to increase with NaCl concentration [10]. These results indicate that the effects of phosphate and NaCl on BuChE-catalysed hydrolysis of substrates are related to ionic strength. In contrast, the buffer-concentration-dependence of re-activation parameters of phosphorylated BuChE at high 2-PAM concentrations would suggest a more complex effect. A lyotropic effect (salting-out) of phosphate ion on oxime re-activation, binding of phosphate to specific sites and/or even formation of complex between phosphate and 2-PAM cannot be ruled out. Moreover, several lines of evidence indicate that the conformation of non-aged phosphorylated cholinesterases is slightly different from that of native ones [46–48]. As a consequence, the conformational plasticity of phosphorylated BuChE is expected to be different from that of the non-inhibited enzyme. Therefore an abnormal response of phosphorylated BuChE to environmental perturbation may also be considered and could explain the unexpected effects of phosphate on re-activation by high concentrations of 2-PAM.

Aging

Our results clearly demonstrate that Asp-70 has an effect on the rate of aging of phosphorylated cholinesterase adducts. Other important residues involved in the aging process of phosphylated cholinesterases have been identified: Glu-199 in soman-phosphonylated *Torpedo* AChE [49] and Glu-202 in human AChE [50] (both are equivalent to Glu-197 in human BuChE) and Trp-84 in soman-phosphonylated human AChE [51] (equivalent to Trp-82 in human BuChE). These residues are in close contact with the alkyl chain (the pinacolyl chain in soman) which is removed from the phosphyl moiety during aging and have been found to be involved in stabilization of the developing

carbonium intermediate. We also found that Trp-82 and Glu-197 play a key role in aging of soman- or DFP-inhibited BuChE (P. Masson, M. T. Froment, C. Bartels and O. Lockridge, unpublished work). Here we are the first to report that a residue located at the rim of the active-site gorge plays a role in aging of diethoxy- or di-isopropyl-phosphorylated BuChE. Understanding how the remote Asp-70 takes part in the dealkylation reaction of organophosphyl conjugates of cholinesterases remains to be solved.

Relevance to people carrying the D70G mutation

In North American and European populations, a single allele of the atypical variant of butyrylcholinesterase (D70G) is carried by 1 in 50 people. Two alleles of D70G are carried by 1 in 2500 people [52]. People who are homozygous for the D70G mutation experience post-anaesthetic apnoea from succinyldicholine and mivacurium [52]. They are unable to breathe for 2 h, whereas the dose of muscle relaxant they received was intended to paralyse them for 3 min. Our results allow us to predict that people homozygous for the D70G mutation will be more sensitive to the toxic effects of positively charged organophosphates (echothiophate). Their BuChE will bind less echothiophate, leaving a larger dose for reaction with AChE. The toxic effects of organophosphates are caused by inhibition of AChE. In contrast, exposure to an uncharged organophosphate such as paraoxon or DFP will be equally toxic to persons with wild-type and atypical BuChE [7,53]. The second-order rate constants for phosphorylation are the same for wild-type and D70G when the organophosphate is uncharged. Previous studies on oxime re-activation of phosphorylated wild-type and D70G mutant BuChE gave contradictory results [7,27] because data were collected at single 2-PAM concentrations only. The present results show that the D70G mutant is resistant to oxime re-activation because of a lower binding affinity for 2-PAM. Nevertheless, the person with atypical BuChE may show a faster recovery of BuChE activity after exposure to either echothiophate or paraoxon because atypical BuChE ages slowly compared with wild-type BuChE. The lower rate of aging of the D70G mutant means that there is a longer opportunity for the covalent bond between the enzyme and the phosphoryl moiety to be broken by nucleophilic re-activators.

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