Hydration change during the aging of phosphorylated human butyrylcholinesterase: importance of residues aspartate-70 and glutamate-197 in the water network as probed by hydrostatic and osmotic pressures

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Wild-type human butyrylcholinesterase (BuChE) and Glu-197 → Asp and Asp-70 → Gly mutants (E197D and D70G respectively) were inhibited by di-isopropyl phosphorofluoridate under standard conditions of pH, temperature and pressure. The effect of hydrostatic and osmotic pressures on the aging process (dealkylation of an isopropyl chain) of phosphorylated enzymes [di-isopropylated (DIP)-BuChE] was investigated. Hydrostatic pressure markedly increased the rate of aging of wild-type enzyme. The average activation volume ($\Delta V^\ddagger$) for the dealkylation reaction was -170 ml/mol for DIP wild-type BuChE. On the other hand, hydrostatic pressure had little effect on the aging of the DIP mutants ($\Delta V^\ddagger = -2.6$ ml/mol for E197D and -2 ml/mol for D70G), suggesting that the transition state of the aging process was associated with an extended hydration and conformational change in wild-type BuChE, but not in the mutants. The rate of aging of wild-type and mutant enzymes decreased with osmotic pressure, allowing very large positive osmotic activation volumes ($\Delta V_{\text{osm}}^\ddagger$) to be estimated, thus probing the participation of water in the aging process. Molecular dynamics simulations performed on the active-site gorge of the wild-type DIP adduct showed that the isopropyl chain involved in aging was highly solvated, supporting the idea that water is important for stabilizing the transition state of the dealkylation reaction. Wild-type BuChE was inhibited by soman (pinacolyl methylphosphonofluoridate). Electrophoresis performed under high pressure [up to 2.5 kbar (1 bar = 10^5 Pa)]

showed that the soman-aged enzyme did not pass through a pressure-induced, molten-globule transition, unlike the native wild-type enzyme. Likewise, this transition was not seen for the native E197D and D70G mutants, indicating that these mutants are resistant to the penetration of water into their structure. The stability energetics of native and soman-aged wild-type BuChE were determined by differential scanning calorimetry. The pH-dependence of the midpoint transition temperature of endotherms indicated that the high difference in stabilization energy between aged and native BuChE ($\Delta\Delta G = 23.7$ kJ/mol at pH 8.0) is mainly due to the salt bridge between protonated His-438 and PO^- , with $\text{p}K_{\text{His-438}} = 8.3$. A molecular dynamics simulation on the MIP adduct showed that there is no water molecule around the ion pair. The 'hydrostatic versus osmotic pressure' approach probed the importance of water in aging, and also revealed that Asp-70 and Glu-197 are the major residues controlling both the dynamics and the structural organization of the water/hydrogen-bond network in the active-site gorge of BuChE. In wild-type BuChE both residues function like valves, whereas in the mutant enzymes the water network is slack, and residues Gly-70 and Asp-197 function like check valves, i.e. forced penetration of water into the gorge is not easily achieved, thereby facilitating the release of water.

Key words: hydrostatic pressure, molecular dynamics, osmotic pressure, phosphorylation.

INTRODUCTION

Cholinesterases [acetylcholinesterase (AChE; EC 3.1.1.7) and butylcholinesterase (BuChE; EC 3.1.1.8)] are inhibited by organophosphorus compounds via phosphorylation of their active-site serine [1] (Scheme 1). Resolution of the crystal structure of *Torpedo californica* AChE revealed that the active-site serine (Ser-200) of cholinesterases is located at the bottom of a 20-Å (2-nm) narrow gorge [2]. (Here, and throughout the paper, italicized numbers refer to the numbering of amino acid residues in *T. californica* AChE.) Phosphorylation of AChE at neuronal and neuromuscular synapses is the main cause of symptoms of organophosphorus poisoning [3]. Phosphorylated cholinesterases can be reactivated by nucleophilic agents, e.g. oximes such as 2-

pyridine aldoxime methiodide (2-PAM), which are used as antidotes. However, phosphorylated enzymes can progressively lose their reactivatability by nucleophilic reactivators. This process, termed 'aging', is due to the dealkylation of a branched alkoxy chain (-OR) on the phosphorus atom (Scheme 1) [1,4]. The mechanism of the dealkylation reaction and the molecular basis of the resistance of aged cholinesterases to reactivators are not completely elucidated [5–9]. Although there is solid evidence for a 'push-pull' mechanism [7,10], some features are still controversial [11–13]. The unimolecular scission of the C–O bond in the alkoxy chain involves a carbocationic transition state, which leads to the release of a carbonium ion and the formation of a negatively charged monophosphoryl ester of serine. Several residues in the active-site gorge are involved in the

Abbreviations used: BuChE, butyrylcholinesterase; AChE, acetylcholinesterase; 2-PAM, 2-pyridine aldoxime methiodide; DFP, di-isopropyl phosphorofluoridate; DIP, di-isopropylphosphoryl; MIP, monoisopropylphosphoryl; DSC, differential scanning calorimetry; H-bond, hydrogen bond; SBMD, Stochastic Boundary Molecular Dynamics.

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a, free enzyme; b, organophosphate; c, phosphylated enzyme; d, carbocationic intermediate; e, dealkylation products; f, aged enzyme.

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(25 °C) high-pressure steel vessel [23]. At t_0 , the DFP-phosphorylated enzyme solutions were introduced in polypropylene microtubes stopped by a latex membrane that transmits pressure. The microtubes were then immersed in the high-pressure vessel and submitted to pressure for a time t_{aging} . The pressure was generated by compression of a hydraulic fluid [23]. Sealing of the high-pressure vessel and compression time took approx. 3 min. After t_{aging} , the pressure was released in less than 1 min and 2-PAM (1 mM final concentration) was immediately added to the depressurized enzyme solutions. Since the pH of the phosphate buffer decreased with pressure (-0.44 unit/kbar), the observed rate constants of aging ($k_{a,\text{obs}}$) were corrected, because aging is accelerated when the pH is decreased [25] according to the equation:

$$k_a = k_{a,\text{obs}}/[1 + \Delta[H^+]/K_i] \quad (2)$$

where $\Delta[H^+]$ is the increase in H^+ concentration with pressure and K_i is the ionization constant of His-438, whose protonated form plays a crucial role in the dealkylation reaction. The pK value of His-438 was assumed to be identical for wild-type and mutant enzymes; for the DIP adduct, the pK of His-438 was assumed to be 0.5 unit higher compared with free enzyme [7]. The pH-dependence of dealkylation of soman-phosphorylated wild-type and E197D BuChEs yielded pK values of between 7.3 and 8.0 for His-438 [10]. Accordingly, the pK value of His-438 for DIP adducts of wild-type and mutant enzymes was set to 7.35. The pressure (P) dependence of the aging rate constants (k_a) allowed activation volumes ($\Delta V^\ddagger$) to be determined for the dealkylation reaction:

$$(\partial \ln k_a / \partial P)_T = -\Delta V^\ddagger / RT \quad (3)$$

where R is the gas constant and T is the absolute temperature.

Aging of DFP-phosphorylated enzymes under osmotic pressure

Osmotic stress provides a convenient method for probing the participation of water in macromolecular systems [26,27]. Osmotic pressure was generated by osmolytes added to the DFP-phosphorylated enzyme solutions at t_0 . After t_{aging} , 2-PAM (1 mM final concentration) was added to osmolyte-containing solutions of phosphorylated enzyme. Osmolytes used were fructose, sucrose and dextran of M_r 500 000 in 50 mM sodium phosphate, pH 8.0. The concentrations of osmolytes used were in the range 0–9% (w/v) for fructose, 0–3% (w/v) for sucrose, and 0–6% (w/v) for dextran. Osmotic pressure of osmolyte-containing solutions was measured using a freezing-point depression osmometer. Viscosity of solutions was determined using a falling-ball viscosimeter.

In osmotic-stress experiments, the hydration layer of the protein acts like a dialysis membrane. Increasing Π (i.e. decreasing water activity) outside the hydration layer tends to strip water molecules off the hydration layer, which in turn tends to decrease the hydration of the protein. Thus the effect of Π on k_a allowed an osmotic activation volume ($\Delta V_{\text{osm}}^\ddagger$) to be determined:

$$(\partial \ln k_a / \partial \Pi)_T = -\Delta V_{\text{osm}}^\ddagger / RT \quad (4)$$

Although osmotic pressure reverses the effects of hydrostatic pressure on proteins [28], $\Delta V_{\text{osm}}^\ddagger$ is not related to $\Delta V^\ddagger$ determined from the dependence of kinetic constants on hydrostatic pressure. Hydrostatic pressure acts on intramolecular distances and density, and thus affects the compressibility of the system, whereas osmotic pressure causes only the removal of water molecules from the system. Therefore $\Delta V_{\text{osm}}^\ddagger$ was used to probe hydration changes accompanying the aging reaction, and to estimate the change in the number of water molecules (n_w) in the solute-inaccessible space (i.e. the active-site gorge and the enzyme

surface) as a function of Π [26,27]. Assuming no change in water density, $n_w = \Delta V_{\text{osm}}^\ddagger / v_w$, where v_w is the molecular volume of water ($30 \text{ \AA}^3$).

Molecular dynamics simulations

To understand $\Delta V^\ddagger$, $\Delta V_{\text{osm}}^\ddagger$ and behavioural differences between wild-type and mutant enzymes, molecular dynamics simulations were performed. The three-dimensional model of wild-type human BuChE used for the calculations was kindly provided by Dr. Harel [29]. All calculations were performed on a SGI Power Indigo 2 workstation and a SGI Power Challenge $4 \times R8000$ advanced workstation. The DIP enzyme employed was the same as that used in our previous study [8]. For construction of the DFP-aged enzyme (MIP adduct), the O-isopropyl bond was simply broken and the resulting isopropyl group deleted. Charges for the dealkylated phosphorylated serine were derived from *ab initio* calculations using the program DMol 2.3.5 (MSI; San Diego, CA, U.S.A.). The aged enzyme structure was then minimized for 200 steepest-descent steps using the molecular mechanics program CHARMM 23.2.

Stochastic Boundary Molecular Dynamics (SBMD) runs [30] were performed on DIP- and MIP-BuChE using a paralleled version of CHARMM 23.2 [31] (MSI). For non-bond interactions, a cut-off of 11 Å was applied with a switching function between 8 and 10 Å. The dielectric constant was set to 1. The reaction region was defined by a 23-Å sphere centred on the Oδ2 atom of residue Asp-70. A 2-Å layer surrounding the reaction region served as the buffer region; the remaining atoms of the protein constituted the reservoir region. All non-hydrogen atoms of the protein in the buffer region were harmonically constrained using a boundary force. We used the simplified Debye–Waller factor values of Nakagawa et al. [32] in our calculations. The force was weighted by a switching function that decreased from 0.5 to 0 when proceeding from the buffer/reservoir boundary to the reaction region [30]. Therefore all atoms in the reaction region were allowed to move freely. Solvation of the active site was achieved by using a pre-equilibrated sphere (25-Å radius) centred on the Oδ2 atom of residue Asp-70. Water molecules overlapping the enzyme (cut-off of 2.8 Å) were deleted. Four successive random rotations of this sphere were applied to minimize the remaining holes in the covalent adduct. For the solvent boundary forces, a radial distribution function centred on the Oδ2 atom of residue Asp-70 was applied to the oxygen atoms [33]. The frictional coefficients related to the stochastic forces were assigned as follows: 62 ps for the heavy atoms in the protein and 200 ps for water oxygens [30,33]. SHAKE [34] was used to constrain bonds with hydrogen atoms, allowing a 2 fs time step. The SBMD simulation was performed by equilibrating each system at 300 K during 30 ps. Production dynamics of 1 ns were then performed. Coordinates were saved every 4 ps in each simulation.

Stability of soman-aged BuChE

Wild-type BuChE was inhibited by racemic soman in 50 mM phosphate, pH 7.0. Under these conditions, the aging reaction was completed in less than 30 min. The stability of soman-aged wild-type BuChE was investigated by studying heat and pressure denaturation using DSC and electrophoresis under hydrostatic pressure. DSC was performed at different pH values [18,35], and in different solvents (water and $^2\text{H}_2\text{O}$) using a micro-DSC III high-sensitivity scanning calorimeter (Setaram, France). The protein concentration was 1–2 mg/ml, and the scan rate was 0.1 °C/min. DSC was used to obtain quantitative information on the energetics of stabilization of aged enzyme. Electrophoresis

of native and aged BuChE under high hydrostatic pressure was performed in capillary gels of different acrylamide concentrations, as described previously [36,37]. Electrophoresis allowed us to construct Ferguson plots, i.e. a plot of the logarithm of mobility of tetramers (m , relative migration distance) against acrylamide concentration ($\%A = 4$ to 6.5%), at different pressures:

$$\log m = \log Y_0 - K_r \cdot A \quad (5)$$

where the retardation coefficient, K_r , depends on the molecular radius and conformation of the protein [38]. Secondary plots (K_r against P) were also drawn.

RESULTS AND DISCUSSION

Kinetics of aging under hydrostatic pressure

Hydrostatic pressure increased considerably the rate of aging of DIP wild-type BuChE, but produced almost no effect on the rate of aging of DIP mutant enzymes. Pressure produced the same effect on both natural and recombinant DIP wild-type BuChE: for both types of enzyme, pressure accelerated the rate of aging to the same extent, but did not induce dissociation of tetramers. This indicates that differences in glycosylation between natural and recombinant enzymes do not affect either the pressure stability of the enzyme quaternary structure or the dependence of the aging reaction on pressure. In fact, it has long been known that removal of the sialic acid residues of human BuChE has no effect on the kinetic properties of either the wild-type or mutant enzymes [39,40]. Recent studies showed that neither the composition nor the size of attached glycans affect catalytic or inhibitory properties of recombinant cholinesterases [41,42]. The oligosaccharide units influence the pharmacokinetics of both tissue-derived and genetically engineered cholinesterases [43–47], and are also important for enzyme protection against irreversible heat inactivation [41].

The pressure-induced acceleration of the rate of aging of the phosphorylated wild-type enzyme was of such great magnitude that no measurement was possible beyond 1 kbar (Figure 1). Under standard conditions (P_0 , 25°C), the aging half-time ($t_{1/2}$)

Figure 1 Hydrostatic pressure-dependence of the rate of aging (k_a) of DFP-phosphorylated recombinant wild-type BuChE ($\bullet$) and its D70G ($\blacktriangle$) and E197D ($\blacksquare$) mutants at pH 8.0 and 25°C

For further details, see the Materials and methods section.

Table 1 Effects of hydrostatic pressure on the half-time of aging of recombinant wild-type and mutant DIP-BuChEs at pH 8.0 and 25°C

Results are shown as means $\pm$ S.E.M. for at least three independent determinations.

	$t_{1/2}$ (min) at P_0^*	$t_{1/2}$ (min) at 1 kbar	$\Delta V^\ddagger$ (ml/mol)
Wild-type	60 ± 5	$0.05 \pm x^\dagger$	-170 ± 120
E197D	360 ± 100	320 ± 5	-2.6 ± 0.4
D70G	490 ± 80	450 ± 5	-2 ± 0.3

* Data obtained from [8]; $x^\dagger$ is the interval time: 3.6×10^{-4} min for $\Delta V^\ddagger = -290$ ml/mol and 7.5 min for $\Delta V^\ddagger = -50$ ml/mol.

of DIP wild-type of 1 h was 6- and 8-fold faster than that of DIP E197D and D70G mutants respectively [8]; at 1 kbar, the aging $t_{1/2}$ for wild-type BuChE was estimated to be ≈ 0.1 min, i.e. 2500–3700-fold faster than for mutants (Table 1). The average pressure-acceleration factor of the aging process (higher than 400 at P_0 and 1 kbar for DIP wild-type BuChE) is due to a highly negative activation volume for the dealkylation (DIP $\rightarrow$ MIP) of the adduct. As shown in Table 1, $\Delta V^\ddagger$ for the dealkylation of DIP wild-type BuChE is -170 ± 120 ml/mol. In contrast, $\Delta V^\ddagger$ is slightly negative for the E197D and D70G mutants (≈ -2 ml/mol), and yet wild-type and mutant enzymes catalysed the same unimolecular reaction. Therefore, in order to interpret the differences in magnitude of $\Delta V^\ddagger$ between wild-type and mutants, it must be borne in mind that activation volume (the difference between the partial molar volume of the transition state and the partial molar volume of the DIP adduct) is a composite parameter, resulting from several additive contributions [22–24]:

$$\Delta V^\ddagger = \Sigma \Delta V_{\text{int},i}^\ddagger + \Delta V_{\text{conf}}^\ddagger + \Delta V_{\text{w}}^\ddagger \quad (6)$$

where $\Sigma \Delta V_{\text{int},i}^\ddagger$ is the volume change associated with the stretching and partial breakage of the covalent bond (i.e. C–O) and formation and disruption of non-covalent bonds around the isopropyl chain to be released during the activation step, $\Delta V_{\text{conf}}^\ddagger$ is the volume change associated with enzyme conformational changes, and $\Delta V_{\text{w}}^\ddagger$ is the volume change due to hydration/water-structure alterations around reacting groups.

The average $\Delta V_{\text{int}}^\ddagger$ for the cleavage of a single bond (C–O) is $+10$ ml/mol [48], and electrostriction of water around the developing carbocation contributes about -20 ml/mol to $\Delta V_{\text{w}}^\ddagger$ [49]. Since rotation around a single bond is associated with a small positive activation volume ($+2$ to $+10$ ml/mol) [44], $\Delta V_{\text{conf}}^\ddagger$ is expected to be positive. Thus the highly negative value of $\Delta V^\ddagger$ for DIP wild-type BuChE suggests that the dealkylation transition state is associated with an extended conformation/hydration change. Although fourth-derivative UV spectra of aged wild-type BuChE indicate that the conformational change upon aging must be very limited (results not shown), gel-electrophoresis data suggest an increase in the hydrodynamic radius of aged BuChE (e.g. see Figure 4). Thus, although $\Delta V_{\text{conf}}^\ddagger$ cannot be estimated experimentally, it can be stated that there is a conformational contribution to the overall $\Delta V^\ddagger$. However, because the partial molar volume of water ($v_{\text{w},N}$) is 18 ml/mol at P_0 and 25°C , the highly negative value of $\Delta V^\ddagger$ for dealkylation of DIP wild-type BuChE suggests that several water molecules are involved in the stabilization of the carbocationic dealkylation transition state of DIP wild-type BuChE. Thus, on the basis of the dependence of the rate of aging on pressure, the negative $\Delta V_{\text{w}}^\ddagger$ appears to provide the dominant contribution to activation volume for aging of phosphorylated wild-type BuChE. In con-

Figure 2 Osmotic pressure dependence of the rate of aging (k_a) of DFP-phosphorylated wild-type BuChE (●), and its D70G (▲) and E197D (■) mutants at pH 8.0 and 25 °C

Upper panel: osmotic pressure generated by fructose; lower panel: osmotic pressure generated by sucrose.

trast, the small negative values of $\Delta V^\ddagger$ for dealkylation of the DIP E197D and D70G mutants suggest that contribution of $\Delta V_w^\ddagger$ to these activation volumes is small.

With respect to phosphorylated mutants, it is noteworthy that the mutation E197D (loss of one methylene group) slows the rate

of aging at P_0 by a factor of only 6, but almost eliminates the responsiveness of the DIP E197D conjugate to hydrostatic pressure. In human AChE, the mutation E202D slows the rate of aging of DFP-phosphorylated enzyme by a factor of 17 [6]. The carboxy group of residue Glu-202(199), equivalent to Glu-197 in human BuChE, contributes to stabilization of the carbocationic transition state, but this carboxylate was also proposed to be involved in the hydrogen-bond (H-bond) network, together with Glu-450(443), Tyr-133(130) and water molecules [6,12,50]. In the E202D mutant, the different orientation of the carboxylic acid side chain precludes the formation of the H-bond network. A similar change in the positioning of the Asp-197 side chain is seen in the modelled E197D mutant of BuChE. Thus the E197D mutation might cause disruption of the H-bond network at the bottom of the active site, and render the active-site gorge of mutant less solvated than that of the wild-type enzyme. The residue Asp-70, located at the rim of the active-site gorge [2,29], does not interact with the DIP moiety, and yet it controls the dealkylation reaction. The indirect effect of the mutation D70G on the aging process was interpreted in terms of change in the conformational state of Trp-82(84), owing to motion of the Ω -loop (C65–C92) [8]. However, the small value of $\Delta V^\ddagger$ for dealkylation of the DIP D70G conjugate suggests that the loss of the Asp-70 carboxylate (which is H-bonded to Tyr-332 and water molecules) in the D70G mutant has caused disruption of the H-bond/water network at the top of the active-site gorge.

Kinetics of aging under osmotic pressure

Osmotic pressures lower than 10 bar, generated by low concentrations of fructose or sucrose (up to 9%, w/v), had an opposite effect to that of hydrostatic pressure on the rate of aging: Π strongly decreased the rate of aging (Figure 2), yielding highly positive $\Delta V_{\text{osm}}^\ddagger$ values for DIP wild-type BuChE and both DIP mutants (Table 2). Using dextran 500000 as the osmolyte (up to 6%, w/v), $\Delta V_{\text{osm}}^\ddagger$ was found to be ≈ 2000 ml/mol ($n_{w,0} = 110$) for DIP wild-type BuChE, and > 3500 ml/mol ($n_{w,0} > 200$) for both DIP mutant enzymes. Other sugars produced a similar effect. The calculated $t_{1/2}$ values at $\Pi = 10$ bar generated by fructose or sucrose (Table 2) indicate that mutant enzymes are far more sensitive to osmotic pressure than is wild-type BuChE. Osmotic stress caused a considerable decrease in the rate of aging of mutants ($k_a = \ln 2/t_{1/2}$), whereas it produced only a small effect on the rate of aging of DIP wild-type BuChE ($\Pi = 10$ bar decreased by twice this rate). Viscosity measurements showed that sugars up to a concentration of 9% (w/v) did not increase significantly the viscosity of the buffer [e.g. the relative viscosity of buffer containing 12% (w/v) fructose compared with

Table 2 Effect of osmotic pressure generated by fructose (a) and sucrose (b) on the half-time of aging of DIP-BuChE at pH 8.0 and 25 °C

	$t_{1/2}$ (min) at P_0^*	$t_{1/2}$ (min) at $\Pi = 10$ bar	$\Delta V_{\text{osm}}^\ddagger$ (ml/mol)	$n_{w,0}$
(a)				
Wild-type	60	120	+1700	90
E197D	360	10500	+8100	450
D70G	490	12000	+7700	430
(b)				
Wild-type	60	100	+1300	70
E197D	360	3700	+5600	310
D70G	490	6200	+6100	340

* Data obtained from [8].

Figure 3 Side view of the solvated active-site gorge of the DIP wild-type BuChE conjugate

Water molecules are represented as spherical balls; the isopropyl chain to be dealkylated (shown as IP) faces Trp-82; the other IP chain, close to Trp-231, is the alkyl chain that will remain on the phosphorus atom after aging. Also shown are His-438 and Glu-197, which are involved in carbocation formation.

buffer without additive, $\rho_r = 1.33$]. In contrast, dextran 500000 generated a moderate Π , but caused a dramatic increase in medium viscosity [with dextran 6% (w/v), $\rho_r = 8.4$]. This indicates that the viscosity of the osmolyte-containing buffer did not contribute towards a decrease in k_a . Thus the highly positive $\Delta V_{\text{osm}}^\ddagger$ values extrapolated to Π_0 for the dealkylation of DIP enzymes indicate an extended release of water from both the active-site gorge and the protein surface in the bulk solvent. Although the calculated numbers of water molecules ($n_{w,0}$) do not correspond to the number of water molecules involved in stabilization of the transition state, osmotic-stress experiments provided evidence that water is needed for the aging process to occur. The fact that mutant enzymes were more sensitive to osmotic stress than wild-type BuChE ($t_{1/2, \text{mutants}} \gg t_{1/2, \text{wild-type}}$ and $\Delta V_{\text{osm}, \text{mutants}}^\ddagger > \Delta V_{\text{osm}, \text{wild-type}}^\ddagger$) supports the hypothesis that the water network in the gorge of mutant enzymes is less structured than in wild-type BuChE. Moreover, the fact that more water molecules were released from phosphorylated mutant enzymes than from phosphorylated wild-type BuChE suggests that residues Glu-197 and Asp-70 are important for the conformational stability of BuChE, and that the mutations E197D and D70G affect indirectly the solvent-exposed surface of the mutant enzymes.

Hydration of the active-site gorge of wild-type BuChE

Molecular dynamics simulations allowed us to relate kinetic data to hydration changes in the enzyme active-site gorge of wild-type BuChE. The gorge of solvated DIP wild-type BuChE appeared to contain about 28 molecules of water after 1-ns molecular dynamics (Figure 3). This number of water molecules is in

Figure 4 Ferguson plot analysis of gels after electrophoresis under hydrostatic pressure

Change in the retardation coefficient, K_r , of native (●, broken line) and soman-aged methylphosphonyl BuChE tetramer (■, continuous line) as a function of hydrostatic pressure in 8.26 mM Tris/0.1 M glycine, pH 8.3, at 10 °C.

agreement with the theoretical number expected from the following estimation: the volume of the active-site gorge of *T. californica* AChE is about 300 Å³ [51,52], and it contains about 13 molecules of water in the crystal structure [53]; therefore the volume of the active-site gorge of modelled human wild-type BuChE, estimated to be approx. 500 Å³ [52], should thus accommodate at least 22 molecules of water. Molecular dynamics resulted in stacking of the isopropoxy chain to be dealkylated on to the Trp-82 indole ring. This isopropoxy chain was found to be highly solvated, water molecules being located near to the breakable bond (C–O). This supports the results of kinetic analyses performed under hydrostatic and osmotic pressures, indicating that water is involved in the dealkylation transition state.

Molecular dynamics simulations were performed on both mutants, E197D and D70G. Unfortunately, we were unable to see significant changes that would have supported experimental data. A tentative explanation for this could be that the mutation-induced change in the water network is so extensive that SBMD is not suitable for simulating the modifications encountered by the enzyme and the water molecules. For instance, enzyme-surface residues that might be important for the trafficking of water molecules within the active-site gorge were not taken into account in SBMD simulations. Molecular dynamics simulations with periodic boundary conditions should circumvent this problem.

Stability of soman-aged wild-type BuChE

Gel electrophoresis under conditions of high hydrostatic pressure, and DSC, provided new evidence that aged wild-type BuChE is more stable than the native (non-phosphonylated) one. As shown in Figure 4, Ferguson-plot analyses of gels after electrophoresis under pressure showed that the retardation coefficient (K_r) of soman-aged wild-type BuChE tetramer decreased slowly with increasing pressure up to $P = 2.5$ kbar, whereas K_r of native wild-type BuChE increased transiently at $P \approx 1.7$ kbar. A swelling of the hydrodynamic radius of native BuChE was attributed to a pressure-induced, molten-globule transition, causing penetration of water into the enzyme structure [36]. It should be

Figure 5 DSC thermograms showing denaturation endotherms of native (a) and soman-aged (b) wild-type BuChE in 50 mM sodium phosphate pH 8.0

Enzymes prepared in buffer made up with water are shown as the continuous curves; broken curves (a' and b') represent enzymes prepared in buffer made up with heavy water.

Figure 6 Difference in the midpoint transition temperature (ΔT_m) between soman-aged and native wild-type BuChE as a function of pH

For further details, see the Materials and methods section.

noted that K_r of aged BuChE at P_0 is 40% higher than that of native enzyme (Figure 4). Since K_r is related to the protein molecular radius R ($K_r^{1/2} \propto kR$), a 40% increase in K_r indicates that the hydrodynamic volume of the aged enzyme is about 1.6-fold greater than that of the native enzyme (assuming sphericity), and/or that the enzyme undergoes a conformational change upon the aging reaction. However, the aged enzyme is not a molten globule, because it is more stable than the native enzyme.

In 50 mM sodium phosphate, pH 8.0, the T_m of the soman-aged enzyme was found to be 73 °C, whereas the T_m of the native wild-type BuChE was 62.8 °C (Figure 5). The difference in T_m between both enzyme species, ΔT_m , was therefore 10.2 °C at pH 8.0. DSC at different pH measurements allowed us to investigate the pH-dependence of the transition temperature midpoint (T_m) of native and soman-aged wild-type BuChE. ΔT_m was plotted as a function of pH (Figure 6); this was found to decrease abruptly above pH 8.0. The transition midpoint of the curve corresponds to a pK of 8.3 ± 0.1 . This value confirms that aged cholinesterases

are mainly stabilized by a salt bridge involving a protonated group [His-438(440)] of high pK [5,7]. This agrees with X-ray crystallographic structures of MIP- and methylphosphonyl (soman-aged)-AChE conjugates, showing that the distance between PO^- and protonated His-440 $N\epsilon 2$ is 2.7 and 2.6 Å respectively, with the angle between groups ($\approx 170^\circ$) favouring the formation of H-bonds [19]. The apparent free energy of the interaction involved in the enhanced stabilization of soman-aged BuChE can be estimated from the increase in free energy of denaturation at T_m of the aged enzyme: $\Delta\Delta G_d = \Delta G_{d,aged} - \Delta G_{d,native}$. Because $\Delta G_d = 0$ at T_m , the Gibbs–Helmholtz equation may be approximated by the relationship [54]:

$$\Delta\Delta G_d = \Delta T_m \cdot \Delta H_{m,native} / T_{m,native} \quad (7)$$

where ΔH_m is the enthalpy of denaturation at T_m (780 kJ·mol⁻¹ in 50 mM sodium phosphate, pH 8.0, as calculated from DSC endotherms). $\Delta\Delta G_d$ was determined as 23.7 kJ·mol⁻¹ at pH 8.0. It should be noted that a single buried salt bridge can contribute up to 12–20 kJ·mol⁻¹ towards protein stability [55–57]. Thus the high $\Delta\Delta G_d$ value in the present study indicates that both electrostatic and other weak interactions contribute to stabilization of the aged state. Results of a previous pressure/solvent denaturation study showed that the remaining alkyl chain on the phosphorus atom plays a role in the resistance to denaturation of aged BuChE [17]. The dehydration of the bottom of the active-site gorge is expected to strengthen hydrophobic interactions in this area. However, an increase in the hydrodynamic volume of the aged enzyme suggests that summation of numerous other weak interactions over the entire enzyme molecule might contribute towards an increase in the stability of the aged enzyme. DSC of native and soman-aged wild-type BuChE, in a solution of 50 mM sodium phosphate prepared in ²H₂O, showed a similar stabilizing solvent isotope effect on both enzyme forms, amounting to an increase in T_m of about 1–1.5 °C (Figure 5). This indicates that the solvent does not contribute to the enhanced stability of the aged state, which supports the conclusion that interactions responsible for the overstability of the aged state are not in contact with the solvent.

Molecular dynamics simulations performed in 1 ns on the DIP and MIP adducts of wild-type BuChE showed that an extended hydration change occurs during the aging process. Results of simulations on the MIP adduct support the above conclusions for soman-aged wild-type BuChE. It was shown that the salt bridge between PO^- and protonated His-438 was stable during the time course of the simulation. Formation of the salt bridge was accompanied by the dehydration of the bottom of the gorge, with water molecules being relocated towards Trp-82 and the gorge entrance (Figure 7). Furthermore, no water molecules were seen in the vicinity of the remaining isopropoxy chain, which interacts with Trp-231. However, although the volume available in the MIP adduct is larger than that in the DIP adduct, the solvation of the aged enzyme gorge appeared to be similar to that in the non-aged conjugate. The presumed low dielectric constant of the salt-bridge environment might explain both the high pK of protonated His-438 [58] and the heat stability of aged BuChE, which is similar to that of salt-bridge-stabilized thermophilic proteins [59].

Stability of native E197D and D70G mutants

Osmotic stress suggested that the E197D and D70G mutations affect the conformational stability of BuChE. Gel electrophoresis of native tetrameric forms of E197D and D70G mutants under hydrostatic pressure in the pressure range from 1 to 2.5 kbar did

Figure 7 Side view of the solvated active site of MIP-wild-type BuChE conjugate

Water molecules are represented by spherical balls. The remaining isopropyl chain (MIP) faces Trp-231; the negatively charged oxygen on the monophosphate moiety forms a salt bridge with protonated His-438 (note the absence of water molecules around this ion pair).

not reveal the presence of the molten-globule transition, compared with native tetramer of wild-type BuChE (results not shown). A similar observation was made for E202Q and D74N mutants of dimeric human AChE (C. Cléry and P. Masson, unpublished work). Moreover, the heat stability of 'atypical' human BuChE, i.e. the D70G mutant, was reported to be higher than that of wild-type BuChE [60]. Similarly, the heat stability of the E202Q and D74N human AChE mutants has been found to be higher than the stability of wild-type AChE (A. Shafferman, personal communication). These results indicate that homologous mutants of BuChE and AChE are resistant to hydrostatic pressure/heat-induced penetration of water.

Figure 8 Proposed hoop-net model of the BuChE active-site gorge

Residues Asp-70 (rim of the gorge) and Glu-197 (bottom of the gorge) control the water molecules (V) traffic between the bulk solvent and the gorge cavity.

Conclusion

The resistance of mutant enzymes to pressure-denaturation corroborates the results regarding the effect of pressure on the aging rate of the DIP E197D and DIP D70G BuChE mutants, and supports the conclusion that homologous residues Glu-199 and Asp-72 in the active-site gorge of cholinesterases control the enzyme's conformational plasticity via the water/H-bond network of the gorge. A simplified schematic representation of the dynamics of water molecules in the cholinesterase active-site gorge can be tentatively proposed in which residues Asp-70 and Glu-197 act as two-way valves controlling the dynamics of water molecules between the bulk solvent and the interior of the gorge, determining the organization of the water/H-bond network (Figure 8). In mutant enzymes, the water/H-bond network is slack, i.e. mutated residues act as 'one-way check valves' in a hoop-net functioning in the wrong direction: water molecules can be released easily from the active-site gorge, but the forced penetration of water into it is difficult.

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