The pH Dependence of Dealkylation in Soman-Inhibited Cholinesterases and Their Mutants: Further Evidence for a Push−Pull Mechanism†

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ABSTRACT: Bimolecular rate constants for the inactivation of recombinant (r) human (Hu) butyrylcholinesterase (BChE) with P(S)C(S)- and P(S)C(R)-2-(3,3-dimethylbutyl) methylphosphonofluoridate (soman) are $(92 \pm 7) \times 10^6$ M^{-1} min^{-1} and $(13.7 \pm 0.8) \times 10^6$ M^{-1} min^{-1} at pH 7.4, $\mu = 0.1$ M and 25 °C. Mutations of E197(*199*) to D or Q and W82(*84*) to A result in reductions in the rate constants for inactivation with P(S)C(S)-soman 4.3-, 11.8-, and 263-fold and with P(S)C(R)-soman by 6.5-, 47.3-, and 685-fold, respectively. The pH dependence of dealkylation (aging) in r mouse (Mo) acetylcholinesterase (AChE) and rHu BChE and their mutants inactivated with P(S)C(S)- and P(S)C(R)-soman was compared. Best-fit parameters for the asymmetric bell curves for the adducts of wild-type Mo AChE are $pK_1 = pK_2 = 4.0-4.9$ and $pK_3 = 5.2-6.6$. These p*K*s are consistent with the involvement of two carboxylic acids, possibly E202(*199*) and either E334(*327*) or E450(*443*), and H447(*440*)H^+ in the dealkylation of AChE. E202Q MoAChE inactivated with the soman diastereomers yielded $pK_3 = 5.5-5.8$. Nearly symmetric pH curves for soman-inhibited wild-type and E197D Hu BChE gave $pK_2 = 3.7-4.6$ and $pK_3 = 7.3-8.0$, but much lower, $pK_3 \sim 5$, for the corresponding adduct of the E197Q mutant. Dealkylation in soman-inhibited BChE is consistent with the participation of one carboxylic acid side chain and H438(*440*)H^+. Maximal rate constants for dealkylation (k_{max}) are 1−6 min^{-1} for AChE and 2 min^{-1} for BChE at 25 °C. The W82 to A mutation in BChE results in the largest reduction, 2500−6000-fold, in the rate constant for dealkylation. The reduction in the rate constants for dealkylation in the E197 mutants is highly pH dependent. The solvent isotope effects at the pH maxima are 1.3−1.4, indicating unlikely preprotonation or proton in "flight" at the enzymic transition states. The new results support the push−pull mechanism of dealkylation in soman-inhibited cholinesterases proposed previously.

Scientific interest in the inhibition of cholinesterases (ChEs)[1] by 2-(3,3-dimethylbutyl) methylphosphonofluoridate (soman) has been sustained by the unusual efficiency of the unnatural, yet enzyme-catalyzed processes involved. A chief characteristic of the soman-inhibited ChEs is the resilience to enzyme-catalyzed dephosphonylation due to the onset of competing and rapid dealkylation (*2*−*26*). Both phosphonylation and the ensuing aging reaction, or loss of the pinacolyl group, in the phosphonyl fragment occur with the participation of acid/base catalysis (*2*−*4*, *6*, *9*, *11*−*14*, *16*, *17*). The ChE-promoted dealkylation is at least 10 orders of magnitude faster than the nonenzymic equivalent (*12*, *13*). Due to the great efficiency of the chemical transformations and the rapid route of entry into vertebrates, soman is one of the most potent nerve agents known to man. Application of soman as a nerve gas against civilian or military populations further necessitates efforts toward a full elucidation of the mechanisms of each phase of the inhibition process.

Mechanistic studies of the early phases of the inactivation of ChEs by soman were carried out in these laboratories, and the results of these studies were reported in a series of papers (*5*, *6*, *8*, *27*−*33*). A feature article (*2*) summed up the main conclusions in which the underlying reason for the irreversible inhibition was proposed to be differences in the stereochemical arrangement at the transition states of the reactions of the natural substrate and the organophosphorus

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[1] Abbreviations: ChE, cholinesterase; AChE, acetylcholinesterase (acetyl hydrolase); *Ee*, *Electric eel*; *Tc*, *Torpedo californica*; FBS, fetal bovine serum; BChE, butyrylcholinesterase; rHu, recombinant human; rMo, recombinant mouse; ATC, acetylthiocholine; BTC, butyrylthiocholine; ONPB, *o*-nitrophenyl butyrate; HI-6, 1-(2-hydroxyiminomethyl-1-pyridinium)-1-(4-carboxyamino-pyridinium)-dimethyl ether chloride; 2-PAM, 2-(hydroxyiminomethyl)-1-methylpyridinium iodide; sarin, 2-propyl methylphosphonofluoridate; soman, 2-(3,3-dimethylbutyl) methylphosphono-fluoridate; DFP, diisopropylfluorophosphate; TAPS, *N*-tris[hydroxymethyl]methyl-3-amino-propanesulfonic acid; TRIS, Tris-(hydroxymethyl) aminomethane; BIS-TRIS PROPANE, 1,3-bis[tris(hydroxymethyl)methyl-amino]propane, BSA, bovine serum albumine.

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compounds. The stereochemical relationship between ligands in pentacoordinate phosphonyl transients and the H of the catalytic triad (S200, H440, and E327 in *Torpedo californica* (*Tc*) acetylcholinesterase (AChE)) disfavors the proton-transfer sequence requisite in acylation, deacylation, and other nucleophilic displacement reactions of the catalytic S in serine hydrolases (*34*−*36*). The consequence is that the catalytic H remains protonated after leaving group departure and *the acid/base apparatus is thus impaired*: this might be termed suicide inhibition. A significant implication of this mechanism is the lack of general base catalysis of the hydrolysis of the phosphonylated enzyme adducts and thus a near absence of dephosphonylation. Recent work with the G117H mutants of butyrylcholinesterase (BChE) has shown that a strategically placed H can restore the general base catalytic feature of the enzyme and thus enhance the rate of dephosphonylation from a number of adducts (*37*, *38*). The article of 1988 (*2*) also speculated on the need for a catalytic triad involving a carboxylic acid and the presence of a W in the binding region. These were in fact found in the crystal structure of the *Tc* AChE.

Immediately after the X-ray coordinates of *Tc* AChE became available (*39*), computational chemistry was used to identify the active-site residues that may promote the reaction at each stage. The first presentation of the structural model explained aging by a push−pull mechanism involving $H440H^+$, E199, and W84 residues (*3*). H440 was fully protonated in the model to correspond to the neutron structure of the monoisopropyl phosphate adduct of trypsin (*40*) and results of other NMR studies (*41*−*44*). This model indicated that one methyl group of the pinacolyl residue of soman binds to W84 while at least one methyl group and the Cα are in the vicinity of E199. An identical mode of interaction between methyl groups of *m*-(*N*,*N*,*N*-trimethylammonio)-trifluoroacetophenone in the transition state analogue inhibitor and W84 and E199 was later found by X-ray crystallography of the inhibited *Tc* AChE (*45*). The model has since been well-supported by studies of the aging reaction with soman-inhibited E202(*199*)Q[2] (*20*, *21*), E202(*199*)D, and E202(*199*)A mutants of AChE (*21*) and DFP-inhibited E197(*199*)Q, E197(*199*)D, and E197(*199*)G mutants of Hu BChE (*26*). An important test of the mechanism has been the pH dependence of the dealkylation reaction in soman-inactivated wild-type AChEs (*12*, *13*). The dependence of the rate constants for dealkylation on pH is bell-shaped and consistent with the push−pull mechanism. A peculiar characteristic of the pH profiles is the very steep rise in the rate of aging with pH between 3.5 and 5.0 for the dealkylation in soman-inhibited *Electric eel* (*Ee*) and fetal bovine serum (FBS) AChE. A similar observation has been reported by Selwood et al. on reactions of *Ee* AChE with substrates that have large acyl groups (*46*).

The solvent isotope effects for dealkylation in soman-inhibited AChE at pH maxima are 1.1−1.3, indicating that neither proton transfer with a large amplitude (*36*) at the transition state nor preprotonation (*47*) is likely. Protonation followed by rate-determining formation of a secondary carbenium ion are the elementary steps often quoted to explain the aging reaction (*14*, *22*). However, a nearly concerted mechanism derived from the structural and mechanistic studies referred to above provides a more coherent explanation (*12*, *13*) for all known experimental observations including studies with mutants. An interplay between the pinacolyl fragment and E199 and aromatic residues, W84 and F330, initiates a methyl migration nearly concerted with C−O bond breaking. This ameliorates charge separation. It appears that the enzyme stabilizes the transition state for dealkylation by ∼14 kcal/mol with respect to an appropriate nonenzymic reaction by avoiding the formation of at least one intermediate. The collapse of the tertiary cation is also a critical facet of this mechanism since it avoids sticking of the carbenium ion to the negatively charged catalytic residues. The tertiary cation rearranges into neutral and volatile products (*14*) due to rapid proton loss to a nearby basic residue, possibly the carboxylate ion of E199.

The obvious question remained, however, whether the unique dependence of the reaction on pH would be altered in mutants of E199. In the following we wish to provide further examples of bell-shaped pH dependence of the aging reaction in recombinant mouse (rMo) AChE and recombinant human (rHu) BChE. The very sharp rise in the rate of dealkylation is also observable in Mo AChE within pH 3.5 and 5.0. The data set in this pH range allowed a fit to a model for two ionizing groups influencing the rate of dealkylation in soman-inhibited wild-type Mo AChE and in the previously studied Ee and FBS AChE. The pH dependence of the dealkylation reaction in rHu BChE and its E197Q and E197D mutants show significant differences and also deviate from AChE. An inspection of the pH profiles should be quite convincing of the need for a full characterization of these reactions for a meaningful comparison. Dealkylation in soman-inhibited W82(*84*)A mutant of rHu BChE is >2500 times slower, above pH 5.5, than in wild-type Hu BChE and it has the same dependence on pH as the wild-type enzyme, since the mutation involves a change in a non-ionizing residue. The new results pertaining to the characteristics of dealkylation of soman-inhibited rHu BChE are of special practical interest since Hu BChE and its mutants are prime candidates for detoxification and decontamination of soman inflicted human subjects and biological or inanimate objects. Most significantly, the results of these studies provide new insight into the modes of participation of catalytic residues and species dependence in the dealkylation of soman-inhibited ChEs.

MATERIALS AND METHODS

Materials. Recombinant wild-type and E202Q mutant of Mo AChE were expressed, purified, and characterized with respect to catalytic parameters as described (*48*). One nanomole of wild-type AChE was equivalent to 132 units, and one nanomole of E202Q AChE was equivalent to 60 units. Recombinant wild-type, E197D, E197Q, and W82A mutants of Hu BChE were expressed in CHO K1 cells in serum-free medium and partially purified on procainamide Sepharose affinity gel as described (*49*). One nanomole of wild-type BChE was equivalent to 68 units, and one nanomole of E197D, E197Q, and W82A mutant BChEs was equivalent to 8, 5, and 25 units, respectively. The Ellman reagents were from Sigma Chemical Co. (St. Louis, MO). Soman was obtained from the Chemical Research, Develop-

[2] The italicized number in parentheses following an amino acid residue refers to the homologous amino acid position in the sequence of *Tc* AChE (*1*).

ment, and Engineering Center (Aberdeen Proving Ground, MD). Soman used in these experiments was 98.6% pure when analyzed by [^{31}P] nuclear magnetic resonance. The two P(S)-diastereomers of soman[3] were obtained as described earlier (*50*). Concentrations of soman solutions were determined by titration of the solution with a known amount of FBS AChE and measurement of residual activity (1 nmol of FBS AChE is equivalent to 400 units). The oximes, HI-6 and 2-PAM, were obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (Washington, DC). Deuterium oxide (99.9%) was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Enzyme Assays. AChE activity was determined in 0.05 M sodium phosphate buffer, pH 8.0, at 25.0 ± 0.1 °C by the Ellman assay (*51*). BChE activity for the wild-type, E197D, and E197Q enzymes was determined similarly using BTC as the substrate instead of ATC. The activity of the W82A mutant of rHu BChE was followed by measuring the hydrolysis of ONPB as described (*52*).

Inhibition of BChE with Soman. Inhibition of wild-type Hu BChE (0.1 units/mL in 50 mM sodium phosphate, pH 7.4, containing 0.05% BSA) by soman was initiated by adding one of the two P(S)-diastereomers of soman (10−25 nM) and measuring enzyme activity at various time intervals. A similar procedure was used for E197D (0.1 units/mL), E197Q (0.1 units/mL), and W82A BChE (1.0 unit/mL) except that the concentration of the two P(S)-diastereomers used was 50−250 nM, respectively. Experiments were carried out at 25.0 ± 0.1 °C with at least four different concentrations of soman. The apparent bimolecular rate constants for the inhibition reactions measured under second-order conditions were determined by nonlinear regression of the $\ln\{BChE_t/(OP_0 - [BChE_0 - BChE_t])\}$ versus time data pairs at different inhibitor concentrations (*53*), where $BChE_t$ is the enzyme concentration at time t, $BChE_0$ is the initial enzyme concentration at time $t = 0$, and OP_0 is the initial concentration of OP.

Reactivation of Soman-Inhibited BChE with 2-PAM. Human wild-type (8.0 units/mL), E197D (10 units/mL), E197Q BChE (12 units/mL), and W82A BChE (4.0 units/mL) in 100 mM TAPS, pH 9.0, were inhibited with stoichiometric amounts of one of the P(S)-diastereomers of soman for 15 min at 4.0 ± 0.1 °C. Reactivation was initiated by mixing 10 μL of the soman-BChE conjugate with 90 μL of sodium phosphate, pH 8.0, containing 2-PAM at a final concentration of 1 mM at 25.0 ± 0.1 °C. Aliquots were removed from the reactivation mixture at various time intervals and assayed for enzyme activity using the Ellman method (*51*). Data for the time course of reactivation reactions were analyzed by nonlinear regression analysis using the equation (*53*):

$$(BChE_{reac})_t = (BChE_{reac})_\infty[1 - e^{-kt}] \qquad (1)$$

where $(BChE_{reac})_t$ is the concentration of reactivated enzyme at time t, $(BChE_{reac})_\infty$ is the maximum concentration of reactivatable enzyme, and k is the pseudo-first-order rate constant for reactivation.

[3] The P(S) configuration has been assigned to the levorotatory diastereomers of soman based on chemical correlation (*17, 50*).

pH Rate Profile for the Aging of AChE and BChE. Wild-type and E202Q Mo AChE (25 units/mL) in 100 mM TAPS, pH 9.0, were inhibited with stoichiometric amounts of one of the P(S)-diastereomers of soman for 30 min at 4.0 ± 0.1 °C. Fifty microliters of the soman−AChE conjugate solution was diluted into 1.0 mL of one of the buffer solutions at different pH values at 25.0 ± 0.1 °C, and the pH of all samples was monitored using the pH meter. Buffer solutions used in these experiments were 0.05 M formate, citrate, acetate, phosphate, and BIS-TRIS PROPANE, at $\mu = 0.1$ (NaCl). Parallel samples without soman were used to monitor the stability of enzyme at each pH. Thirty-microliter aliquots were removed at various time intervals and transferred to tubes containing 20 μL of 5 mM HI-6 in 0.2 M TAPS, pH 9.0. Samples were incubated overnight at room temperature before assay for AChE activity using the Ellman method (*51*).

A similar protocol was used for studying the aging of rHu BChE. E197D (12 units/mL), E197Q (10 units/mL), and W82A (4 units/mL) BChE in 100 mM TAPS, pH 9.0, were inhibited with stoichiometric amounts of one of the P(S)-diastereomers of soman for 30 min at 4.0 ± 0.1 °C. Fifty microliters of soman−BChE conjugate was diluted into 0.5 mL of one of the buffer solutions at different pH values at 25.0 ± 0.1 °C, and the pH of all samples was monitored using the pH meter. Due to the fast rate of aging and small extent of reactivation, the preparation of adducts of wild-type Hu BChE (100 units/mL) with the P(S)-diastereomers of soman and the aging reactions at different pH were carried out at 4.0 ± 0.1 °C. For all enzymes, the aging reaction was followed by transferring 30-μL aliquots at various time intervals to tubes containing 20 μL of 2 mM 2-PAM in 0.5 M TAPS, pH 9.0. Samples were incubated overnight at room temperature before assay for BChE activity using the Ellman method (for wild-type, E197D, and E197Q BChE). For W82A BChE, activity was measured using ONPB as the substrate.

Enzyme activity time pairs for at least 4 half-lives were fit to the first-order rate law using GraFit (*54*), and 2−4 repeats were averaged at each experimental pH value. The pH rate profile was fit to an asymmetric bell corresponding to a three-pK model for wild-type Mo AChE, to a symmetric bell with two inflection points corresponding to two-pK models for wild-type, E197D, and E197Q BChE, and to a sigmoid corresponding to a one-pK model for E202Q Mo AChE and W82A Hu BChE.

RESULTS

The Effect of E197 and W82 Mutations on Phosphonylation of BChE by P(S)C(S)- and P(S)C(R)-Diastereomers of Soman. To examine the role of E197 and W82 in the stereoselectivity of BChE for the two P(S)-diastereomers of soman, we compared the bimolecular rate constants for the phosphonylation of wild-type and mutant BChEs by the two P(S)-diastereomers (Table 1). P(S)C(S)-soman is more potent than P(S)C(R)-soman for inhibiting wild-type and mutant BChEs. These results are in agreement with those reported previously for horse and Hu BChE (*15*, *55*) and rHu BChE (*26*). A 6.7-fold difference in bimolecular rate constants for the inactivation of wild-type BChE by the two P(S) diastereomers of soman has been observed. The stereoselectivity of phosphonylation is greater in the mutants

Table 1. Bimolecular Rate Constants for the Inhibition of Hu BChE by Diastereomers of Soman[a]

	P(S)C(S)-soman		P(S)C(R)-soman	
enzyme	$k \times 10^{-6}$ (M^{-1} min^{-1})	k_0/k^b	$k \times 10^{-6}$ (M^{-1} min^{-1})	k_0/k^b
wild type	92 ± 7	1	13.7 ± 0.8	1
E197D	20 ± 3	4.3	2.1 ± 0.4	6.5
E197Q	7.8 ± 0.7	11.8	0.29 ± 0.04	47.3
W82A	0.35 ± 0.04	263	0.020 ± 0.003	685

[a] Measured in 0.05 M phosphate buffer, pH 7.4, at 25.0 ± 0.1 °C. [b] k_0 is the bimolecular rate constant for wild-type Hu BChE, and k is the bimolecular rate constant for mutant BChE.

than in the wild-type BChE with the E197Q mutation in the lead. The stereoselectivity is 9.6, 27, and 17.5 for E197D, E197Q, and W82A BChE, respectively. The difference in the bimolecular rate constants for wild-type and E197Q BChE reacting with P(S)C(S)-soman and P(S)C(R)-soman suggests that the loss of charge on E197 decreased the reactivity of P(S)C(R)-soman for mutant BChE more than that of the P(S)C(S)-diastereomer. The greatest effect, however, is on the rate constant for phosphonylation of W82A BChE with decreases of 263- and 685-fold for the P(S)C(S)- and P(S)C(R)-diastereomers of soman, respectively.

The Effect of E197 and W82 Mutations on the Reactivation of BChE Inhibited with the P(S)C(S)- and P(S)C(R)-diastereomers of Soman. The maximum reactivation of P(S)C(S)-soman-inhibited BChE with 2-PAM was 40% for the wild-type, 50% for the E197D, and 80% for the E197Q BChE relative to the control. The maximum reactivation of P(S)C(R)-soman-inhibited BChE with 2-PAM was 40% in all cases. The reactivation from the P(S)C(R)-soman-inhibited wild-type, E197D, and E197Q BChE with 2-PAM was about half as fast as from the P(S)C(S)-soman-inhibited enzymes. Reactivation of wild-type BChE and E197D BChE inhibited with either of the two P(S)-diastereomers was 3-fold faster than reactivation of E197Q BChE from the corresponding adducts (Table 2). These results show that mutation of E197 affects the rate of reactivation as well as the extent of reactivation, especially for P(S)C(R)-soman-inhibited BChE.

Aging in Diastereomers of Soman-Inhibited ChEs. The dealkylation reaction in soman-inactivated rMo AChE was not quite as fast as the corresponding reaction in *Ee* AChE and FBS AChE (*12*) and was measured at 25 °C. In contrast, dealkylation from soman-inhibited BChE was too fast for the sampling technique at 25 °C. Consequently, the dealkylation reaction for Hu BChE was carried out at 4 °C as reported earlier (*12*). The first-order rate constants for dealkylation in the adducts of rMo AChE and rHu BChE inhibited by the P(S)C(S) and P(S)C(R) diastereomers of soman are shown, as a function of pH, in Figure 1. Bell-shaped figures (not shown) were obtained for E197Q and E197D BChE. Also shown in the figure are the rate constants for denaturation of the enzymes which were nearly identical for wild-type and mutant enzymes. Denaturation in the ChEs from mammalian sources was more pronounced than in *Ee* AChE and interfered with accurate determination of the rate constants between pH 3.5 and 4.0. Small corrections, <5%, were applied to the rate constants for dealkylation by subtracting the value of the rate constant for denaturation. As the correction increases with decreasing pH, the errors in the rate constant for dealkylation become intolerable.

The shape of the pH profile for dealkylation of soman-inhibited Mo AChE is best described as a distorted bell with a maximum between pH 5.0 and 5.5, and it is very similar to the shape of the pH profile for the FBS AChE (*12*). Because of the significant contribution of denaturation at pH <3.5, the lower limit of the dealkylation reaction cannot be established with certainty. Yet, with a large number of data points measured between 3.5 and 5.0 for the dealkylation of soman-inhibited wild-type Mo AChE, it was possible to fit the data to a three-pK model according to eq 2.

$$k = \frac{L}{1 + 10^{(pK_{1,2} - pH)} + 10^{2(pK_{1,2} - pH)} + 10^{(pH - pK_3)}} \quad (2)$$

In eq 2, L designates a common upper limit of the sigmoids while the lower limits are set to 0, two identical pKs describe the steep slope of the ascending leg, and one pK is associated with the descending leg of the pH profile. This fit actually decreased the value of the reduced chi squares by 40% with respect to a two-pK model. A fit of three independent pKs might be more desirable but would require more accurate data below pH 3.5, which is not warranted due to the interference by denaturation. The calculated pKs and limiting rate constants for dealkylation in soman-inhibited ChEs from different sources are listed in Table 3.

The pH curve is much broader for wild-type Hu BChE (Figure 1B) than for AChEs and similar to that reported for cycloheptyl methylfluorophosphonate-inhibited BChE (*56*). Because the plateau is much broader in the pH profile for dealkylation in soman-inhibited Hu BChE, the decline in the value of the rate constant in the lower pH range could not be characterized to the extent it was for Mo AChE: a two-pK model, described by eq 3, was used for fitting.

$$k = \frac{L}{1 + 10^{(pK_1 - pH)} + 10^{(pH - pK_2)}} \quad (3)$$

While only a single ionizing residue can be calculated from the data on BChE for the acid limb above pH 4, the participation of another and more acidic carboxylic acid ionizing at lower pH is of course possible.

The pH dependence of the rate constants for dealkylation in the soman-inhibited E202Q Mo AChE and W82A BChE

Table 2. Rate Constants for the Reactivation of Soman-Inhibited Hu BChE by 1 mM 2-PAM[a]

	k_{obs} (min^{-1})			
inhibitor	wild-type	E197D	E197Q	W82A
P(S)C(S) soman	0.21 ± 0.20	0.23 ± 0.06	0.07 ± 0.01	0.028 ± 0.004
P(S)C(R) soman	0.12 ± 0.08	0.12 ± 0.03	0.04 ± 0.01	0.07 ± 0.01

[a] Measured in 0.05 M phosphate buffer, pH 8.0, at 25.0 ± 0.1 °C.

Table 3. Maximal Rate Constants (k_{max}) and pK Values Calculated for Dealkylation in Diastereomeres of Soman-Inhibited Cholinesterases[a]

adduct, configuration of soman	k_{max} (min^{-1})	pK_1 (and pK_2)	pK_3	$k(H_2O)/k(D_2O)$
Mo AChE, P(S)C(S)	2.4 ± 0.5	4.5 ± 0.1	5.2 ± 0.2	1.3 ± 0.1
Mo AChE, P(S)C(R)	1.0 ± 0.1	4.1 ± 0.1	5.8 ± 0.1	
Mo AChE, E202Q, P(S)C(S)	0.071 ± 0.008		5.8 ± 0.2	1.4 ± 0.2
Mo AChE, E202Q, P(S)C(R)	0.021 ± 0.003		6.2 ± 0.2	
Hu BChE, P(S)C(S)[b]	2.0 ± 0.2	4.2 ± 0.1	7.5 ± 0.1	~1.3
Hu BChE, P(S)C(R)[b]	1.8 ± 0.4	3.7 ± 0.4	7.8 ± 0.4	
Hu BChE E197D, P(S)C(S)	0.056 ± 0.003	4.4 ± 0.1	8.0 ± 0.1	
Hu BChE E197D, P(S)C(R)	0.056 ± 0.003	4.7 ± 0.0	8.1 ± 0.1	
Hu BChE E197Q, P(S)C(S)	0.31 ± 0.03	4.6 ± 0.1	5.0 ± 0.1	
Hu BChE E197Q, P(S)C(R)	0.22 ± 0.03	4.9 ± 0.2	4.9 ± 0.2	
Hu BChE W82A, P(S)C(S)	0.0008 ± 0.0002		7.5 ± 0.6	
Hu BChE W82A, P(S)C(R)	0.0003 ± 0.0001		7.3 ± 0.4	
Ee AChE, P(S)C(S)[b,c]	6.3 ± 0.6	4.3 ± 0.1	6.0 ± 0.1	
Ee AChE, P(S)C(R)[b,c]	5.1 ± 0.4	4.3 ± 0.1	6.6 ± 0.2	
FBS AChE, P(S)C(S)[b,c]	3.1 ± 0.4	4.8 ± 0.1	5.0 ± 0.1	
FBS AChE, P(S)C(R)[b,c]	3.1 ± 0.4	4.8 ± 0.1	5.3 ± 0.1	

[a] Values calculated at μ = 0.1 M (NaCl) and at 25.0 ± 0.1 °C. Three-pK model; pK_1 = pK_2 for AChEs and two-pK model for BChE. [b] Values are extrapolated from those measured at 4.0 ± 0.1 °C. [c] Recalculated from the data in ref 12.

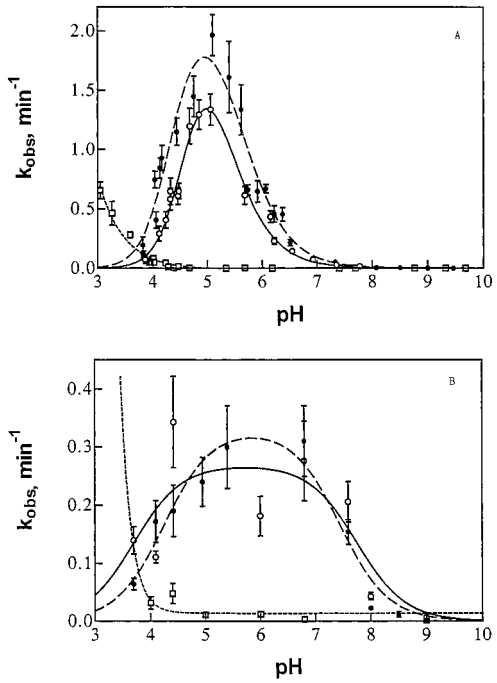


FIGURE 1: (A) pH dependence of the rate constants for dealkylation of wild-type Mo AChE inhibited with P(S)C(R) soman (○) and P(S)C(S) soman (●) and denaturation of the protein (□) at 25.0 ± 0.1 °C. (B) pH dependence of the rate constants for dealkylation of wild-type Hu BChE inhibited with P(S)C(R) soman (○) and P(S)C(S) soman (●) and denaturation of the protein (□) at 4.0 ± 0.1 °C.

above pH 4.0 were fitted to a one-pK model as in eq 4.

$$k = \frac{L}{1 + 10^{(pH - pK)}} \quad (4)$$

The effect of the E197Q mutation in Hu BChE on the pH dependence of the dealkylation reaction is markedly different from the others. The bell curve is narrower than those observed for wild-type, E197D, and W82A Hu BChE and more similar to the curves obtained with AChE with a down slope beginning at pH 4.8.

The rate constants for the aging of soman-inhibited wild-type rHu BChE are ~2 min^{-1} and 1.8 min^{-1} at pH 6.5 and 25 °C calculated with $\Delta H^{\ddagger}$ = 14.3 kcal/mol (*12*). There is no notable difference in the rates of dealkylation between the diastereomeric adducts of Mo AChE or Hu BChE, suggesting that chirality at Cα has a negligible effect on the rate or the mechanism of aging in the adducts of P(S) diastereomers of soman. There is, however, a 3-fold difference in the dealkylation rates between the diastereomers of the soman-inhibited E202Q Mo AChE. The adducts of the mutant enzymes formed with P(S)C(S)-soman generally age faster than the diastereomers formed with P(S)C(R)-soman.

Solvent isotope effects for the wild-type ChEs and the E202Q Mo AChE were 1.3−1.4 at the pH of maximal rate constants and identical to those observed for the aging in soman-inhibited *Ee* and FBS AChE (*12*) and in sarin-inhibited *Ee* AChE (*33*). These small solvent isotope effects indicate that it is the motion of heavy atoms rather than that of protons that constitutes the reaction coordinate of this reaction.

DISCUSSION

Inactivation of Hu BChE with the P(S)C(S)- and P(S)C(R)-Diastereomers of Soman. Racemic soman is a mixture of four diastereomers due to the presence of two stereogenic centers; at the P atom and the Cα atom of the pinacolyl group. Previous inhibition studies with stereoisomers of soman also have shown that, whereas the stereoselectivity of ChEs for soman is 10^4 favoring the P(S) configuration (*17*), the P(S)C(S)-soman is only a slightly more potent inhibitor than the P(S)C(R)-diastereomer. Bovine erythrocyte AChE exhibited a 6-fold difference in the bimolecular rate constants for the two P(S)-diastereomers as compared to a 1.6-fold difference observed for *Ee* AChE (*17*, *57*). The stereoselectivity is small in the wild-type Mo AChE (*24*) and Hu BChE, but increases in the reaction involving the mutants. Replacement of E197, the residue next to the active-site S198(*200*) in Hu BChE, by D or Q results in a 4.5- and a 11.8-fold decrease in the rate of phosphonylation with P(S)C(S)-soman compared to a 6.5- and a 47-fold decrease with P(S)C(R)-soman. These results suggest that the loss of charge in E197Q decreases the reactivity of soman for mutant BChE and increases the stereoselectivity between the P(S)-diastereomers of soman. The effect of this mutation is greater in BChE than in Mo AChE (*24*). Phosphonylation

is surprisingly slower, 263−685-fold, in the W82A mutant of Hu BChE than in the native enzyme. Removal of the indole ring of W82 results in a great reduction in binding of the pinacolyl group of soman to BChE. Inactivation by racemic soman is only 16-fold slower in the W86(*84*)A mutant of rHu AChE than in the native enzyme (*22*).

Reactivation of Hu BChE Inhibited with the P(S)C(S)- and P(S)C(R)-Diastereomers of Soman. Phosphonylated BChE can be reactivated with 2-PAM provided it has not undergone aging by dealkylation. Reactivation of P(S)C(S)-soman and P(S)C(R)-soman inhibited wild-type, E197D, E197Q, and W82A BChE with 1 mM 2-PAM showed different rates and extent of reactivation. The results in Table 2 are similar to those obtained for the reactivation of soman-inhibited rMo (*24*) Hu (*58*), *Ee* (*12*, *18*, *59*), and Plaice AChE (*60*) with HI-6. The results are also consistent with in vitro studies with Hu, *Ee*, and Plaice AChE, which have demonstrated that reactivation with HI-6 of AChE is more effective from P(S)C(S)-soman-inhibited adducts than from P(S)(R)-soman-inhibited adducts (*15*, *17*, *57*, *61*). An explanation of the phenomenon has been given by molecular modeling studies, which show that steric hindrance between the methyl group at Cα in P(S)C(R)-soman and $H440H^+$ can reduce the efficiency of nucleophilic reactivation of adducts of P(S)C-(R)-soman with AChE compared to P(S)C(S)-soman with AChE (*4*). It is also noteworthy that the W82A mutation reduces the rate constant for reactivation of BChE from the adduct with P(S)C(S)-soman five times more than from the adduct with P(S)C(R)-soman. Presumably, weaker binding in the soman-inhibited adduct with S configuration at Cα affects the alignment for nucleophilic attack at P by 2-PAM.

Dealkylation in Diasteromeric Adducts of Soman-Inhibited Mo AChE and Hu BChE: Comparison of the pH Profiles. The dependence of dealkylation in soman-inhibited ChEs on pH between 3.5 and 10 has been reported earlier for bovine serum AChE (*16*), *Ee*, and FBS AChE (*12*) and now for Mo AChE and Hu BChE under comparable conditions. Dealkylation has partly been characterized for other soman-inhibited ChEs at pH > 5.5 (*20*−*22*, *24*).

A clear understanding of bell-shaped pH rate profiles may be best served by dissecting the curve into its components: that is, two sigmoids or titration curves. The ascending leg originates from the increase in rate with increasing basicity due to the rising concentration of the conjugate base form of an acid. The descending leg of the curve can be derived from the effect on rate of declining concentration of a weak conjugate acid of a base. Each titration curve yields an inflection point corresponding to the p*K* of an ionizing residue catalyzing the reaction.

The full pH dependence of the log of the corrected rate constants is compared for wild-type and E202Q Mo AChE in Figure 2A. Dealkylation in soman-inhibited Mo AChE depends on two residues ionizing to their conjugate bases with p*K*s near 4.0. The participation of base catalysis in the dealkylation of soman-inhibited E202Q Mo AChE cannot be established. The p*K* of the residue acting as an acid catalyst is nearly the same at ∼6 in wild-type and E202Q Mo AChE.

The same comparison is made for Hu BChE in Figure 2B. The symbols for the wild-type Hu BChE indicate rate constants calculated for 25 °C from data obtained at 4 °C and using $\Delta H^{\ddagger} \sim 14.3$ kcal/mol (*12*). This temperature

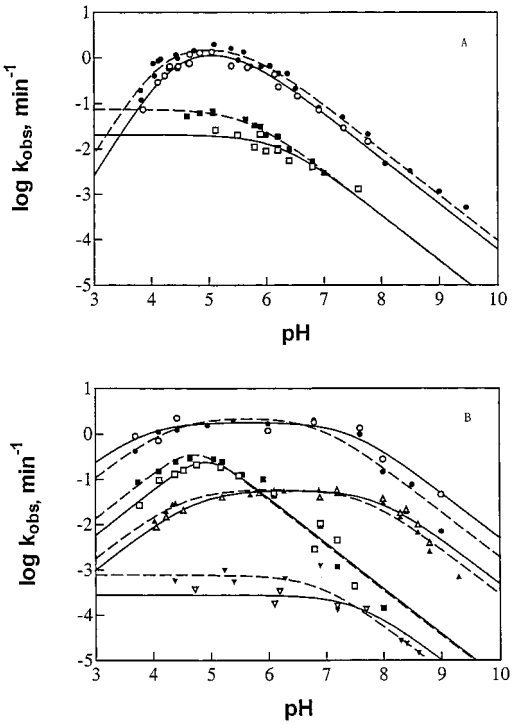


FIGURE 2: (A) Log *k* of dealkylation versus pH profiles for rMo AChE inhibited with P(S)C(R) soman (○) and P(S)C(S) soman (●); for the E202Q mutant of Mo AChE inhibited with P(S)C(R) soman (□) and P(S)C(S) soman (■). (B) Log *k* of dealkylation versus pH profiles for rHu BChE inhibited with P(S)C(R) soman (○) and P(S)C(S) soman (●); for the E197Q mutant of Hu BChE inhibited with P(S)C(R) soman (□) and P(S)C(S) soman (■); for the E197D mutant of Hu BChE inhibited with P(S)C(R) soman (△) and P(S)C-(S) soman (▲); for the W82A mutant of Hu BChE inhibited with P(S)C(R) soman (▽) and P(S)C(S) soman (▼).

dependence translates into a 7-fold increase in the maximal rate constants when going from 4 to 25 °C. The curves are broad because the p*K* values of the basic and acidic groups catalyzing dealkylation are quite far apart. All curves can be fitted with p*K* values between 3.8 and 4.3 for the general base catalyst and with p*K* values 7.3−7.8 for wild-type Hu BChE and ∼8 for the E197D mutant indicating catalysis by a weak acid as well. A conspicuous deviation from this trend is presented by the E197Q mutant in a narrow bell curve resembling the AChE curves and giving an upper p*K* value of ∼5.

The *lower kinetic pK values* are typical of enzymic carboxylic acids, the best candidates for promoting dealkylation in the ChEs being the conjugate base of E199 and E327 or E443. The E197(*199*)Q BChE still shows a dependence on an ionizing carboxylate, either E325(*327*), which is essential for the stability of $H438(440)H^+$, (*2*, *7*−*11*) or E447(*443*), shown to have a considerable effect on dealkylation in soman-inhibited Hu AChE (*21*).

The *higher kinetic pKs* calculated from the curve fitting have been attributed to the participation of the catalytic $H440H^+$ in the dealkylation reaction (*12*, *16*, *56*). A surprisingly low p*K*, ∼6, has been measured for H440 in the dealkylation reaction in a number of AChEs (*16*, *56*, *62*). This p*K* value is similar to intrinsic p*K* values of the catalytic H440 for acylation and deacylation in the reaction of substrates of AChE (*63*−*66*). In contrast, the p*K* values of the catalytic H in the dealkylation in soman-inhibited BChE are two units higher than in AChE. A recent report (*67*) on

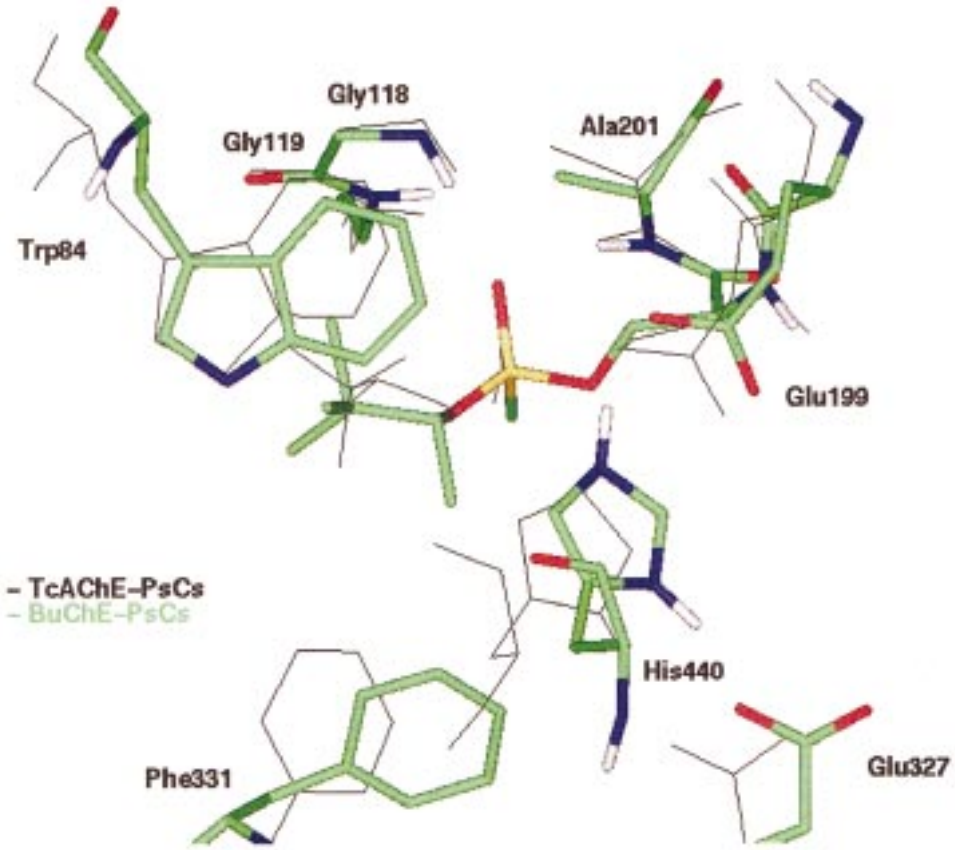


FIGURE 3: A comparison of stereochemical relationships at the active sites of adducts of P(S)C(S)-soman with AChE, licorice representation, and BChE, thin line.

a measurement of the p*K* of H438(*440*) in dealkylated soman-inhibited Hu BChE, a phosphonate monoester anion, gave a value of 8.3, not much higher than the kinetic p*K* values which are for the phosphonate diester. The p*K* value of the catalytic H in other covalently modified serine hydrolases has been known to rise substantially as the negative charge accumulates during C−O bond breaking in alkyl side chains of the modifier (*40*−*44*).

Using the Poisson−Boltzmann formalism, a calculation of the p*K* of catalytic residues in *Tc* AChE gave a high p*K*, 9.3, for H440 in the native enzyme, while the p*K* dropped to 6.7 when the substrate was inserted into the active site (*68*). A p*K* of 9.3 in native ChEs seems too high, but considering the vicinity of carboxylates, E327, E199, and E443 (*39*), it is conceivable that $H440H^+$ enjoys exceptional stability (*45*, *69*−*71*). The authors (*68*) attributed the large drop in p*K* to the mitigating effect of the positive charge of ATC on the high electron density in ionized carboxylates. However, the intrinsic p*K* values obtained from pH dependence of k_{cat}/K_m values with a number of substrates, neutral as well as charged, all range between 5.8 and 6.5 (*65*). In addition, the p*K* values for the enzyme−substrate complex, reflected in k_{cat}, are also in this range. Thus the screening effect cannot be confirmed by experimental evidence.

The p*K* of H438(*440*) in Hu BChE is in better agreement with the calculated p*K*, because it is generally higher than H447(*440*) in AChEs. A greater stabilization of $H438H^+$ than H447(*440*)H^+ by surrounding carboxylates may be the reason for this difference. The large drop in the p*K* to the value of ∼5, which is observed upon removal of electron density from E197 in E197Q Hu BChE, can then be rationalized by the loss of the stabilizing effect of the negative charge of E197 on the protonated form of H438. Such a large drop in the p*K* of H447(*440*) does not occur possibly due to negligible change in the electronic environment of the catalytic H in Mo AChE upon mutation of E202 to Q.

In Figure 3, a comparison is offered of the active-site residues in question in the two enzymes, *Tc* AChE and BChE (*72*, *73*). A segment of the backbone including the oxyanion hole has been superimposed in the two structures to deduce the differences in the stereochemical relationships between the pinacolyl methylphosphonyl fragment and the protein. It should, however, be pointed out that the BChE structure had been created using homology modeling to the *Tc* AChE X-ray structure (*74*). Residues, W82, F329, and the catalytic triad, all appear shifted farther from the phosphonate fragment in the BChE structure than in the AChE structure. Masson and co-workers have recently reported on careful molecular dynamics studies of the adduct of BChE with DFP. The loose binding of the isopropyl group to aromatic residues in these adducts has been pointed out by the authors (*26*). It has also been suggested that the pinacolyl group of soman would more effectively interact with W82 in Hu BChE than the isopropyl group of DFP (*26*).

pH-Dependent Reduction in the Rate Constants for Dealkylation in the Soman-Inhibited Mutants of AChEs and BChEs. The E202 to Q mutation reduces the maximal rate constant for dealkylation 18-fold in the P(S)C(S)-soman-inhibited Mo AChE and 47-fold in the P(S)C(R)-soman-inhibited AChE adduct with respect to the wild-type rMo AChE at pH 5.0. The drop in the rate constant for

Table 4. Rate Reduction, k_0/k, for Dealkylation in Soman-Inhibited Mutant RHu BChEs

		k_0, (min^{-1})	k_0/k		
inhibitor	pH	wild type	E197D	E197Q	W82A
P(S)C(S)	5−6	2 ± 0.2	36 ± 5	6 ± 0.5	2500 ± 700
P(S)C(S)	8	0.18 ± 0.02	8 ± 2	450 ± 100	3200 ± 600
P(S)C(R)	5−6	1.8 ± 0.2	32 ± 5	8 ± 0.7	6000 ± 1000
P(S)C(R)	8	0.4 ± 0.05	14 ± 2	900 ± 100	6000 ± 1000

dealkylation becomes equal, ∼15-fold, between pH 5 and 9. A similar trend is discernible in the data published for the racemic soman-inhibited Hu AChE (*21*), that is, the reduction in the rate constant for dealkylation can be calculated as 260-fold at pH 6.0 and 137-fold at pH 8.0. In contrast, the analogous comparison for *Tc* AChE (*20*) gives a 17-fold rate reduction at pH 6.0 and a 600-fold reduction at pH 8.0: just opposite to the two cases above. Figure 2B portrays the log of the rate constants for dealkylation in soman-inhibited wild-type Hu BChE and its mutants as a function of pH, while Table 4 provides a comparison of rate reductions due to mutation in the wild-type rHu BChE, in the maximal rates, at pH 5.0 or 6.0 and at pH 8.0. The pH dependence of the effect of mutation is quite astonishing; the difference in rate constants decreases for the E197D BChE between pH 5 and 8, which is similar to rMo and Hu AChEs. However, the difference in the rate constants for dealkylation between soman-inhibited wild-type and E197Q BChE increases from pH 5 to 8, which is just the opposite to all other cases but similar to *Tc* AChE.

The reduction in the rate of dealkylation in soman-inhibited W82A Hu BChE with respect to the wild-type enzyme is 2500 in the adduct with the P(S)C(S)-isomer of soman, and it is 6000 in the adduct with the P(S)C(R)-isomer of soman at pH 6.0. The mutation of W82 does not change the pH dependence of the reaction since it does not involve a change in an ionizing group.

The importance of W84 in the catalytic activity of ChEs has been demonstrated for many reactions. It has been especially noted in binding of cationic ligands (*39*, *45*, *66*, *70*, *75*) and in the dealkylation reaction (*2*, *3*, *9*, *22*, *25*) where positive charge development at the transition state is a main characteristic. A stabilizing effect of π electrons of the aromatic indole ring on the accumulating positive charge at the transition state of dealkylation in soman-inhibited ChEs is very likely since the effect of W86A mutation on the rate constant is 1000−6000-fold for Hu AChE as well (*22*, *25*, *26*). However, the effect of this mutation is also considerable on reactions of neutral substrates (*12*, *13*). In this work, the W82(*84*)A mutation caused 10 times larger rate reduction in dealkylation than in phosphonylation. Phosphonylation not only does not involve cation formation but proceeds through a transition state with the likely accumulation of some negative charge. The very large effect of the W82A mutation, 260−680-fold reduction in the rate constant observed in the phosphonylation of Hu BChE by soman, can only be interpreted as a loss of significant binding of the pinacolyl moiety to the indole ring, which serves as a "wall". A similar role of W residues at other active sites can be shown for many enzymes, for example, W215 in serine proteases (*34*). When taking this effect into account, the unique cation π effect amounts to approximately 10-fold in rate or 1.4 kcal/mol in transition state stabilization, whereas binding of the pinacolyl group is worth 3−4 kcal/mol. It appears that the distance between the pinacolyl fragment and the indole ring of W82 is about 1.5 Å longer in BChE than in AChE. Yet, the rate constant for dealkylation is nearly the same in AChEs and BChE. If indeed the cation π interaction is a critical stabilizing force at the transition state of dealkylation, then it is probably supplemented by F329- (*331*), which may move closer to the phosphonate fragment in the soman-inhibited BChE structure than in the AChE structure.

Table 5. First-Order Rate Constants for the Dealkylation in Soman-Inhibited Cholinesterases at the pH of Maximal Rate[m]or as Reported

source	k (min^{-1})	T (°C)	μ (M)	pH	reference
Ee AChE	4.8	25	0.84	5[m]	14
	1.0	25	0.84	7	14
	0.96	4	0.1	5[m]	12
	(6.3)[a]	(25)			
Tc AChE	0.34	25	0.064	6[m]	20
muscles of plaice AChE	0.335	22	0.262	6.1	61
	0.071	22	0.064	7.4	61
rMo AChE	1.2	25	0.1	5[m]	this work
rat brain AChE	0.26	37	0.064	7.3	76
FBS AChE	0.48	4	0.1	5[m]	12
	(3.1)[a]	(25)			
bovine erythrocyte AChE	0.08	25	0.064	7.5	15
	0.115	25	0.064	7.4	77
	∼0.7	5	0.155	5[m]	16
	(∼5)[a]	(25)			
rHu AChE	1.7	24	0.064	6[m]	21
rHu BChE	0.22	4	0.1	6	this work
	(2)[a]	(25)			

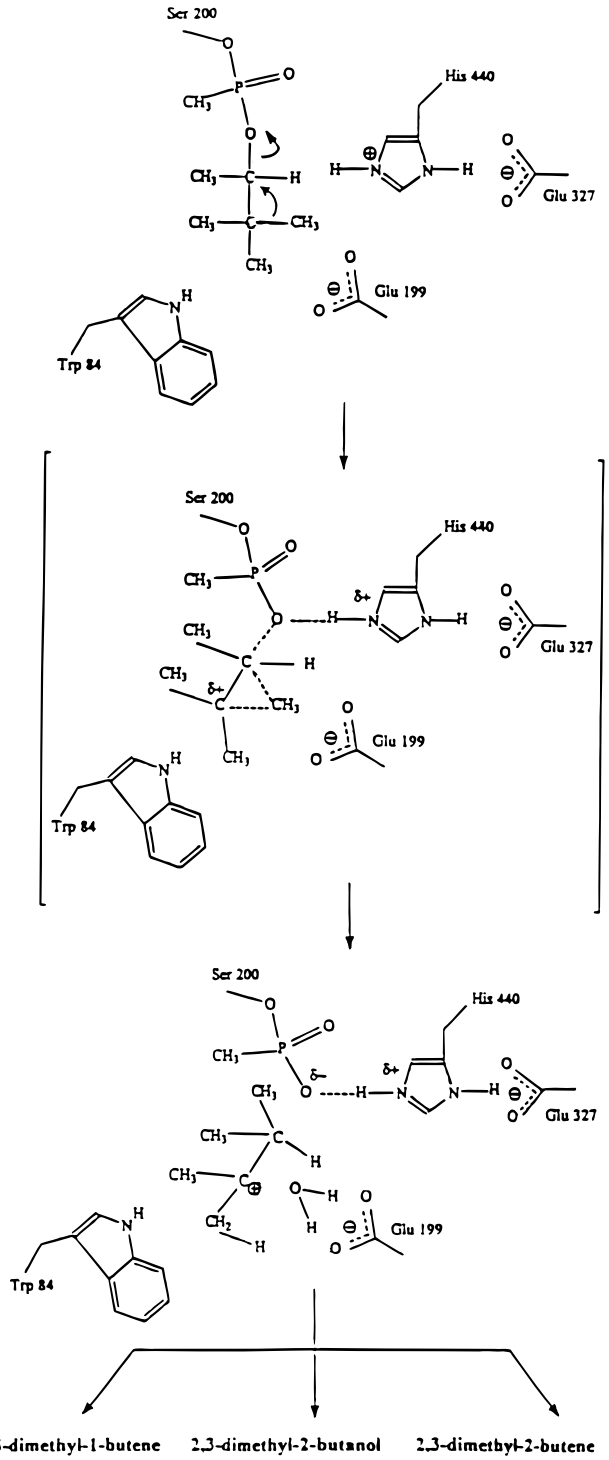
[a] Value extrapolated to 25 °C.

The Species Dependence and Stereospecificity of Dealkylation in Soman-Inhibited ChEs. Rate constants for dealkylation have been reported for a number of soman-inhibited ChEs, but they were measured at different pH, ionic strength, and temperature. While, as shown above, this may render most comparisons tenuous, a summary of the data is provided in Table 5. The largest rate constants are for the *Ee* AChE. The stereospecificity of dealkylation in soman-inhibited ChEs is small in general except for the E199Q mutants.

Dealkylation in soman-inhibited ChEs is an astonishing case of enzyme efficiency in catalyzing a reaction so unlike the reaction the enzymes evolved to catalyze. Phosphonylation and dephosphonylation are frequently compared to the nucleophilic double displacement at carbonyl carbon which is the hallmark of serine hydrolase function. Dealkylation in soman-inhibited ChEs, in contrast, occurs with the loss of electrons in the reacting fragment and the transfer of electrons to the enzyme active site. ChEs having high electron density at their active site in the native state further enhance their electron density by covalently binding a negatively charged fragment at the end of the reaction. The electrostatic catalysis initiating the *push−pull action of the enzyme* results in the departure of products, leaving enhanced electron density behind, which is suicidal for enzyme function.

A Self-Consistent Mechanism of Dealkylation in Soman-Inhibited ChEs. The extensive pH profiles for the dealkylation reaction in soman-inhibited ChEs presented in this study as well as in a previous study (*12*) support the push−pull mechanism proposed earlier (*3*, *4*, *9*, *12*, *13*). Scheme

Scheme 1



1 illustrates the essence of the mechanism: The impetus for methyl migration stems from the electrostatic and steric push from the anionic binding site including E199 and W84 in the ground state. Concerted with methyl migration from Cβ to Cα, the C−O bond breaks without sharp charge polarization at the transition state and provides the soft interactions which are the hallmark of enzyme catalysis (*35*). H440H$^+$ and the electropositive oxyanion hole provide the pulling effect to the C−O bond breaking and the ensuing development of the negative charge on the phosphonate monoester anion. It appears that the enzyme stabilizes the transition state for dealkylation by ~14 kcal/mol with respect to an appropriate nonenzymic reaction by avoiding the formation of at least one intermediate (*12*). Methyl migration in organic reactions is perceived to occur from the secondary carbenium ion toward formation of a tertiary carbenium ion thermodynamically downhill by ~10 kcal/mol. In the tertiary carbenium ion the center of positive charge on the alkyl fragment is on Cβ which is only 4 Å from the N in the indole ring of W84, whereas Cα is ~7 Å from the same point; thus electrostatic stabilization by aromatic π electrons of the positive charge on Cβ is more substantial than on Cα.

The collapse of the tertiary cation[4] is also a critical facet of this mechanism since it avoids the sticking of a carbenium ion to the negatively charged carboxylate side chains (E199, E327, E443). The first intermediate formed is the tertiary carbenium which then rapidly rearranges into neutral products. The catalytic function of E199 is apparently 2-fold: electrostatic catalysis in stabilizing the developing positive charge at the transition state for the formation of the tertiary carbenium ion and general base catalysis without which the carbocation rearrangements may not occur as readily.

The earlier (oxonium ion) mechanism (*14*) often cited has lately been claimed to be supported by hyperconjugation with H−C or Cγ-Cβ electrons (*22*). The question is the following: If the C−C electrons are mobile, why would methyl migration not occur to avoid the formation of a high-energy intermediate? Indeed, there were essentially no products isolated that originate from the secondary cation (*14*). Rapid preprotonation followed by rate-determining formation of a secondary carbenium ion would be associated with an inverse solvent isotope effect, whereas the observed values are between 1.1 and 1.4 for the maximal rate constant for aging in soman-inhibited ChEs.[5] The pH dependence of the reaction, as pointed out above, is consistent with p*K* values of the catalytic H in the phosphonate diester, which is two p*K* units higher in Hu BChE than in AChE. It is quite unlikely that the p*K* of H would drop due to preprotonation of the oxygen, as in the oxonium ion, since the differential calorimetric measurements for aged soman-inhibited Hu BChE (*67*), and the earlier neutron diffraction data and a number of NMR investigations (*40*−*44*) support a serine

[4] Since reaction progress was monitored by measurements of reactivatable enzyme concentration, it is almost certain that events beyond breaking of the C−O bond are not represented by the kinetic data of our work. C−O bond breaking is irreversible for practical purposes. It is difficult to imagine any internal return if the neutral phosphonate monoester is the product of this step (protonation is assumed). If proton transfer has not yet occurred, as we suggest, the anion of the phosphonate monoester may be more prone to trap the carbenium ion: but the product of aging is the monoester anion−HisH$^+$ ion pair. It is the leaving group, the phosphonate monoester anion, that is trapped in this reaction. Since there is no evidence of protein alkylation or electrostatic binding of the carbenium ion, it seems to just collapse.

[5] The isotope effect near one alone does not distinguish between a late transition state for rate-determining oxonium formation and no proton transfer at all, but certainly precludes stepwise preprotonation followed by rate-determining carbenium formation. Other circumstantial evidence militating against this mechanism follows. (1) The residues that can stabilize the positive charge are significantly closer to Cβ than to the pinacolyl oxygen. 2) The argument for oxonium formation is much weakened by the fact that NMR and X-ray evidence support the existence of a phosphonate monoester anion−histidinium cation pair in the product. Why should the proton shuttle in a thermodynamically unfavorable step and back in a subsequent step? This dilemma is reminiscent to that of proton relay catalysis in serine proteases.

methylphosphonate anion−histidinium ion pair product of dealkylation below pH ∼8−9.

It is often said about mechanistic claims that a mechanism can never be proven only discounted if in conflict with experimental facts. A nearly concerted methyl migration with C−O bond breaking is consistent with experimental observations and conforms to the maxim of Occam's razor.

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