Unique Push−Pull Mechanism of Dealkylation in Soman-Inhibited Cholinesterases†

Carol Viragh, Rinat Akhmetshin, and Ildiko M. Kovach*

Department of Chemistry, The Catholic University of America, Washington, D.C. 20064

Clarence Broomfield

U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland 21010-5425

Received November 6, 1996; Revised Manuscript Received April 22, 1997[⊗]

ABSTRACT: The pH-dependence and solvent isotope effects of dealkylation in diastereomeric adducts of *Electric eel* (*Ee*) and fetal bovine serum (FBS) acetylcholinesterase (AChE) inactivated with P(−)C(+) and P(−)C(−) 2-(3,3-dimethylbutyl) methylphosphonofluoridate (soman) were studied at 4.0 ± 0.2 °C. The rate constant versus pH profiles were fit to a bell-shaped curve for all adducts. Best fit parameters are pK_1 4.4−4.6 and pK_2 6.3−6.5 for *Ee* AChE and pK_1 4.8−5.0 and pK_2 5.8 for FBS AChE. The pKs are consistent with catalytic participation of the Glu199 anion and $HisH^+440$. Maximal rate constants (k_{max}) are $13-16 \times 10^{-3}$ s^{-1} for *Ee* AChE and 8×10^{-3} s^{-1} for FBS AChE. The solvent isotope effects at the pH maxima are 1.1−1.3, indicating unlikely proton transfer at the enzymic transition states for the dealkylation reaction. Slopes of log rate constant versus pH plots are near 1 at 25.0 ± 0.2 °C between pH 7.0 and 10.0. In stark contrast, the corresponding adducts of trypsin are very stable even at 37.0 ± 0.2 °C. The rate constants for diastereomers of soman-inhibited trypsin at 37.0 ± 0.2 °C are pH independent and $\sim 10^4$ times smaller than k_{max} for analogous adducts with AChE. Dealkylation in soman-inhibited AChEs is estimated to occur at $>10^{10}$ times faster than a plausible nonenzymic reaction. Up to 40% of the catalytic acceleration can be attributed to an electrostatic push, and an electrostatic pull provides much of the balance. The results of this work together with results of a product analysis by Michel et al. (1969) can be explained by an initial and rate-determining methyl migration from Cβ to Cα. This is driven by the high electron density of residues (Glu199 and Trp84) at a crowded active site and may be concerted with C−O bond breaking. The positive charge at the rate-determining transition state is distributed between Cβ and His440. A tertiary carbocation may have a fleeting existence before it is trapped by water or neighboring electrons which is likely to be promoted by Glu199 as the proton acceptor.

The aging reaction or loss of the pinacolyl group in 2-(3,3-dimethylbutyl) methylphosphonofluoridate (soman)-inactivated[1] cholinesterases has been known to be remarkably efficient (Kovach, 1988a,b, 1993; Qian & Kovach, 1993; Bencsura et al., 1995a,b; Enyedy et al., 1996; Michel et al., 1967; Schoene et al., 1980; Benschop et al., 1984; Schoene, 1978; Keijer & Wolring, 1969; Segall et al., 1993). In fact, the dealkylation reaction in soman-inhibited AChE is difficult to monitor by conventional techniques at room temperature and pH <7. The first paper on a study of the pH dependence of the aging reaction in soman-inhibited *Ee* AChE at room temperature reported an increase in the rate constants with decreasing pH between 5.5 and 8.5 (Michel et al., 1967). The most compelling evidence for the intervention of a carbocation intermediate on the reaction path was reported in this paper; the isolation of predominantly 2,3-dimethyl-2-butanol and ∼40% alkene products using countercurrent distribution of the labeled products and identification of them by scintillation counting provided the basis for the claim. By comparison, the acid-catalyzed decomposition of ethyl pinacolyl methylphosphonate at 100 °C in 50% dioxane and 50% water in the presence of 1 N phenylsulfonic acid occurred with a rate constant of 1.2×10^{-4} s^{-1} and produced ∼30% alkenes (Cadogan et al., 1969).

More extensive studies of the pH dependence of the rate constants for dealkylation in the adduct of bovine erythrocyte AChE with cycloheptyl methylphosphonofluoridate were carried out at room temperature (Keijer et al., 1974). First-order dependence of dealkylation on H^+ concentration between pH 7.5 and 9.0 and a leveling off of the rate below pH 6.0 are clearly discernable on the curves constructed from these data. A break in the pH-rate profile of these reactions was attributed to the catalysis of dealkylation by an unionized carboxylic acid residue with an estimated p*K* of 5.8 (Keijer et al., 1974). Subsequent investigations of organophosphorus inhibition of AChE revealed that a protonated His residue plays a critical role in the aging process (Beauregard et al.,

† This work was supported by the U.S. Army Medical Research and Material Command under Contract DAMD-17-C-91-1064.

⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1997.

[1] Abbreviations: AChE, acetylcholinesterase (acetyl hydrolase); bis-tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; 18-crown-6, (1,4,7,10,13,16-hexaoxacyclooctadecane); DMSO, dimethylsulfoxide; *Ee*, electric eel; FBS, fetal bovine serum; FFAP, free fatty acid phase (chromosorb with 80/100 solid coated with 10% nitrophthalic acid on Carbowax 20 M); GLC, gas−liquid chromatography; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-2-ethanesulfonic acid; HI-6, 1-(2-hydroxyiminomethyl-1-pyridinium)-1-(4-carboxyaminopyridinium)-dimethyl ether HCl; Hu, human; MUGB, 4-methylumbelliferyl-p-guanidinobenzoate hydrochloride; 2-PAM, 2-(hydroxyiminomethyl)-1-methylpyridinium iodide; PEG, polyethylene glycol; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; Porapak Q, a nonpolar porous polymer sorbent of small pore size composed of ethyl vinyl benzene cross linked with divinyl benzene; pNP, 4-nitrophenol; sarin, 2-propyl methylphosphonofluoridate; soman, 2-(3,3-dimethylbutyl) methylphosphonofluoridate; TAPS, *N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid; *Tc, Torpedo californica;* Tris, tris(hydroxymethyl)-aminomethane; Triton X-100, *tert*-octylphenoxypoly(oxyethanol); vdW, van der Waals; Z, carbobenzoxy.

S0006-2960(96)02764-X CCC: $14.00

1981). Schoene et al. (1980) reported a bell-shaped pH dependence of the rate constant for the aging of soman-inhibited bovine erythrocyte AChE at 5 °C. Slopes of the curve between pH 4 and 5 and pH 7 and 8 seem to approach unity, indicating a first-order dependence on increasing and decreasing concentrations of H^+, respectively. Although the authors estimated p*K* values of 4.5 and 6.0 at half maximal velocities on asymmetric bell curves, limiting values of rate constants are not discernable on these curves. Soman-inhibited plaice cholinesterase was also shown to age as a function of increasing H^+ concentration up to pH ~5.0 at 25 °C (Bucht & Puu, 1984). More recent studies of the adducts of *Tc* AChE with the cyclopheptyl analog of soman showed a sigmoid dependence of the rate constants for aging on pH between 5 and 8 and a sharp inflection point at pH 6.1, which was attributed to catalysis by $HisH^+$ at the active site (Berman & Decker, 1986). This study included the ionic strength dependence of the reaction; it was significant at pH below 6 indicative of electrostatic stabilization of the incipient carbocation. Except for this case, neither an unequivocal inflection point(s) nor limiting rate constants at either end of the pH scale has been established for the pH dependence of the rate constants for the aging reaction studied to date.

Since the X-ray-structure (Sussman et al., 1991; Bourne et al., 1995) of *Tc* AChE became available, we have investigated the interactions in the adducts of aged soman-inhibited *Tc* AChE by computational techniques (Qian & Kovach, 1993; Bencsura et al., 1995a,b, 1996; Enyedy et al., 1996) to provide the structural framework for our own observations of the dependence on pH of the aging reaction in diastereomers of soman-inhibited cholinesterases. Subsequently, we proposed the "push−pull" mechanism involving the negatively charged Glu199, Trp84, and other aromatic residues and the electropositive region of the active site, *i.e.*, the protonated $HisH^+440$ and oxyanion hole, to be the origin of the aging reaction in cholinesterases (Qian & Kovach, 1993; Bencsura et al., 1995a,b, 1996; Enyedy et al., 1996). The Glu199Gln mutant of *Tc* AChE (Saxena et al., 1993, 1996) and the Glu202Gln mutant of Hu AChE (Ordentlich et al., 1993) have indeed shown much diminished capacity for dealkylation when inhibited with soman. While this paper has been in review, a large effect of the Trp86Ala mutation in Hu AChE on the aging reaction in soman-inhibited enzymes was published (Shafferman et al., 1996). The authors attributed the aging phenomenon in soman-inactivated AChEs to the cation-π effect and misinterpreted the model we proposed for the mechanism of aging.

In this paper, we wish to give experimental support of the push-pull mechanism of dealkylation in soman-inhibited AChE. To this end, two of the most active diastereomers of soman P(−)C(−) and P(−)C(+) were used for the generation of diastereomeric adducts of *Ee* and FBS AChE to study the full pH dependence and solvent isotope effect of the reaction. Analogous diastereomers of soman-inactivated porcine trypsin were also studied for comparison to assess how the composition of the active site, especially the electrostatic makeup of the two enzymes, affects the occurrence and mechanisms of dealkylation in the two types of enzymes. The loss of reactivatable trypsin from its soman-inhibited forms occurs with a ~5.6 kcal/mol higher energy barrier than those of reactivatable AChE from any adduct studied. Moreover, an S_N1-type hydrolysis of analogs of Ser covalently modified with soman could not be brought about in aqueous solutions even at acidic or basic extremes. Nucleophile-driven P−O bond fission in these compounds was accomplished at ~37 °C, in 0.6−1.2 M $NaOCH_3/CH_3$-OH in 4 days. These reactions then bear no resemblance to the enzyme-catalyzed dealkylation process. A structural interpretation of the mechanistic studies of the AChE-catalyzed reactions is based on our computational studies of the aging reaction (Qian & Kovach, 1993; Bencsura et al., 1995a,b,1996; Enyedy et al., 1996).

MATERIALS AND METHODS

Materials. Enzymes were Sigma products except for the FBS AChE which was a gift from Dr. B. P. Doctor, Director, and Dr. A. Saxena, Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC. (*Z*)-*N*-Phe-Val-Arg-*p*-nitroanilide (pNA) HCl, MUGB, and the Ellman reagents were from Sigma. HI-6, 2-PAM, and the diastereomers of soman were from the U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground. Solvents, buffer salts, and other reagents were analytical grade and were used as purchased. Deuterium oxide (99.9%) was purchased from Aldrich Chemical Co.

Model Compounds. Ethyl n-Propylphosphonochloridate. Ethyl-*n*-propylphosphonochloridate was prepared by an adaptation of reported methods (Lieske et al., 1969; Hovanec et al., 1977; Lacy & Lowe, 1980). The *n*-propylphosphonodichloridate (Aldrich), 0.11 mol, was stirred vigorously in 50 mL of pentane under N_2 while 0.095 mol each of dry lutidene and ethanol, in 50 mL of pentane, was added dropwise over a 1 h period. Stirring was continued for 48 h and then the solid was filtered and washed well with pentane under reduced pressure and the product distilled to give 10.0 g (67%) of an oil, bp 75 °C, 3.24 mm Hg. The distilled product was estimated to be 80% of the ethoxy compound by NMR. NMR: CCl_4, 2% TMS, δ 0.8−1.4 (m, 3H, CH_3), 1.4−2.9 (m, 4H, CH_2-CH_2 + 3H, CH_3 ethyl), 3.85 (d, 2H, OCH_2), J (P-H) = 11 Hz.

Ethyl Pinacolyl n-Propylphosphonate. Pinacolyl alcohol 7.2 mmol in 5 mL of dry pentane, was stirred with 4.7 mmol (1.3 equiv) of NaH (washed with pentane) overnight. To this mixture, 3.6 mmol of ethyl *n*-propylphosphonochloridate in pentane was added dropwise and the mixture was allowed to stir overnight. The reaction mixture was centrifuged, then the supernatant was removed. The residue was filtered and washed with pentane. The pentane solution of products was treated with a small amount of Dowex 50 × 8 (50 mesh Bio-Rad), and the solvent removed under reduced pressure. The pinacolyl alcohol free product distilled at 95 °C, 1.2 mm Hg. GLC analysis showed no evidence of the alcohol. NMR: CCl_4, 2% TMS, δ 0.9−1.4 (m, 3H, CH_3), 0.92 [s, 12 H, $CCH_3C(CH_3)_3$], 1.4−2.9 (m, 4H, CH_2CH_2 + 3H, CH_3 ethyl), 3.85 (d, 2H, OCH_2 + 1H, OCH), J (P−H) = 11 Hz.

Dipinacolyl n-Propylphosphonate. This compound was prepared from pinacolyl alcohol and n-propylphosphonodichloridate as described above. NMR: CCl_4, 2% TMS, δ 0.8−1.4 (m, 3H, CH_3), 0.92 [s, 24 H, $[CCH_3C(CH_3)_3)]_2$], 1.4−2.9 (m, 4H, CH_2CH_2), 3.85 [d, 2H, $(OCH)_2$], J (P−H) = 11 Hz.

Stock Solutions, Reagent, and Buffer Solutions. Stock solutions were prepared for AChE at 1 μM and trypsin at 40 μM. About 1−2 mL volumes of the enzyme solutions

were inhibited with 1 and 40 μL, respectively, of a 10 mM solution of the soman diastereomers. The trypsin adduct solutions were preserved with 0.05 M Triton X-100. Excess of soman was removed on a Sephadex 25-G column at pH 9.5. The solutions were frozen after a few minutes and stored in a deep freezer. Solutions of AChE adducts all contained >98% inhibited enzyme and trypsin solutions were inhibited to 92% with the P(−)C(+) and 66% with the P(−)C(−) diastereomer of soman. Enzyme stability was monitored under the conditions of the experiments; a decline in enzyme activity was observed for AChE at 4.0 °C and below pH 4.5 and for trypsin at 37 °C and pH >9.0. A slight correction was applied to the reactions of trypsin. Buffer solutions were prepared by weight in water or heavy water, and the pH was measured at 25 °C. Buffers used in the aging experiments were 0.1 M formate, acetate, HEPES, PIPES, Bis-Tris propane and Tris in H_2O and D_2O for AChE, and 0.1 M Bis-Tris propane containing 0.05% Triton X-100 for trypsin. Buffer solutions for experiments with AChE were at μ = 0.1 (KCl). Solutions for reactivation were 0.005 M HI-6 in pH 9, 0.2 M TAPS buffer for AChE and 0.1 M 2-PAM in pH 6−8, and 0.1 M Bis-Tris propane buffer containing 0.05% Triton X-100 for trypsin.

Enzyme Assays. AChE activity was determined in pH 7.6, 0.1 M HEPES buffer, μ = 0.1 (KCl) at 25.0 ± 0.2 °C by the Ellman assay (Ellman et al., 1961). Trypsin activity was monitored by a spectrophotometric assay at 405 nm, with 2 × 10^{-4} M (*Z*)-*N*-Phe-Val-Arg-pNA in pH 8.2, 0.05 M Tris buffer containing 0.01 M $CaCl_2$, 0.005 M NaCl, and 2% DMSO at 25.0 ± 0.2 °C.

Studies of Soman-Inhibited AChE. A three-step protocol for monitoring the aging reaction was followed in all cases: 1) the dealkylation reaction was carried out in a small vial in the required medium in a carefully thermostated reactor at 4.0 ± 0.2 °C and at 25.0 ± 0.2 °C at pH > 7.0; 2) aliquots were drawn from the vessel in a regular time-course and the reaction was quenched by dilution into a prethermostated reactivation mixture at 38.0 ± 0.2 °C; and 3) after an hour the fully reactivated enzyme was assayed for activity. The rate constants measured for reactivation with HI-6 are tabulated in Table 1. An aliquot of the aging mixture was also drawn for assaying for spontaneous recovery of enzyme activity.

Enzyme activity-time pairs for at least 4 half lives were fit to the first-order rate law using GraFit (Leatherbarrow, 1992) and 2−4 repeats were averaged at each experimental pH value. The weighted average of first-order rate constant - pH data pairs obtained at 4.0 °C were fit to a bell-shaped two-pK model. The higher pK (pK_2) was constrained at values between 5.6 and 6.6 in a series of calculations to find the best fit in three cases. The data at 25.0 °C were fit by linear least squares.

Studies of Soman-Inhibited Trypsin. Due to the sluggish decline in reactivatable trypsin, the aging reaction from the adduct formed with the P(−)C(+) soman diastereomer was followed by a sampling technique at 37.0 ± 0.2 °C similar to that described for AChE. Nucleophilic reactivation at 37.0 ± 0.2 °C was slower still from the adduct formed with the P(−)C(−) diastereomer of soman. Therefore, the competing reactions, aging, and nucleophilic reactivation were followed from one reaction mixture and individual rate constants were elucidated from the observed kinetic rate constants and the composition of products. In a typical protocol, 975 μL of the 0.1 M 2-PAM solution and 25 μL of the soman-inhibited trypsin stock solution of either diastereomer were mixed and the timer was started. Aliquots of 20 μL were extracted at specified time intervals, diluted and assayed for reactivated trypsin. Thus, studies of the P(−)C(+) diastereomeric adducts were carried out by two different techniques and the results were consistent. Spontaneous reactivation and denaturation of trypsin was monitored from independent measurements.

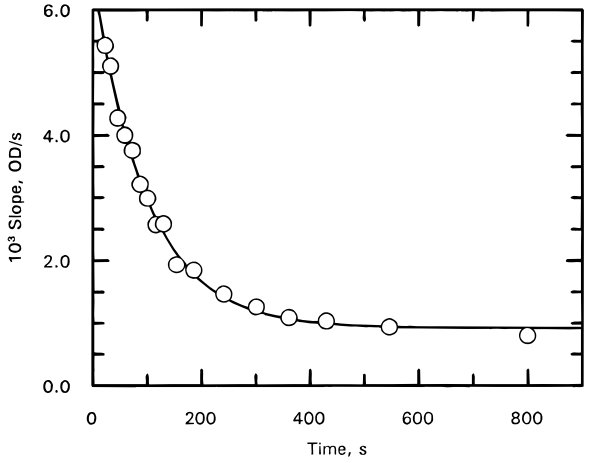


FIGURE 1: Time dependence of the aging reaction in P(−)C(−)-soman-inhibited *Ee* AChE at 4.0 ± 0.2 °C and pH 5.5.

Model Reactions for PO Fission: GLC Experiments. Separation of isopropyl alcohol and its methyl ether from other components was accomplished by the injection of 0.5 μL samples of the supernatant of the reaction mixtures into the Porapak Q column at 120 °C followed by ramping to 140 °C (2 °C/min) at t = 5 min, then to 200 °C (10 °C/min) at 15 min. The N_2 carrier gas flow was 20 mL/min and the elution times were as follows: methanol, 2 min; isopropyl alcohol, 7 min; isopropyl methyl ether, 9 min. Separation was also attained on an FFAP column at 30 °C with ramping to 200 °C (20 °C/min) at t = 10 min with the following retention times: isopropyl methyl ether, 0.7 min; isopropyl alcohol and methanol, 3 min; phosphonates, 16 min.

Separation of pinacolyl alcohol and its methyl ether from other components was accomplished by injection of 1 μL samples of the supernatant of reaction mixtures (filtered through a nylon millipore filter) into the FFAP column. Elution with the N_2 carrier gas (20 mL/min) began at 25 °C and was subsequently ramped to 200 °C (20 °C/min) at t = 18 min. The retention times were as follows: pinacolyl methyl ether, 1.5 min; methanol, 3.5 min; pinacolyl alcohol, 14 min; phosphonates, 27 min.

Instrumentation. NMR spectra were obtained with a Varian T-60 spectrophotometer. GLC measurements were carried out on a Varian Aerograph instrument. A λ-6 Perkin-Elmer spectrophotometer interfaced to a PC and thermostated with a NESLAB RTE-4 circulating bath was used for enzyme assays.

RESULTS

Aging in Diastereomers of Soman-Inhibited AChE. Indeed, the aging reaction in soman-inactivated AChE is remarkably fast and could only be followed at 4.0 ± 0.2 °C at pH below 7.0 (Bencsura et al., 1995b). A typical curve showing declining reactivatable *Ee* AChE as determined by the Ellman assay is shown on Figure 1. Although the extent

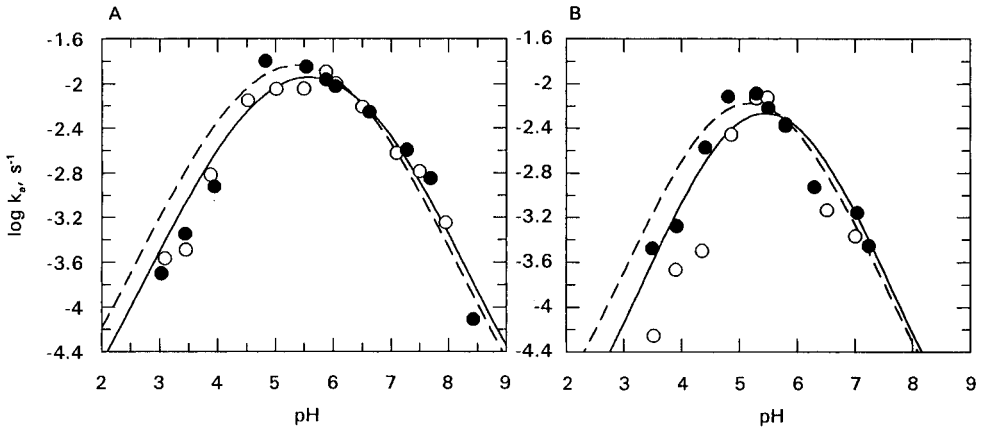


FIGURE 2: pH dependence of the aging reaction in soman-inhibited (A) *Ee* AChE and (B) FBS AChE at 4.0 ± 0.2 °C: P(−)C(+)-soman (●) and calculated line (---); P(−)C(−)-soman (○) and calculated line (—).

of AChE reactivation from the adducts with soman is species dependent, HI-6 was reported to be the most effective nucleophile in all cases (Puu et al., 1986; Sun et al., 1986; Grunwald et al., 1989; Segall et al., 1993). Enzyme recovery was ~95% from the P(−)C(+) soman-inhibited AChE and only ~40% from the P(−)C(−) soman-inhibited AChE immediately after inactivation but slowly diminished during a few weeks of storage at −70 °C. This is very similar to that reported earlier for AChE from other species (Saxena et al., 1997, DeJong & Wolring, 1985; DeJong & Kossen, 1985).

The pH dependence of log rate constants for dealkylation of soman-inhibited AChE on Figure 2, albeit not entirely symmetric, is best described by a bell-shaped curve with a maximum between pH 5 and 5.5 at 4.0 ± 0.2 °C. A decline in the rate of dealkylation is discernable between pH 4 and 5.5 for the adducts of *Ee* and FBS enzymes, respectively. The rate constant for aging trails off for the adducts of the FBS enzyme below pH 4.0, whereas it persists to fall with decreasing pH for *Ee* AChE. The upper leg of the pH profile also differs slightly for the two enzymes. Extension of the studies between pH 7 and 10 at 25.0 ± 0.2 °C showed a continuing decline in the value of the rate constants with slopes 0.80 ± 0.04 and 0.92 ± 0.05 for the P(−)C(+) and P(−)C(−) soman-inhibited *Ee* AChE, and 0.80 ± 0.07 and 0.86 ± 0.02 for diastereomers of the soman-inhibited FBS AChE, respectively (Figure 3). A selfconsistent fitting of all data sets at 4.0 ± 0.2 °C required fitting to a two p*K* model with zero lower limits and upper limits (L) of the two sigmoids set equal as in eq 1:

$$k = \frac{L10^{(\mathrm{pH} - \mathrm{p}K_1)}}{10^{(2\mathrm{pH} - \mathrm{p}K_1 - \mathrm{p}K_2)} + 10^{(\mathrm{pH} - \mathrm{p}K_1)} + 1} \qquad (1)$$

Due to the sharp rise in the sigmoid curves, the higher p*K*s for the FBS AChE had to be constrained to an optimal value obtained by fitting to a range of values between 5.6 and 6.6. The parameters obtained from the best fit of the rate constants to pH for dealkylation of the soman-inhibited AChEs at 4.0 ± 0.2 °C are displayed in Table 2.

There is no notable difference in the rates of dealkylation between the diastereomeric adducts formed with either the *Ee* or the FBS AChE; chirality at C has a negligible effect on the rate or the mechanism in the P(−) diastereomers of soman-inhibited AChEs. Solvent isotope effects were 1.1−1.3 for the maximal rate constants and identical to that observed for the aging in 2-propyl methylphosphonyl adducts of AChE (Kovach & Bennet, 1990). These small normal solvent isotope effects indicate that there is no transfer of protons in the rate-determining step of these reactions.

Our calculation of $\Delta H^{\ddagger}$, from the data at pH 7.0 and 4 and 25 °C, for the aging of diastereomers of soman-inhibited

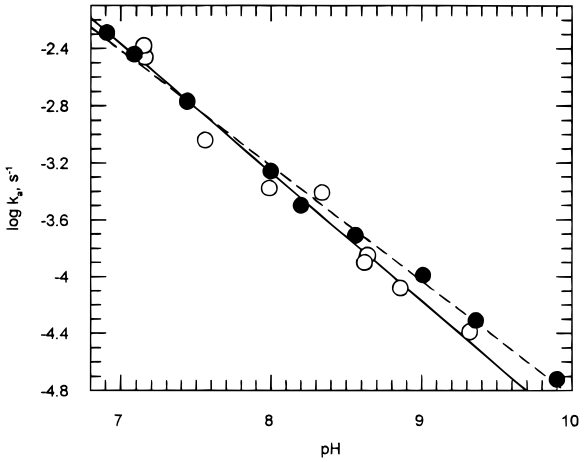


FIGURE 3: pH dependence of the aging reaction in soman-inhibited FBS AChE at 25.0 ± 0.2 °C: P(−)C(+)-soman (●) and calculated line (---); P(−)C(−)-soman (○) and calculated line (—).

Table 1: Second-Order Rate Constants[a] (10 k_r, M^{-1} s^{-1}) for the Reactivation of Soman-Inhibited AChE at pH 10 and 38.0 ± 0.2 °C

AChE	P(−)C(+)	P(−)C(−)
Ee	3.4 ± 0.2[b]	2.6 ± 0.8
FBS	1.1 ± 0.1	0.36 ± 0.02

[a] Average values of three to six measurements. [b] Mean of two measurements.

Table 2: Observed Maximal Rate Constants (k_{max}), Solvent Isotope Effects and p*K*s Calculated for Dealkylation of Diastereomers of Soman-Inhibited AChEs at 4.0 ± 0.2 °C and μ = 0.1 M (KCl)

adduct	10^3 k_{max}, s^{-1}	k_H/k_D	pK_1	pK_2
Ee AChE and Soman P(−)C(+)	16 ± 2	1.1 ± 0.2	4.4 ± 0.2	6.3[a]
Ee AChE and Soman P(−)C(−)	13 ± 1	1.3 ± 0.3	4.6 ± 0.2	6.5 ± 0.2
FBS AChE and Soman P(−)C(+)	8 ± 1	1.1 ± 0.1	4.8 ± 0.2	5.8[a]
FBS AChE and Soman P(−)C(−)	8 ± 1		5.0 ± 0.2	5.8[a]

[a] Best fit values to eq 1.

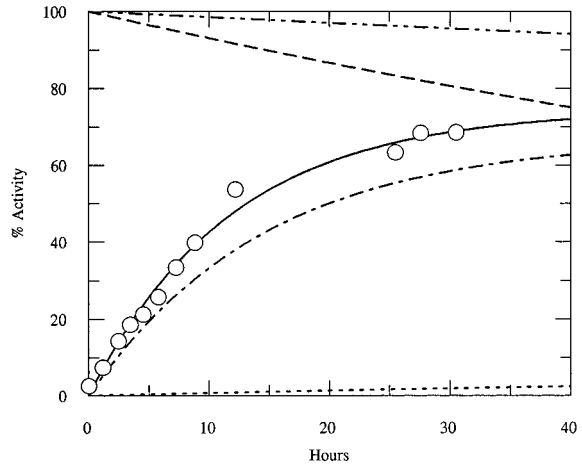


FIGURE 4: Time dependence of aging reaction in P(−)C(+)-soman-inhibited trypsin at 37.0 ± 0.2 °C and pH 8.0: experimental points for reactivatable trypsin are represented by the circles and the corresponding calculated first-order curve is indicated by the solid line. Other lines were calculated from eq 3 for the first-order increase in trypsin activity due to nucleophilic reactivation (− - −) and for decay of trypsin activity due to aging (− − −). The first-order decay of enzyme activity due to denaturation is indicated with (− - - −) and the first-order spontaneous enzyme reactivation from inhibited trypsin in indicated with (---).

FBS AChE is 14.3 ± 0.5 kcal/mol and in excellent agreement with the value of 14.6 kcal/mol measured for the soman-inhibited bovine erythrocyte enzyme by Schoene et al. (1980). An extrapolation of the maximal rate constants in Table 2 to 37 °C using the Eyring equation and $\Delta H^{\ddagger} = 14.3$ kcal/mol gives ∼0.2 s^{-1} for soman-inhibited *Ee* AChE diastereomers and ∼0.15 s^{-1} for the FBS adducts. This amounts to a 17-fold increase in the maximal rate constants at pH 5.5 when going from 4 to 37 °C.

Aging in Diastereomers of Soman-Inhibited Trypsin. Lengthy experiments for the pH dependence of trypsin reactivation from the soman-inhibited adducts at 37.0 ± 0.2 °C had to be corrected from control experiments for a slight denaturation of trypsin shown in the scheme below:

$$[E_{inh}] \xrightarrow{k_r} [E] \xrightarrow{k_d} [E^*] \quad (2)$$

where $[E_{inh}]$ is the initial concentration of inactivated trypsin, [E] is the concentration of free enzyme at time *t*, [E*] is the concentration of denatured trypsin, and k_r, k_a, and k_d are the first-order rate constants for reactivation, aging, and enzyme denaturation, respectively. Recovering enzyme activity can be expressed by eq 3 where $[E_0]$ is the concentration of free enzyme and $[EI_0]$ is the concentration of inactivated enzyme at t = 0:

$$E(t) = \frac{k_s + k_r}{k_d - k_{obs}}[EI_0](e^{-k_{obs}t} - e^{-k_d t}) + [E_0]e^{-k_d t} \quad (3)$$

$$k_{obs} = k_a + k_r + k_s \quad (4)$$

The rate constant for spontaneous reactivation, k_s, was measured independently. Figure 4 gives the time dependence of all simultaneous processes for trypsin denaturation, aging, nucleophilic reactivation, and spontaneous reactivation in P(−)C(+) soman-inhibited trypsin. The pH dependence of the rate constants is discernible from Table 3.

There is a marked difference in the fate of the soman-inactivated AChE and trypsin adducts. Reactivation of trypsin by 2-PAM was very slow, albeit the most efficient from an arsenal of oximes. Similar observations were reported by Grunwald et al. (1989). Trypsin could be reactivated up-to 70% of the activity of the control from the adduct formed with the P(−)C(+) soman diastereomer after 3 days at 37.0 ± 0.2 °C and neutral pH. The decline in reactivatable trypsin in 3 days obeyed first-order kinetic law, but was largely pH independent in both diastereomers.

Model Reactions. Structural analogs, given in Scheme 1, of sarin (I) and soman (II), were generated as described below. The UV−visible spectra of compounds I and II remained intact in aqueous solutions at pH ∼1 and >12 and room temperature for several days. Ethylacetate extracts of the solutions contained no detectable decomposition products on GLC. Then the compounds were subjected to methoxide attack in methanol or acetonitrile, and the appearance of their low molecular weight products was monitored by GLC. As shown in Scheme 1, production of an alcohol would entail P−O bond cleavage, whereas C−O bond cleavage should result in the formation of the methyl ether of the corresponding alcohol.

(I) Isopropyl methyl methylphosphonate was prepared *in situ* from isopropyl methylphosphonochloridate (Aldrich) in methanol with sodium methoxide. The sodium chloride by-product was filtered out and the 0.3 M methanolic solution of I was then reduced to $^1/_2$, and 2 equiv of sodium methoxide was added with 0.1 mol of 18-crown-6 to facilitate the dissolution of sodium methoxide. The mixture was sampled on a Porapak Q column, and the slow appearance of a peak was followed for 50 h. The mixture was then heated to 37 °C and refluxed for 72 h until no more increase in (the isopropyl alcohol) peak intensity was discernable. Addition of 1.5 mol of 18-crown-6 brought about the dissolution of all sodium methoxide, and the clear solution was further kept at 40 °C for several hours. This did not change the peak intensity, nor did it bring out any other peaks. The retention time of the product was identical with that of isopropyl alcohol and spiking with isopropyl alcohol resulted in complete overlap with the product peak. An isopropyl methyl ether standard for GLC was also prepared from sodium isopropoxide in excess isopropyl alcohol and methyl iodide. The retention time of this compound was distinctly different from that of the product of the methoxide displacement reaction.

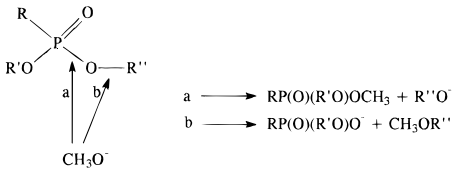
(II) One millimole of ethyl pinacolyl *n*-propylphosphonate and 1 mmol of 18-crown-6 were dissolved in 3 mL dry acetonitrile, and an aliquot was drawn and injected into an FFAP column. One millimole of sodium methoxide was added and the mixture was heated in a bomb to 50 °C for a day. Pinacolyl alcohol was the identified product of this reaction. This reaction was also repeated in dry methanol as follows: a 0.3 M solution of II was stirred with 2 equiv of sodium methoxide solid until it all dissolved. Then the first sample was drawn for GLC analysis, and the mixture was heated to 37 °C in a closed refluxing system. Samples were drawn and analyzed on GLC in 20 min intervals for 5 h and after 24, 48, and 72 h until a change in peak heights became negligible. The major product again was pinacolyl alcohol and a small amount of a volatile component, possibly ethyl methyl ether, that chromatographed with diethylether. Identification of the product is based on peak matching with

Table 3: Rate Constants Observed (k_{obs}), for Dealkylation (k_a), Nucleophilic Reactivation (k_{re}), and Spontaneous Reactivation (k_{sp}) from (A) P(−)C(+) and (B) P(−)C(−) Soman-Inhibited Trypsin at 37 ± 0.2 °C

	10^5 k_{obs},[a,b] s^{-1}		10^5 k_{re} [N],[a,b] s^{-1}		10^5 k_a,[a,b] s^{-1}		10^6 k_{sp},[b,c] s^{-1}	
pH	A	B	A	B	A	B	A	B
6.0	1.1 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	0.2 ± 0.2	2.1 ± 0.1	1.1 ± 0.1
6.5	1.2 ± 0.2	1.0 ± 0.1	0.8 ± 0.1	0.3 ± 0.1	0.5 ± 0.2	0.7 ± 0.1	2.1 ± 0.1	1.2 ± 0.1
7.0	1.5 ± 0.1	1.4 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	2.3 ± 0.1	1.2 ± 0.1
7.5	2.3 ± 0.2	1.8 ± 0.2	2.0 ± 0.1	1.0 ± 0.1	0.4 ± 0.1	0.7 ± 0.2	2.3 ± 0.1	1.2 ± 0.1
8.0	2.5 ± 0.3	2.7 ± 0.1	1.6 ± 0.2	1.1 ± 0.1	0.7 ± 0.3	1.4 ± 0.1	2.3 ± 0.1	1.2 ± 0.1
8.0[d]	2.0 ± 0.1	2.6 ± 0.2	0.7 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	1.6 ± 0.2		

[a] The reaction was followed in 0.1 M 2-PAM solution in 0.1 M Bis-Tris propane buffers and the data were fit to eq 3 with $k_d = 3 \times 10^{-6}$ s^{-1} measured in controls. [b] Mean value of two measurements. [c] Determined independently. [d] Determined in D_2O buffer.

Scheme 1: (a) P−O Bond Fission and (b) C−O Bond Fission in Nucleophilic Reactions of Phosphonate Diesters



I R = CH_3; R' = $CH(CH_3)_2$; R'' = CH_3

II R = $CH_2CH_2CH_3$; R' = $CH(CH_3)C(CH_3)_3$; R'' = CH_2CH_3

III R = $CH_2CH_2CH_3$; R' = R'' = $CH(CH_3)C(CH_3)_3$

pinacolyl alcohol and on spiking with pinacolyl alcohol. A pinacolyl methyl ether standard was also used as described for the isopropyl methyl ether. The retention time of the pinacolyl methyl ether was significantly different from that of the product or pinacolyl alcohol.

(III) Dipinacolyl *n*-propylphosphonate was reacted with methoxide ion in methanol in the manner described for II. This reaction proceeded even slower than the reaction of II, because of the two bulky groups present. A small amount of pinacolyl alcohol was detected after several days at room temperature. A trace amount of the volatile component was produced along with pinacolyl alcohol.

DISCUSSION

pH-Dependence of the Aging Reaction in Diastereomers of Soman-Inhibited Ee and FBS AChE. Extensive pH rate profiles of the dealkylation reaction in diastereomericly pure soman-inhibited AChE from two different species are basically similar between pH 3.5 and 8.0 at 4.0 °C and between pH 7 and 10 at 25.0 °C: they are asymmetric bell-shapes and similar to those reported by Schoene et al. (1980) between pH 4 and 8. However, a quantitative evaluation of p*K*s and an interpretation in physical terms requires circumspection. One problem is the slightly asymmetric nature of the profiles in all cases. Thus, an ideal fit would be to two sigmoid curves of different amplitude and the calculation of six parameters. A more modest approach had to be taken in the data analysis, that is a fit to a true bell-shaped curve.

There is uncertainty in the actual lower limits of the reaction. One might expect the log rate constant of the enzyme-catalyzed reaction to drop continuously with a slope of 1 as the catalytically competent form of the enzyme decreases due to protonation or deprotonation. The rate should level off where the activation energy of the prevailing enzyme-catalyzed process rises over that of a competitive and pH-independent parallel reaction. A nonenzymic process might be on a limiting pathway. It has been shown in this work that either CO or PO fission in phosphonate diester analogs of soman-inhibited Ser is slow without a very good catalyst such as an enzyme.

If in fact the limiting rate constants are higher than that for a nonenzymic process, it would indicate catalysis by an enzyme form of lower activity than the dominant form in the middle pH range. This less active enzyme form may not participate at the atomic level in the catalysis of dealkylation in soman-inhibited AChE. Instead, a residue or a collection of residues providing electrostatic (or hydrophobic) catalysis may promote the reaction in such a case.

Declining Rate with Decreasing pH. It is almost certain that the decline in the rate of aging with decreasing pH beginning at pH 5.5 is due to the involvement of a basic residue that becomes protonated. The declining slope of the log rate constants below pH 5.5 is even greater than 1, which may be an artifact although this trend is also discernable on the pH profile of Shoene et al., (1980). An entirely different technique, pH-stat, was used for data acquisition in that work.

The best candidate for a base catalyst in the vicinity of the phosphonyl fragment is Glu199 (Qian & Kovach, 1993; Bencsura et al., 1995a,b, 1996; Enyedy et al., 1996). Glu199 is 4−5 Å from the Cα and 3−4 Å from a methyl group in Cβ of the pinacolyl moiety in energy-minimized structures of tetracoordinate adducts of soman-inhibited *Tc* AChE. Molecular dynamics (MD) simulations on the 120 ps time scale allowed for relief of the steric strain at the tight active site, and this increased the Glu199−Cα-pinacolyl distance by ∼1 Å. However, MD simulations do not allow for changes in bonding and electron redistribution as in a chemical reaction. *The aging reaction is the consequence of local electron density amplified by soft electrostatic effects in a tight active site.* An electrostatic attraction, 5−10 kcal/mol, between a Glu residue and a fledgling carbocation is all that is needed for catalysis. This interaction energy would arise, for example, between a unit negative charge and a $^1/_2$ positive charge at 4−5 Å distance in a dielectric medium of $\epsilon = 5-10$.

If Glu199 is involved in stabilizing the incipient carbocation, one might expect its effect on the rate constant to be first order in free base with a lower limit approaching the nonenzymic rate of dealkylation. It is not possible to follow the reaction reliably below pH ∼3.5, the point where only ∼10% of the free base is remaining. The log k_a for the aging of the soman-inhibited FBS AChE versus pH curve seems to trail off at pH ∼4. A fit of the rate constant-pH data pairs to two symmetric sigmoid titration curves yields pK_1 values typical for carboxylic acids (Table 2). A limiting rate constant at low pH would be about >200 times smaller than the maximal rate constant but larger than a nonenzymic limit.

Incidentally, a Glu202Gln mutation in Hu AChE (Shafferman et al., 1996) caused a similar reduction in the rate of aging. If another carboxylate ion participates in promoting this reaction, or can take over the role of Glu202 in the Gln202 mutant, that should manifest in the pH dependence of the mutant-catalyzed aging reaction. Experiments are in progress in this laboratory to illuminate this question.

The residual catalysis at low pH might represent electrostatic catalysis by the cation-aromatic-π interaction described by Dougherty (1996). Aromatic amino acid residues that are within 4−6 Å from Cα and Cβ of pinacolyl are Trp84 and Tyr121. The mutation of Trp86 to Phe and Ala caused 20 and ∼1800 reduction, respectively, in the rate constants of aging in soman-inactivated Hu AChE at pH 7 (Shafferman et al., 1996). However, a 10−20 fold rate reduction results in all reactions catalyzed by all Trp86 mutants of AChE, which may be attributed to the lack of binding and poising of a substrate or inhibitor at the active site. The remaining >100-fold reduction observed in the rate constants for aging in soman-inactivated Hu AChE Trp86 mutants may arise from cation-π interactions. If catalysis of dealkylation by Glu199 and Trp84 is additive, their facilitation of the reaction is by $>10^4$.

Is the Catalytic His a Proton Donor in the Reaction? A second and higher p*K* associated with the descending leg of the pH-rate profiles above pH 6 is more difficult to assess. The best fit values are betwen 5.8 and 6.5. The slopes of the log k_a versus pH profiles were consistently between 0.8 and 0.9 between pH 7 and 10 in all cases. An evaluation of earlier data in the literature gave 5 (Bucht & Puu, 1984)−8 (Michel et al., 1967)-fold decrease in the rate of aging between pH 7 and 8. The orientation of active-site residues in the native structure of *Tc* AChE and results of MD simulations suggest that $HisH^+440$ is a very likely participant in the reaction mechanism (Qian & Kovach, 1993; Bencsura et al., 1995a, 1996; Enyedy et al., 1996; Beauregard et al., 1981; Berman & Decker, 1986).

The p*K*s of the catalytic His in phosphonate ester adducts of serine proteases are 0.5 unit higher in the diesters (Adebodun & Jordan, 1989; Robillard & Shulman, 1974; Porubcan et al., 1979; Jordan et al., 1985) and ∼3 units higher in the monoester anions than in the native enzyme (Adebodun & Jordan, 1989, Finucane & Malthouse, 1992). A very recent study of the pH dependence of the low frequency proton NMR chemical shifts of tripeptide ketone adducts of chymotrypsin also showed elevated p*K*s up to 11 of His57 (Cassidy et al., 1997). This is likely to be similar in AChE except with lower p*K*s of His440 (Quinn et al., 1991). The p*K* of the His at the transition state for the dealkylation reaction then might be expected to be between 6 and 10. If the p*K* is closer to 6 than 10, as in the systems in consideration, then the transition state more nearly resembles the diester than the monoester anion of the enzyme with little accumulation of negative charge on the oxygen in the scissile bond. This condition would also imply that *the positive charge is either not fully developed on C*α or spread out in the carbocation-like transition state.

The pH-rate profiles may level off at pH values above the pH range studied. An extrapolation of the pH dependence of the rate constants to pH 11 at 37 °C gives 5×10^{-6}−10^{-5} s^{-1} and well above plausible nonenzymic rate constants under the conditions. If the rate of dealkylation should level off at this value, the conclusion that the ionizing group is not the only catalyst of the reaction is inevitable. It is also likely that the electron-deficient oxyanion hole in the vicinity of the developing oxyanion exerts a stabilizing effect (Bencsura et al., 1995a). The lack of solvent isotope effects indicates that proton transfer at the reaction center in this reaction and in the dealkylation from sarin-inhibited *Ee* AChE (Kovach & Bennet, 1990) is not occurring at the transition state of the rate-determining step or in the preceding step. The incipient anion of the phosphonate monoester is then stabilized by electrostatic forces by $HisH^+$-440 (Kovach, 1988a; Kossiakoff & Spencer, 1981).

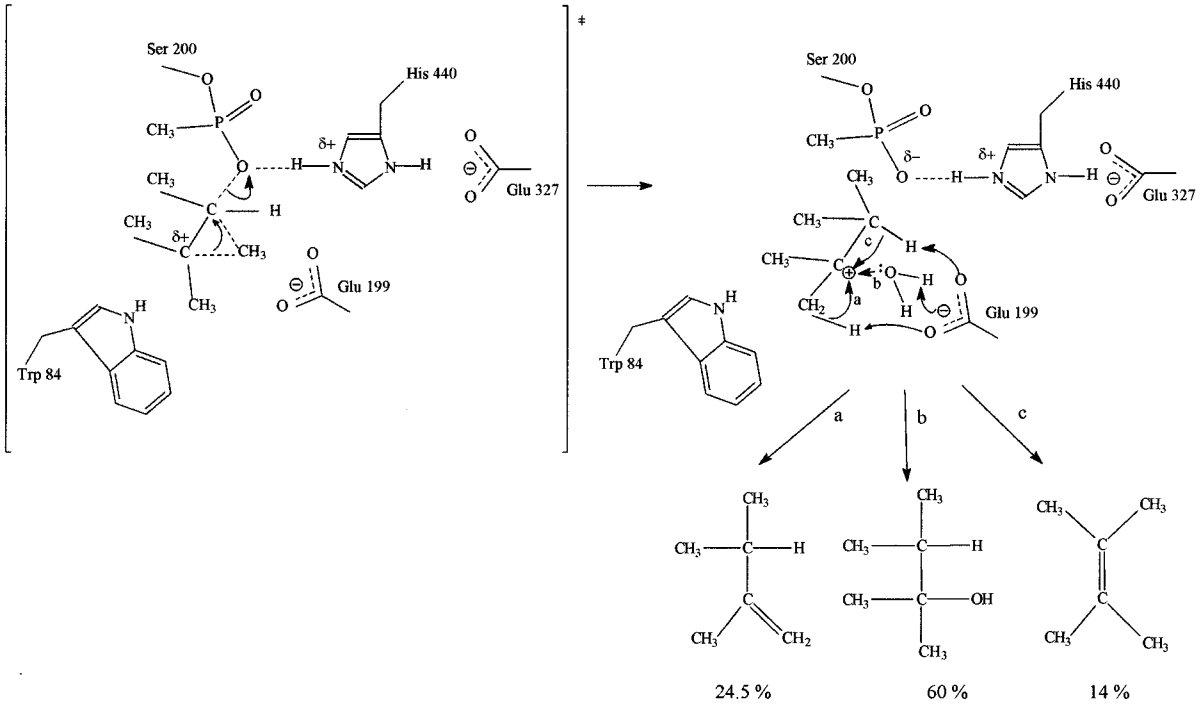
Proposal for the Charge Distribution at the Transition State for the Dealkylation in Soman-Inactivated AChE. It can be inferred from the product distribution of the dealkylation reaction of soman-inactivated AChE, reported by Michel et al. (1967), that the predominant intermediate leading to products is the tertiary cation. In the light of our modern kinetic and structural evidence, a plausible charge distribution at the transition state can now be proposed as in Scheme 2.

Electron pushing from carboxylic acids and aromatic residues, primarily Glu199 and Trp84, enforces the methyl migration from Cβ to Cα with concurrent breaking of the C−O bond. Stabilization of the developing negative charge on the anion of the phosphonate monoester is provided by $HisH^+440$ and the extensive electropositive oxyanion hole region of the active site. The positive charge is spread out from Cβ to $HisH^+440$, which seems most consistent with the participation of soft electrostatic interactions, π electrons, in the stabilization of charge separation. This transition state may be considered nonclassical. If a tertiary carbenium ion develops, it collapses due to trapping by base-catalyzed attack of water and by base-catalyzed removal of a proton from either Cα or Cγ. This results in the formation of 60% 2,3-dimethyl-2-butanol, 24.5% 2,3-dimethyl-1-butene, and 14% 2,3-dimethyl-2-butene.

A stepwise mechanism involving preprotonation of the oxygen in the breaking bond and the rate-determining formation of a secondary carbenium ion (Shafferman et al., 1996) is not easily reconciled with solvent isotope effects near one. Rate-determining C−O bond breaking from a preprotonated phosphonate diester would be expected to occur with an inverse solvent isotope effect (Kresge et al., 1978; Schowen & Schowen, 1982). Preprotonation would also require another transfer of the proton back to the His to reflect the pattern of protonation observed in aged adducts (Kossiakoff & Spencer, 1981). There is no need for a secondary carbocation to develop at all, because a charge-delocalized tertiary carbocation is much better stabilized by constituents of the leaving group binding site in the active-site gorge. It seems much more likely that the high-electron-density-driven methyl migration at the active site actually is the key to the aging reaction in soman-inhibited cholinesterases.

pH-Dependence of the Aging Reaction in Diastereomers of Soman-Inhibited Trypsin. Observed rate constants for declining reactivatability of trypsin from its adducts with soman were nearly pH independent. They were corrected for denaturation ($k_d = 3 \times 10^{-6}$ s^{-1}) and resolved into rate constants for reactivation and aging with decreasing precision in this order because of the dominance of reactivation. However, the rate constants for the aging reaction from the P(−)C(+) diastereomer were essentially the same when the

Scheme 2: Charge Distribution at the Rate-Determining Transition State Supported by Kinetic Results and the Ensuing Events Leading to Observed Product Distribution in the Dealkylation of Soman-Inactivated AChE



reaction was followed from an incubate and the aliquots drawn at regular time intervals were incubated with 0.1 M 2-PAM for overnight at 40 °C. There is little if any pH dependence of the rate constants for aging. The reactions were followed in heavy water and all that can be ascertained about the solvent isotope effects, which are associated with large errors, is that they are probably close to one for either process.

Rate constants for the process giving rise to a decline in reactivatability of trypsin from its adducts with soman are $\sim 10^4$ times slower at 37 °C than the maximal rate constants for the aging reaction in *Ee* and FBS AChE extrapolated to 37 °C ($\Delta H^{\ddagger} = 14.3$ kcal/mol). Dealkylation of other soman-inhibited serine proteases is similarly slow, although quantitative data for it are scarce. We conclude that the decline in the reactivatability of trypsin from its soman-inhibited forms is quite different from aging of the AChE adducts; the lack of pH dependence is inconsistent with S_N1-type dealkylations and may result from general base-catalyzed nucleophilic attack at C or P by water. However, solvent isotope effects of <2 would indicate that proton transfer at the transition state may not be important.

Choosing a Nonenzymic Reference Reaction for the Aging Reaction! Three structural analogs of the pinacolyl methylphosphonate ester of the active-site Ser have been synthesized and found resistant to either acidic or basic conditions in water at room temperature. Subsequently, their chemical stability at the CO or PO bond were estimated in methanol and acetonitrile, two solvents that can provide similar dielectric media to the active sites. A strong base, such as methoxide ion in methanol, effected PO fission in analogs of the phosphonate esters of the active-site Ser in 4 days at 37 °C. If a pseudo-first-order rate constant were estimated for this process, it would be (much) more than 5 orders of magnitude smaller than the maximal rate constant of aging of soman-inhibited AChE at the same temperature. The activation barrier of these reactions are then >7 kcal/mol higher than the barriers for dealkylation in soman-inhibited AChE. An acid-catalyzed CO bond fission should have a much higher energy barrier under comparable conditions. Close analogs of model compounds I and II were studied by Cadogan et al. (1969) in 1 N phenylsulfonic acid, 50% H_2O, and 50% DMSO at 100 °C, and the rate constants for dealkylation were measured to be 1.2×10^{-4} s^{-1}. A rate constant for acid-catalyzed dealkylation of these analogs at pH 5.5 and 100 °C might be expected to be at least 5 orders of magnitude smaller, $\sim 1 \times 10^{-9}$ s^{-1}. By comparison, a hypothetical rate constant for the AChE-catalyzed dealkylation in soman-inactivated AChE at pH 5.5 and 100 °C, would be 11 s^{-1} ($\Delta H^{\ddagger} = 14.3$ kcal/mol). Thus, a good estimate for a nonenzymic reference reaction under optimal conditions for the aging reaction would be at least 10 orders of magnitude slower than the AChE-catalyzed dealkylation. This limit would be indistinguishable from zero on Figure 2. Such *a remarkable efficiency of AChE in catalyzing a chemical transformation so different from the one it evolved to catalyze* is what sustained scientific and practical interest in this reaction for over half a century.

Uniqueness of the CO Bond Fission Occurring in Soman-Inhibited AChE. These studies demonstrate that the dealkylation reaction in soman-inactivated AChE has a unique pH dependence consistent with the participation of electrostatic catalysis by a residue with a pK 4.4−5.0 and by another residue with a p*K* around 6.5; *this mode of catalysis is consistent with S_N1-like CO bond fission and observed only in soman-inhibited AChE*. P−O bond cleavage could be brought about in 1.2 M $NaOCH_3/CH_3OH$ in compound I, and in 0.6 M $NaOCH_3/CH_3OH$ in compound II at 37 °C in 4 days, and not under physiologically viable circumstances. Apparently, aging is more efficient even in soman-inhibited trypsin than under these extreme conditions. Whether dealkylation in soman-inhibited trypsin is by CO fission by an S_N2-like mechanism or P−O fission cannot be completely sorted out. Whatever the mechanism, the trypsin active site

catalyzes removal of the pinacolyl group as efficiently as 0.6 M $NaOCH_3$ in methanol at the same temperature; this might be considered the effective molarity (Jencks, 1969) of the active-site groups in trypsin when catalyzing the dealkylation of the soman-phosphonylated active-site Ser.

AChE can lower the activation barrier of the dealkylation reaction involving the pinacolyl side chain by ~5.6 kcal/mol with respect to trypsin and by >13.6 kcal/mol with respect to a plausible model reaction. We attribute this phenomenon to the unique composition of the catalytic site of cholinesterases; particularly the dry and narrow active site with strong electrostatic forces. Attachment of the phosphonate fragment results in charge redistribution and compartmentation that sustains the push−pull mechanism of aging shown on Scheme 2. The participating residues identified by computational techniques (Qian & Kovach, 1993; Bencsura et al., 1995a,b, 1996) are Glu199 and Trp84 and other aromatic residues stabilizing the carbocation-like transition state. AChE presents a powerful motif in molecular recognition at the active site where catalytic carboxylic acids and aromatic π-electrons are juxtaposed to amplify electron density. An array of electropositive residues including $HisH^+440$ and components of the oxyanion hole provide the countervailing forces. Thus, *C−O bond fission in all of these adducts is likely to be promoted by nearly 4 orders of magnitude by negative electrostatic catalysis and by greater than 5 orders of magnitude by positive electrostatic catalysis and steric strain.*

The catalytic effect of Glu199 stands in stark contrast to the role of the corresponding Asp residues conserved in many serine proteases. Asp194 in serine proteases is engaged in a salt bridge with Ile16, which is formed in the course of zymogen activation to mature enzyme (Fersht, 1985; Polgar, 1987, 1990). In the α/β-hydrolase type enzymes (Cygler et al., 1993), there is no precedent for the occurrence of this mode of activation. The implications of this comparison to enzyme evolution are intriguing.

CONCLUSIONS

The irreversibly bound ligands are good active-site probes of serine hydrolases, because of the extra ligand in phosphonate esters relative to carboxyl esters.

The unique architecture of the active site of cholinesterases and the distribution of local electrostatic forces in phosphonylated cholinesterases promote the S_N1-type dealkylation from soman-inhibited cholinesterases. It is enforced by bifunctional electrostatic catalysis. The positive electrostatic catalyst is likely to be $HisH^+440$ with a pK 5.8−6.5 at the transition state.

The push−pull mechanism of aging observable in cholinesterases is absent in trypsin and other serine proteases and it is >10 orders of magnitude faster than an appropriate reference reaction.

We take note of the conformation that enables *Glu199 to exert a strong electrostatic catalytic effect* on most reactions (Qian & Kovach, 1993; Bencsura et al., 1995b; Quinn et al., 1991). Its basic catalytic power is enhanced by the high π-electron density of neighboring aromatic residues, primarily Trp84.

The aging reaction is initiated by a Glu199- and Trp84-driven methyl migration. The positive charge at the transition state of the rate-limiting methyl migration is spread through Cβ and $HisH^+440$.

ACKNOWLEDGMENT

We gratefully acknowledge helpful discussions and advice from Professor Richard L. Schowen, Department of Chemistry, The University of Kansas. Technical assistance with the synthesis and GLC experiments is acknowledged to Dr. Joan Harmon-Ashley Huber.

SUPPORTING INFORMATION AVAILABLE

Tables of rate constants and solvent isotope effects for Ee AChE and FBS AChE (4 pages). Ordering information is given on any current masthead page.

REFERENCES

Adebodun, F., & Jordan, F. (1989) *J. Cell. Biochem. 40*, 249−260.
Beauregard, G., Lum, J., & Roufogalis, B. D. (1981) *Biochem. Pharmacol. 30*, 2915−2920.
Bencsura, A., Enyedy, I., & Kovach, I. M. (1995a) *Biochemistry 34*, 8989−8999.
Bencsura, A., Enyedy, I., Viragh, C., Akhmetshin, R., & Kovach, I. M. (1995b) in *Enzymes of The Cholinesterase Family* (Quinn, D. M., Balasubramanian, A. S., Doctor, B. P., & Taylor, P., Eds.) pp 155−162, Plenum Press. New York.
Bencsura, A., Enyedy, I., & Kovach, I. M. (1996) *J. Am. Chem. Soc. 118*, 8531−8541.
Benschop, H. P., Konings, C. A. G., VanGenderen, J., & DeJong, L. P. A. (1984) *Toxicol. Appl. Pharmacol. 72*, 61−74.
Berman, A., & Decker, M. M. (1986) *J. Biol. Chem. 261*, 10646−10652.
Bourne, Y., Taylor, P., & Marchot, P. (1995) *Cell 83*, 503−512.
Brooks, C. L., III, Karplus, M., & Pettitt, B. M. (1988) in *Proteins: A Theoretical Perspective of Dynamics, Structure and Thermodynamics* (Prigogine, I., & Rice, S. A., Eds.) pp 38−44, Willey & Sons, New York.
Bucht, G., & Puu, G. (1984) *Biochem. Pharmacol. 33*, 3573−3577.
Cadogan, J. I. G., Eastlick, D., Hampson, F., Mackie, R. K. (1969) *J. Chem. Soc. B 115*, 144.
Cassidy, C. S., Lin, J., & Frey, P. A. (1997) *Biochemistry 36*, 4576−4584.
Cygler, M., Schrag, J. D., Sussman, J. L., Harel, M., Silman, I., Gentry, M. K., & Doctor, B. P. (1993) *Protein Sci. 2*, 366−382.
DeJong, L. P. A., & Benschop, H. P. (1988) in *Stereoselectivity of Pesticides* (Ariens, E. J., van Rensen, J. J. S., & Welling, W., Eds.) pp 109−149, Elsevier, Amsterdam.
DeJong, L. P. A., & Kossen, S. P. (1985) *Biochim. Biophys. Acta 830*, 345−348.
DeJong, L. P. A., & Wolring, G. Z. (1985) *Biochem. Pharmacol. 34*, 142−145.
Dougherty, D. A. (1996) *Science 271*, 163−168.
Ellman, G. L., Courtney, K. D., Andres, V., & Featherstone, R. M. (1961) *Biochem. Pharmacol. 7*, 88−95.
Enyedy, I., Bencsura, A., & Kovach, I. M. (1996) *Phosphorus, Sulfur Silicon Relat. Elem. 109−110*, 249−252.
Fersht, A. (1985) in *The Three-Dimensional Structure of Enzymes*, W. H. Freeman and Company, New York.
Finucane, M. D., Malthouse, J. P. G. (1992) *Biochem. J. 286*, 889−900.
Grunwald, J., Segall, Y., Shirin, E., Waysbort, D., Steinberg, N., Silman, I., & Ashani, Y. (1989) *Biochem. Pharmacol. 38*, 3157−3168.
Hovanec, J. W., Broomfield, C. A., Steinberg, G. M., Lanks, K. W., & Lieske, N. C. (1977) *Biochem. Biophys. Acta 483*, 312−319.
Jencks, W. P. (1969) in *Catalysis in Chemistry and Enzymology*, McGraw-Hill, New York.
Jordan, F., Polgar, L., & Tous, G. (1985) *Biochemistry 24*, 7711−7717.

Keijer, J. H., & Wolring, G. Z. (1969) *Biochim. Biophys. Acta 185*, 465−468.
Keijer, J. H., Worling, G. Z., & DeJong, L. P. A. (1974) *Biochim. Biophys. Acta 334*, 145−155.
Kossiakoff, A. A., & Spencer, S. A. (1981) *Biochemistry 20*, 6462−6474.
Kovach, I. M. (1988a) *J. Enzyme Inhib. 2*, 199−208.
Kovach, I. M. (1988b) *THEOCHEM 47*, 159−169.
Kovach, I. M. (1993) *Phosphorus, Sulfur Silicon Relat. Elem. 75*, 131−134.
Kovach, I. M., & Bennet, A. J. (1990) *Phosphorus, Sulfur, Silicon Relat. Elem. 51/52*, 51−56.
Kresge, A. J., More O'Ferrall, R. A., & Powell, M. F. (1987) in *Isotopes in Organic Chemistry, 7* (Buncel, E., & Lee, C. C., Eds.) Chapter 4, p 183, Elsevier, Amsterdam.
Lacy, C. I., & Lowe, L. M. (1980) *Tetrahedron Lett. 21*, 2017.
Leatherbarrow, R. J. (1992) in *GraFit*, Erithacus Software Ltd., Staines, U.K.
Lieske, C. N., Hovanec, J. W., Steinberg, G. M., Pikulin, J. N., Lennox, W. J., Ash, A. B., & Blumbergs, P. (1969) *J. Agric. Food Chem. 17*, 255−258.
Michel, H. O., Hackley, B. E. J., Berkowitz, L., List, G., Hackley, E. B., Gillilan, W., & Pankau, M. (1967) *Arch. Biochem. Biophys. 121*, 29−34.
Ordentlich, A., Kronman, C., Barak, D., Stein, D., Ariel, N., Marcus, D., Velan, B., & Shafferman, A. (1993) *FEBS Lett. 334*, 215−220.
Polgar, L. (1987) in *Hydrolytic enzymes* (Neuberger, A., & Brocklehurst, K. Eds.) pp 159−200, Elsevier Sci. Pub. Co., Amsterdam.
Polgar, L. (1990) *Mechanisms of Protease Action*; CRC Press, Inc., Boca Raton, FL.
Porubcan, M. A., Westler, W. M., Ibanez, I. B., & Markley, J. L. (1979) *Biochemistry 18*, 4108−4116.
Puu, G., Artursson, E., & Bücht, G. (1986) *Biochem. Pharmacol. 35*, 1505−1510.
Qian, N., & Kovach, I. M. (1993) *FEBS Lett. 336*, 263−266.
Quinn, D. M., Pryor, A. N., Selwood, T., Lee, B. H., Acheson, S. A., & Barlow, P. N. (1991) in *Cholinesterases: Structure, Function, Mechanism, Genetics, and Cell Biology* (Massoulie, J., Bacou, F., Barnard, E., Chatonnet, A., Doctor, B. P., & Quinn, D. M., Eds.) pp 252−257, American Chemical Society, Washington DC.
Robillard, G., & Shulman, R. G. (1974) *J. Mol. Biol. 86*, 541−558.
Saxena, A., Doctor, B. P., Maxwell, D. M., Lenz, D. E., Radic, Z., & Taylor, P. (1993) *Biochem. Biophys. Res. Commun. 197*, 343−349.
Saxena, A., Maxwell, D. M., Quinn, D. M., Radic, Z., Taylor, P., & Doctor, B. P. (1997) *Biochem. Pharmacol.* (in press).
Schoene, K. (1978) *Biochim. Biophys. Acta 525*, 468−471.
Schoene, K., Steinhanses, J., & Wertmann, A. (1980) *Biochem. Biophys. Acta 616*, 384−388.
Schowen, K. B., & Schowen, R. L. (1982) *Methods Enzymol. 87*, 551.
Segall, Y., Waysbort, D., Barak, D., Ariel, N., Doctor, B. P., Grunwald, J., & Ashani, Y. (1993) *Biochemistry 32*, 13441−13450.
Shafferman, A., Ordentlich, A., Barak, D., Stein, D., Ariel, N., & Velan, B. (1996) *Biochem. J. 318*, 833−840.
Sun, M.-C., Li, F.-Z., & Chou, T.-C. (1986) *Biochem. Pharmacol. 35*, 347−349.
Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., & Silman, I. (1991) *Science 253*, 872−879.
Taylor, P., & Radic, Z. (1994) *Annu. Rev. Pharmacol. Toxicol. 34*, 281−320.

BI962764Q